THE IMPACT OF DIETARY INTAKE ON SOX2 PROTEIN LEVELS AND ADULT NEUROGENESIS IN THE AGING ZEBRAFISH BRAIN

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE

OF BILKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF

MASTER OF SCIENCE

IN

NEUROSCIENCE

By

Seçil Gülden

July 2024

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

Michelle Marie Adams (Advisor)

Burcu Ayşen Ürgen

Elif Tuğçe Karoğlu Eravşar

Approved for the Graduate School of Engineering and Science

Orhan Arıkan Director of the Graduate School

ABSTRACT

THE IMPACT OF DIETARY INTAKE ON SOX2 PROTEIN LEVELS AND ADULT NEUROGENESIS IN THE AGING ZEBRAFISH BRAIN

Seçil Gülden

M.Sc. in Neuroscience

Advisor: Michelle Marie Adams

July 2024

Aging in humans is described as the gradual decline in physiological functions due to alterations in complex biological processes. As the lifespan increases and the population shifts towards older age, health problems associated with age become a worldwide problem. Thus, understanding the molecular and cellular changes that contribute to the aging process is essential to ensure healthy aging. Nine hallmarks that describe these changes were identified, which are also observed in the aging brain, leading to structural and cognitive deficiencies. Stem cell exhaustion is one of these hallmarks that explains a loss of stem cell function due to brain aging. A transcription factor, Sox2 is one of the main responsible proteins ensuring the stem cell maintenance through regulation of self-renewal and differentiation. Age-related changes in neural stem cells (NSCs) mediated by Sox2 can lead to a decline in new neuron formation. Neurogenesis is proven to continue in the defined neurogenic niches in the mammalian brain throughout adulthood and is considered to have a crucial role in the healthy functioning of the brain during aging. The regulation of the NSCs in neurogenic niches is achieved through diverse intrinsic and extrinsic factors that are affected by age-related changes. Dietary interventions are thought to modulate these factors and improve the age-related decline. Previous studies show that dietary restriction through lowering the calorie intake improves age-related impairment on neurogenesis, while high-calorie intake has a negative effect. Zebrafish, a small teleost fish, is a highly suitable model organism for investigations on neurogenesis with respect to aging due to exhibiting gradual decline with age, similar to humans and having a high capacity of neurogenesis in a widespread area.

Hence, in the first part of the study, the influence of diet on age-associated changes in the brain was observed by following two short-term opposing dietary interventions. Using zebrafish as a model organism, dietary restriction (DR), over-feeding (OF), and ad libitum (AL) diets were included. These interventions demonstrated that short-term DR, compared to the AL, downregulated Sox2, as evidenced by western blot analysis. This might indicate a decreased self-renewal properties and activation of differentiation of NSCs. Short-term OF, on the other hand, did not change its expression levels. Moreover, aging did not alter the Sox2 expression levels. Further correlational and multivariate analyses were performed combining Sox2 with a proliferation marker proliferating cell nuclear antigen (PCNA) and neuronal lineage markers doublecortin-like kinase 1a (DCAMKL1) and ELAV-like neuron-specific RNA binding protein 3 (HuC). The analysis demonstrated a positive relationship between Sox2 and PCNA indicating their similar pattern in the aging process. Also, DCAMKL1 was shown to have a positive relationship with PCNA and a negative relationship with HuC. The multivariate analyses of all datasets exhibited age-specific effects of OF and DR by decreasing the survival of new neurons in old animals. Also, OF reinforced the commitment to neuronal fate with old age. The second part demonstrated a recombinant protein purification approach using immobilized-metal affinity chromatography to purify the His-tagged Sox2 protein. The recombinant Sox2 protein was successfully purified, and concentration values were measured.

Taken together, these findings show that although older age led to changing dynamics in the neuronal lineage in the zebrafish brain, Sox2 managed to show a persistent expression. Furthermore, the short-term DR was shown to change the Sox2 expression levels, indicating the importance of calorie intake in alterations in neural stem cell properties. In conclusion, this study makes contributions to understanding cellular and biochemical changes occurring in the neurogenic niches of the aging brain and the altered neurogenic capacity due to dietary interventions.

Keywords: brain aging, zebrafish, adult neurogenesis, neural stem cells, dietary interventions

ÖZET

BESLENME ŞEKİLLERİNİN ZEBRABALIĞI BEYİN YAŞLANMASINDA SOX2 PROTEİN SEVİYESİ VE YETİŞKİN NÖROGENEZ ÜZERİNE ETKİSİ

Seçil Gülden

Nörobilim Lisansüstü Programı, Yüksek Lisans

Tez Danışmanı: Michelle Adams

Temmuz 2024

İnsanlarda yaşlanma, karmaşık biyolojik süreçlerdeki değişikliklere bağlı olarak fizyolojik işlevlerde kademeli bir azalma olarak tanımlanmaktadır. Yaşam süresinin uzaması ve nüfusun ileri yaşlara doğru kaymasıyla birlikte yaşa bağlı sağlık problemleri dünya çapında bir sorun haline gelmektedir. Bu nedenle yaşlanma sürecine sebebiyet veren moleküler ve hücresel değişikliklerin anlaşılması, sağlıklı yaşlanmanın sağlanması açısından önemlidir. Yaşlanma sürecinde gözlenen dokuz ayırt edici özellik belirlenmiş ve bu özellikler beyin yaşlanmasında da gözlenmiş olup yapısal ve bilişsel bozukluklara yol açmaktadır. Kök hücre tükenmesi, beyin yaşlanmasına bağlı olarak kök hücre fonksiyonundaki kaybı açıklayan bu ayırt edici özelliklerden biridir. Bir transkripsiyon faktörü olan cinsiyet belirleyici bölge Y-box 2 (Sox2), kendini yenileme ve farklılaşmayı düzenleyerek kök hücrenin devamlılığını sağlayan ana proteinlerden biridir. Sox2'nin aracılık ettiği nöral kök hücrelerde yaşa bağlı değişiklikler, yeni nöron oluşumunda bir düşüşe yol açabilir. Nörogenezin, memeli beynindeki tanımlanmış nörojenik nişlerde yetişkinlik boyunca devam ettiği kanıtlanmıştır ve yaşlanma sırasında beynin sağlıklı işleyişinde çok önemli bir role sahip olduğu düşünülmektedir. NSC'lerin nörojenik nişlerdeki düzenlenmesi, yaşa bağlı değişikliklerden etkilenen çeşitli içsel ve dışsal faktörler aracılığıyla sağlanır. Diyet müdahalelerinin bu faktörleri modüle ettiği ve yaşa bağlı düşüşü iyileştirdiği düşünülmektedir. Önceki çalışmalar, kalori alımını azaltarak diyet kısıtlamasının, nörojenez üzerinde yaşa bağlı bozulmayı iyileştirdiğini, yüksek kalorili alımın ise olumsuz bir etkiye sahip olduğunu gösteriyor. Küçük bir teleost balığı olan zebra balığı, insanlara benzer şekilde yaşla birlikte kademeli bir düşüş sergilemesi ve geniş bir alanda yüksek nörojenez

kapasitesine sahip olması nedeniyle yaşlanma açısından nörogenez araştırmaları için oldukça uygun bir model organizmadır.

Bu nedenle, çalışmanın ilk bölümünde, iki kısa süreli birbirine zıt diyet müdahalesi izlenerek diyetin beyinde yaşa bağlı değişiklikler üzerindeki etkisi gözlemlendi. Zebra balığı model organizma olarak kullanılarak, diyet kısıtlaması (DR), aşırı besleme (OF) ve isteğe bağlı (AL) diyet çeşitleri deneye dahil edildi. Bu müdahaleler, AL ile karşılaştırıldığında kısa süreli DR'nin, western blot analiziyle kanıtlandığı üzere Sox2 ekspresyonunun azalmasına yol açtığını gösterdi. Bu durum, kök hücrelerin kendini yenileme özelliklerinin azaldığına ve farklılaşmanın aktive olduğuna işaret etti. Kısa süreli OF için ise Sox2 protein düzeylerinde bir değişiklik gözlenmedi. Ek olarak yaşlanma, Sox2 ekspresyon düzeylerini değiştirmedi. Daha sonrasında, bir proliferasyon belirteci olan proliferatif hücre nükleer antijeni (PCNA) ile nöronal soy belirteçleri olan doublecortin benzeri kinaz 1a (DCAMKL1) ve ELAV benzeri nöron-spesifik RNA bağlayıcı protein 3 (HuC) de dahil edilerek korelasyonel ve çok değişkenli analizler yapıldı. Analiz, Sox2 ve PCNA arasında yaşlanma sürecinde birbirlerine benzer bir yol aldıklarına işaret eden pozitif bir ilişki olduğunu gösterdi. Ayrıca DCAMKL1'in PCNA ile pozitif, HuC ile ise negatif bir ilişkiye sahip olduğunu gösterdi. Tüm veri setlerinin çok değişkenli analizleri, OF ve DR'nin yaşa bağlı etkilerinin olduğunu, yaşlı hayvanlarda yeni nöronların hayatta kalma oranını azaltmaları ile gözlendi. Ayrıca OF, yaşlılıkla birlikte nöronal kadere bağlılığı güçlendirdi. Çalışmanın ikinci bölümünde, His etiketli Sox2 proteinini saflaştırmak için immobilize metal afinite kromatografisi kullanan bir rekombinant protein saflaştırma metodu sundu. Rekombinant Sox2 proteini başarıyla saflaştırıldı ve konsantrasyon değerleri ölçüldü.

Birlikte ele alındığında, bu bulgular, ilerleyen yaşın zebra balığı beynindeki nöron soyunda değişen dinamiklere yol açmasına rağmen Sox2'nin ekspresyonunun sabit olarak süregelmeyi başardığını gösteriyor. Buna ek olarak, kısa süreli DR'nin Sox2 ekspresyon seviyelerini değiştirdiği gösterildi; bu da kalori alımının nöral kök hücrelerin özelliklerinin üzerindeki değişmelerde önemli rolü olduğunu gösteriyor. Sonuç olarak, bu çalışma, yaşlanan beynin nörojenik nişlerinde meydana gelen

hücresel ve biyokimyasal değişikliklerin ve diyet müdahaleleri nedeniyle değişen nörojenik kapasitenin anlaşılması konusunda katkı sağlamıştır.

Anahtar sözcükler: beyin yaşlanması, zebrabalığı, yetişkin nörogenez, nöral kök hücrele, diyet müdahaleleri

ACKNOWLEDGMENTS

First of all, I would like to express my gratitude to my supervisor, Prof. Dr. Michelle Adams, for her guidance and support throughout my master's studies.

I would like to thank the members of my thesis committee, Asst. Prof. Burcu Ayşen Ürgen and Dr. Elif Tuğçe Karoğlu Eravşar for taking their time to attend my defense.

I want to thank all the members of Adams Lab: Büşranur Şeker, Fulya Kızıldağ, Naciye Bozkurt, Narin Ilgım Ardıç Avcı, Naz Mengi Çamur, Sanem Sobacı, Serena Sevdiye Aktürk, and Zeynep Karaer.

I want to mention my gratitude to not only my colleague but also my dear friend Sanem for showing her constant support. I am lucky to have met such a like-minded person at the beginning of my master's studies. I also want to express my appreciation to my dear friends Esnan Ece, Zeynep, and Ozan. Just spending time with them, talking and laughing together, made these last few years better for me. Lastly, I am deeply thankful to my dear friend Ece Sezer for always being there for me from our undergrad times as roommates to our lives as new adults. I always feel her care and support even from almost 10.000 km away.

I am especially thankful for my partner in crime, Büşranur. Joining this lab is one of my core memories because it led me to meet her. I am forever grateful for her caring words, eternal support, and countless hours talking about anything and everything. I am lucky to have her as my companion through this journey.

I cannot express enough gratitude to my dear family for always being there for me. I am deeply thankful for having such caring parents, Sevgi and Mehmet, who supported me in pursuing my goals and encouraged me to keep going and believe in myself when I doubted my abilities. I also want to thank my dear sister Selin for not only being the best sibling a person could ask for but also for being my best friend throughout my life. Her love and guidance made me who I am today.

I want to acknowledge the Scientific and Technological Research Council of Türkiye (TÜBİTAK) (Project no: 20AG003) for the financial support in the experiments.

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AD: Alzheimer's Disease

AL: *Ad libitum*

ALS: Amyotrophic Lateral sclerosis

AMPK: AMP-activated protein kinase

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

BMP: Bone morphogenetic protein

BrdU: Bromodeoxyuridine

BSA: Bovine Serum Albumin

CA: cornu ammonis

CNS: Central nervous system

CR: Caloric restriction

DCAMKL1: Doublecortin-like kinase 1a

DCX: Doublecortin

DDR: DNA damage response

dpf: days post-fertilization

DR: Dietary restriction

DRG: Dorsal root ganglia

DTT: dithiothreitol

ELAV: Embryonic lethal abnormal visual

ENS: Enteric nervous system

ESCs: Embryonic stem cells

FGF: Fibroblast growth factor

FOXO: Mammalian forkhead members of the class O

GCL: granule cell layer

HRP: Horseradish Peroxidase

HuC: ELAV-like neuron-specific RNA binding protein 3

IF: Intermittent fasting

IGF1: insulin-like growth factor 1

IIS: insulin/insulin-like growth factor 1 signaling

IL-1β: interleukin-1 β

IL-6: interleukin-6

IMAC: Immobilized-metal affinity chromatograpy

iPSCs: Induced pluripotent stem cells

IPTG: isopropyl β-D-1-thiogalactopyranoside

Klf4: Krüppel-like factor 4

LB: Luria Bertani

LF: Lipofuscin

LIF: Leukemia inhibitory factor

MRI: Magnetic resonance imaging

mTOR: mammalian target of rapamycin

NPs: Neural progenitors

NSCs: neural stem cells

NTA: Nitrilotriacetic acid

Oct4: Octamer-binding transcription factor 4

OF: Overfeeding

ORF: Open reading frame

ORN: Olfactory receptor neuron

PCA: Principal component analysis

PCNA: Proliferating cell nuclear antigen

PD: Parkinson’s disease

PI3K: Phosphoinositide 3-kinase

PVDF: polyvinylidene difluoride

RGL: Radial glial-like

RIPA: Radioimmunoprecipitation assay

RMS: Rostral migratory stream

ROS: reactive oxygen species

SA-β-gal: senescence-associated β-galactosidase

SDS-PAGE: sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SGZ: subgranular zone

Shh: Sonic hedgehog

SIRT1: Sirtuin 1

Sox2: Sex-determining region Y box 2

SVZ: subventricular zone

TAPs: Transit-amplifying progenitors

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline-Tween 20

TH: Tyrosine hydroxylase

TNF-α: tumor necrosis factor-α

VEGF: vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Brain Aging

In today's society, it is becoming evident that a demographic shift is occurring towards older age. People aged 65 and above are the most rapidly growing age group. As of 2020, individuals 60 years and older are found in the population more than children under five years old. In little over five years, one-sixth of the world will be aged 60 years or older, according to the findings of the World Health Organization [1]. This shift, referred to as population aging, first became noticeable in high-income, developed countries, but now developing countries are becoming the subject of it, and the old population will reside in low- and middle-income countries by 2050 [2]. Therefore, understanding the mechanism of the aging process to ensure a healthy population aging is more crucial than ever. From a biological perspective, aging can be described as molecular and cellular damage that accumulates in the body over time, causing a gradual decline in physiological functions and increasing the risk of developing diseases [3].

Aging can be observed in different organs, particularly the central nervous system. It is an especially vulnerable system to the decline of processes related to aging since neurons have a high energy demand [4]. With increasing age, neural functions deteriorate, causing physiological alterations, cognitive impairment, and weakened immune response. Brain aging is a main risk factor for many diseases associated with neural degeneration, such as Alzheimer's Disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and stroke [5].

During aging, brain volume and weight decrease. White matter is more affected by this shrinkage, especially in the prefrontal cortex, as shown by magnetic resonance imaging (MRI) studies [6]. Neural loss is thought to play a main role in this loss. According to the observations, different neuron types and brain regions are affected differently by this change. In some regions, no significant changes occur in cell number, whereas in the hippocampal cortex, for example, the number of granule and pyramidal neurons decreases after age 60 [7]. Myelin sheath starts to

deteriorate. The vascular system gets impaired, and blood pressure gets higher, resulting in a high risk of stroke and ischemia [8].

The morphology of synapses and dendritic spines are altered. As a compensatory mechanism for synaptic loss, dendritic sprouting can be observed. These changes can also impact the synaptic plasticity [9]. Calcium influx into neurons and signaling pathways mediated by calcium are impaired in the aged brain, disrupting calcium homeostasis. This can also influence the plasticity of synapses [10]. Lipofuscin (LF) pigment granules accumulate gradually over time in post-mitotic neurons, which is one of the most common features observed in aging [11].

An age-related decline in cognitive function occurs, especially memory impairment. Even though it is most prominently seen in patients with AD, episodic memory gets impaired starting from middle age in healthy individuals as well [8]. How fast information is processed and short-term memory recall are altered as a person ages [10]. Additionally, semantic memory initially improves with age but starts to decline at very old ages. Neurotransmitters are considered to play a role in the decrease in cognitive performance. For instance, it is observed that the activity of dopamine and serotonin receptors falls with age, affecting the neural networks and synapses. They are also thought to impact neurogenesis, thereby causing neural loss. Other influences are hormones and growth factors, such as estrogen and brain-derived neurotrophic factor (BDNF). According to studies, estrogen therapy might act as a protective agent against the negative effects of AD [12][13].

1.1.1 Hallmarks of Brain Aging

Comprehensive investigations at molecular and cellular levels shed light on the mechanisms that drive aging. As a result, nine hallmarks have been described as the common regulators of mammalian aging [14]. Most of these hallmarks are also observed in the aging brain. Only telomere shortening and senescence are not well-established, although studies support that they have a role in the aging of the nervous system [15]. The other seven hallmarks are genomic instability, epigenetic alterations, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, stem cell exhaustion, and altered intercellular communication.

1.1.1.1 Genomic Instability

DNA is exposed to damage by errors in DNA replication, reactive oxygen species (ROS), and external factors such as radiation. Normally, damaged DNA is corrected through various DNA repair mechanisms, but the DNA damage response (DDR) pathways also become less functional as the body ages. In addition to that, neurons are more prone to damage since they are post-mitotic cells and can not repair the DNA through homologous recombination [16]. Accumulation of unrepaired DNA in cells causes genomic stability, thereby disrupting the transcription process and eventually leading to cell death. Altered transcription also diminishes the expression of genes related to synaptic plasticity and survival of the neuron [15].

1.1.1.2 Epigenetic Alterations

Epigenetic alterations can be identified as post-translational modifications, such as changes in DNA methylation, acetylation, and phosphorylation, as well as post-transcriptional modifications, such as microRNA activities. Changes in the DNA methylation at specific sites are referred to as DNA methylation clocks, and these are considered to serve as biomarkers of aging. These alterations in epigenetic mechanisms change gene expression and lead to cognitive deficits in the aging brain [17].

1.1.1.3 Loss of Proteostasis

Proteostasis in cells ensures the clearance of damaged, misfolded, and dysfunctional proteins. Proteotoxic stress occurs when protein degradation machinery fails to work properly, which is a hallmark of brain aging. Many age-related disorders in the brain are the consequence of abnormal protein aggregation. In mice, aged glial cells are detected to have an accumulation of myelin fragments, which also promotes neurodegeneration [18].

1.1.1.4 Dysregulated Nutrient Sensing

There are several nutrient-sensing pathways in the body, such as insulin/insulin-like growth factor 1 (IGF1) signaling (IIS), AMP-activated protein kinase (AMPK), the mammalian target of rapamycin (mTOR), and sirtuins signaling pathways [19]. The

role of the IIS pathway in the brain is to convey information from the body regarding energy intake to the brain. The brain modulates eating behavior; high insulin levels cause decreased eating, whereas low insulin levels cause increased eating behavior. In aging, the responsiveness of these energy metabolism-regulating pathways decreases, and can no longer respond to nutrient signals efficiently. Furthermore, IGF1 levels decline significantly in an age-related manner, as reported in studies with mice [20].

1.1.1.5 Mitochondrial Dysfunction

Mitochondria is an organelle that plays a role in providing energy for cellular function through oxidative phosphorylation. Mitochondrial function is highly important for neurons since they have high energy demands. The organelle is found along the axons and dendrites and has an important role in electrochemical neurotransmission and axonal transport. With increasing age, mitochondria become dysfunctional through mitochondrial DNA damage, increased oxidative stress, morphological transitions, depolarization of the membrane, and impairment of the electron transport chain. As a result, it can affect neuronal function and cause axonal degeneration [21].

1.1.1.6 Stem Cell Exhaustion

Although most mammalian brain cells are post-mitotic, there are neuroprogenitor cells that can produce new neurons in specific regions, such as the subventricular zone (SVZ), the dentate gyrus of the hippocampus, and the olfactory bulb. Granule neurons in the dentate gyrus have highly important functions in learning and memory processes. During the course of aging, the neurogenesis in these regions decreases, leading to impairments in cognitive and olfactory functions. Neural progenitors (NPs) become less functional with time due to several changes, such as excessive DNA damage, dysregulated mitochondrial metabolism, oxidative stress, and chronic inflammation [21],[22].

1.1.1.7 Altered Intercellular Communication

Communication between cells is also altered with age. In the brain, synaptic connections are impaired, and neural function declines. Furthermore, pro-inflammatory cytokines are released from the glial cells, mostly microglia, causing age-related neuroinflammation. On top of this, clearance mechanisms of metabolic byproducts are disrupted. In the brain, waste disposal is done by the glial-lymphatic system and transport pathways across the blood-brain barrier (BBB). These mechanisms lose efficiency in time, resulting in waste accumulation [23].

1.1.1.8 Telomere Shortening & Cellular Senescence

Telomeres are short repeating DNA sequences at the ends of chromosomes, maintaining genomic stability, but they shorten gradually after each cell division. Telomerase reverse transcriptases function to prevent this shortening. As mentioned previously, neurons are post-mitotic, so they should not go through telomere attrition. However, neural stem cells (NSCs) in proliferative regions of the brain are shown to be vulnerable to it. High telomerase activity is crucial for proliferative activity, differentiation, survival of newborn neurons, and overall neurogenesis. Research done with telomerase-deficient mice found that hippocampal neurogenesis declined, and learning and memory were impaired. In another study with telomerase-deficient mice, restoration of telomerase activity inhibited neurodegeneration and increased the number of progenitors and newborn neurons [24]. Shortening reaches a critical point during aging, so cells exit the cell cycle and become senescent. Cellular senescence can occur in neural stem and progenitor cells, resulting in irreversible cell-cycle arrest along with high oxidative stress, impaired DNA repair response, and chronic neuroinflammation. Furthermore, findings are showing that senescence-associated β-galactosidase (SA-β-gal) activity can be observed in post-mitotic neurons [11].

1.2 Adult Neurogenesis

Neurogenesis is a complex process of generating and incorporating mature and functional neurons into the brain circuitry [25]. It starts with the division of stem cells, leading to the extension of the neural stem cell pool. After that, these precursor

cells are specified into committed progenitors. It is followed by a migration and differentiation of the progenitor into post-mitotic neurons. These immature neurons then go through a survival phase to transform into mature neurons. The surviving mature neurons are ultimately integrated into the pre-existing circuitry [26].

It was commonly believed that in mammalian brains, the production of new neurons only occurred during embryonic development and did not persist into adulthood. Evidence gathered in the past decades challenged this belief and showed that neurogenesis continues during adulthood in specific mammalian brain regions. In the 1960s, Joseph Altman and Gopal Das revealed the first evidence of neurogenesis continuing in adult mammalian brains [27]. Since it was difficult to observe and detect proliferating cells in vivo, the research on humans was generally based on investigating the postmortem brain tissues. However, the accuracy of the studies on postmortem tissues was questioned since there were many parameters to be optimized. In 1998, a study presented compelling evidence for adult neurogenesis in humans incorporating thymidine analog bromodeoxyuridine (BrdU) to detect the cycling cells in the brains of cancer patients [28]. Later, the radiocarbon dating method was utilized to show newly born neurons' presence throughout human adulthood [29].

However, it is also argued that mature neurons can dedifferentiate and re-express the markers of progenitors and immature neurons through an event called de-maturation. Thus, detecting the expression of these markers should not be considered conclusive evidence for adult neurogenesis [30]. Accordingly, a recent study stated no evidence of neurogenesis past adolescence [31]. In contrast, several recent studies were arguing that neurogenesis continues in certain regions until 90 years of life while observing a slight drop in the levels with increasing age [32]. Although the utilization of newer technologies allowing for direct observation of the cells is needed for a better understanding of the extent of neurogenesis, the majority of studies favor the existence of neurogenesis, even later in life.

1.2.1 Adult Neurogenesis in Mammals

In mammals, neurogenesis is commonly observed in two different niches: the dentate gyrus of the hippocampus and the SVZ. Recently, the olfactory bulb was

found to show neurogenesis [33] (Figure 1.1). Some studies are showing that neurogenesis might be taking place in other brain areas, such as the cerebral cortex, striatum, amygdala, substantia nigra, and spinal cord. However, further studies are needed to validate these findings [34].

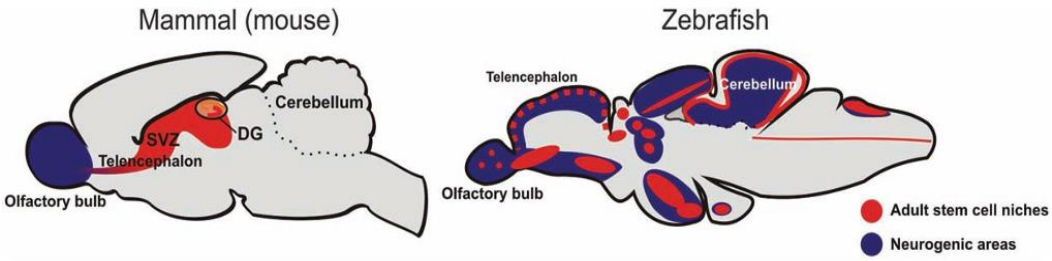

Figure 1.1 Comparison of neurogenic niches in the mammalian and zebrafish brain. Adapted from [35].

Progenitor cells are located in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus [36]. Progenitors in this niche are not true stem cells since they have a more limited fate. The majority of the NSCs are found in a quiescent state, meaning they are dormant with a low basal metabolic rate and not actively dividing. Through the regulation of cell-intrinsic and extrinsic factors, NSCs transition from the quiescent to the activated state. Activated NSCs can either symmetrically divide for self-renewal, sustaining their pluripotency, or asymmetrically divide to generate progenitor cells. Progenitor cells have a more restricted proliferation capability than NSCs and can differentiate into neurons or glia [37]. Through a few rounds of rapid cell division, they give rise to neuroblasts, progenitors that are committed to a neuronal fate. The GABAergic signal is later transmitted to the neuroblasts, differentiating them into immature neurons. During the maturation phase of these neurons, only about half survive, and the other half go through cell death. After two weeks, surviving neurons travel a short distance to the granule cell layer (GCL) and integrate into the neural network by developing axons and dendrites. The GABAergic signal continues to promote their maturation, helping the formation of synapses and the dendritic spine along with axon elongation until they become functional mature neurons of the GCL. New granule cells obtain the synaptic input from the entorhinal cortex and relay it to the cells in the cornu ammonis (CA) 1 and CA3 regions through their axons. The function of this circuitry is highly important

for learning and memory [38]. Until middle age, the total number of cells in the circuitry increases with the addition of the newly generated granule cells [36].

The second neurogenic niche is the SVZ of the lateral ventricle walls. NSCs in this niche, also known as type B cells, are slow-cycling, have astrocyte-like characteristics, and can differentiate into neurons and glia. They give rise to fast-cycling transit-amplifying progenitor (TAP) cells. TAP cells (type C cells) differentiate into migrating neuroblasts (type A cells). Migration occurs anteriorly through the rostral migratory stream (RMS) to the olfactory bulb [39]. There, neuroblasts undergo differentiation into interneurons, which regulate the spatial and temporal coding of olfactory [25].

Progenitor cells in the basal layer of the olfactory epithelium are also observed to have adult neurogenesis. They generate new olfactory receptor neurons (ORNs), replacing the old neurons in the area every few weeks since they have a short lifespan. In contrast, there is a selective replacement of neurons in the dentate gyrus and SVZ [40].

1.2.2 Adult Neurogenesis in Zebrafish

Different vertebrate species possess proliferation zones and neurogenic capacities to different extents. The mammalian species have evolved in a way that they strictly control the cell proliferation process, thereby restricting proliferation and neurogenesis in the adult brain. In contrast, teleost fish continue to grow throughout adult life and show widespread neurogenesis by continuously producing new neurons in many brain areas [35]. Zebrafish, a teleost fish, have 16 distinct proliferating regions along the rostrocaudal brain axis, as identified by the thymidine analog injection [41].

The telencephalon of zebrafish has two different niches: the dorsal telencephalon (pallium) and the ventral telencephalon (subpallium). The dorsal part is homologous to SGZ, and the ventral part is homologous to SVZ. It has radial glial-like (RGL) cells and is positive for glial markers such as GFAP, similar to the dentate gyrus. A small portion of them are PCNA-positive slow-cycling RGL cells (Type 2 cells), and the rest are PCNA-negative quiescent RGL cells (Type 1 cells). They

differentiate into neuroblasts (Type 3 cells), and then these cells migrate and generate HuC-positive newborn neurons. In the ventral part, there are neuroepithelial-like progenitors that do not show a glial-like phenotype. Most of them are PCNA-expressing proliferating cells and produce HuC-expressing neurons [42].

In the cerebellum, neuroepithelial-like progenitors first give rise to intermediate progenitors. They migrate into the granule cell layer (GCL) and give rise to granule neurons in a similar fashion to the mammalian dentate gyrus of the hippocampus. Some other important neurogenic niches in zebrafish include the olfactory bulb, optic tectum, habenula, and spinal cord [43].

1.2.3 Markers of Adult Neurogenesis

As mentioned in the previous section, there are some proteins expressed during the process of neurogenesis that can help identify the presence of different cell types. For this thesis study, the cellular biomarkers that are shown in Figure 1.2 are investigated.

Sex-determining region Y box 2 (Sox2) is a transcription factor that plays a critical role in the self-renewal and differentiation of NSCs and is used as a marker for the presence of NSCs [44]. Proliferating cell nuclear antigen (PCNA) is a protein that functions in DNA replication. It is expressed throughout the cell cycle process and used as a marker for actively cycling cells. In the brain, it can indicate the presence of proliferating NSCs [45]. Doublecortin (DCX) is a protein present in the migrating neuroblasts and immature neurons. It is not expressed in the newborn neurons in the neocortex [45]. There is a homolog of this protein, doublecortin-like kinase 1a (DCAMKL1), in the zebrafish brain [46]. Embryonic lethal abnormal visual (ELAV)-like neuron-specific RNA binding protein 3, HuC is an RNA-binding protein playing a role in neuronal differentiation (Zhu et al., 2006). It is commonly present in the early post-mitotic neurons [47].

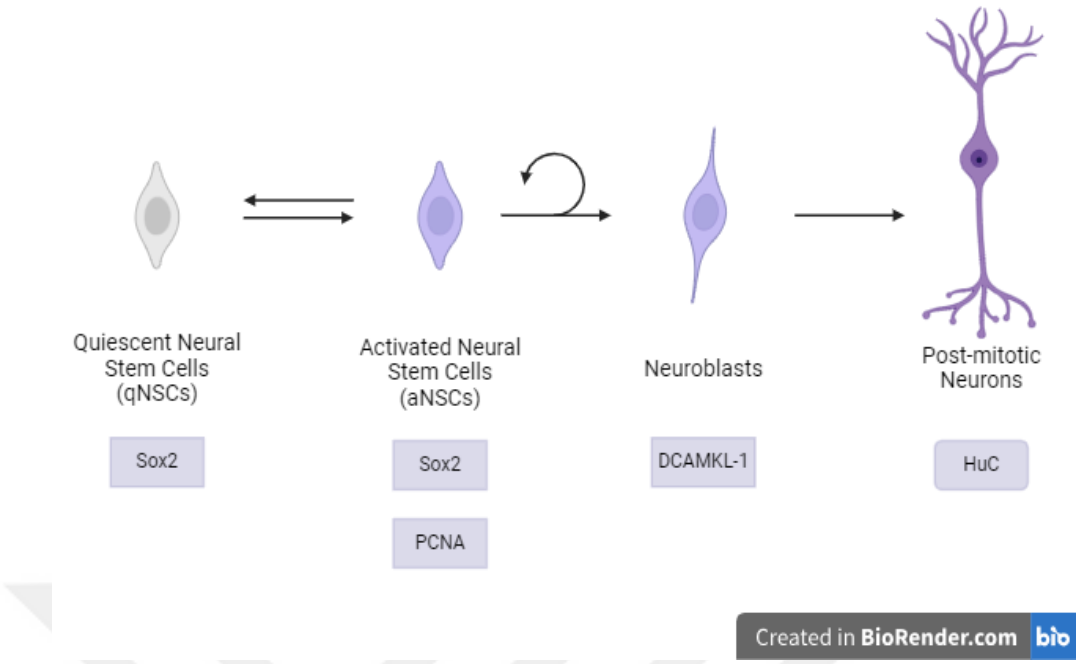

Figure 1.2 Illustration showing the neuronal lineage and the corresponding markers that are used in this study. Created with BioRender.

1.2.4 Age-Related Changes and Regulations in Adult Neurogenesis

There is a significant decline in neurogenesis with increasing age in rodents [38]. BrdU labeling revealed a prominent decrease in the number of new neurons in the hippocampus and SVZ of rodents from adolescence to middle age. From middle age to late age, the decline was less dramatic [48]. Changes in neurogenesis can result from the regulation of different stages of the process: expansion of the neural stem cell pool, quiescence and activation balance, proliferation rate, commitment to a neuronal fate, migration rate, survival rate of the immature neurons, and maturation by forming connections with other neurons [49].

Stem cell pool along with their daughter cells reside in the neurogenic niches which are specialized microenvironments providing communication between the surrounding brain regions and the rest of the body while also protecting the cells from outside factors. A neurogenic niche contains glial cells, endothelial cells, and blood vessels with a specialized extracellular matrix. Local cues or various factors coming from other brain regions or circulating in the blood can regulate the

neurogenesis in the niche [25]**.** In addition to the cell-intrinsic changes of NSCs, the components of the niche and the signals coming from the outside regions or circulating in blood can all have a part in regulating the process of neurogenesis. With increasing age, signals coming from the niche can get impaired, or NSCs ability to properly respond to these signals is altered. A study showed that removing the NSCs from the aged mice and transferring them in vitro prevented the decline in their proliferation capacity. Accordingly, it led to the belief that the age-related decline in NSCs was mainly due to changes in the niche [50]

Some important signaling pathways regulating the activity of progenitor cells in the neurogenic niches are the Notch, fibroblast growth factor (FGF), bone morphogenic protein (BMP), Wnt, and Sonic hedgehog (Shh) signaling pathways. Notch signaling is the key NSC quiescence-facilitating pathway. Notch is expressed in high levels in NSC, helping to maintain the quiescence of NSCs. When the Notch signaling is absent, the NSCs transition to the cycling state, and proliferation is increased. Similarly, BMP signaling inhibits neurogenesis by causing a decrease in the proliferation levels. An antagonist of BMP, Noggin enhances neurogenesis when expressed in cells. On the other hand, FGF and Wnt are expressed in proliferating cells, promoting the cell cycle activity of NSCs. Thus, age-related changes in the activity of these signaling pathways impact neurogenesis. Moreover, the release of neurotrophic and growth factors such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) declines with age, affecting the function of the niche [51][52]. Moreover, an age-related decrease in the release of neurotransmitters (NTs) such as dopamine causes a reduction in the number of progenitors in SVZ [53]**.** In the aged brain, microglia activity is elevated, resulting in the secretion of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β), and IL-6. Release of of these cytokines induces quiescence and disrupts the self-renewal of NSCs. This shrinks the NSC pool and decreases neurogenesis [54]**.** Lastly, changes in the genome stability and telomere length due to aging and epigenetic regulations also have an important role in diminished neurogenesis.

1.3 The sex-determining region Y-Box 2 (Sox2)

Sox2 belongs to the SRY-related HMG-box (SOX) family of transcription factors. In 1990, the SOX family of proteins was discovered, and four years later, Sox2 was identified through its role in regulating the activity of fibroblast growth factor 4 (FGF4) in embryonic cells [55]. It has an essential role in determining cell fate during embryonic development from the oocyte stage to the development of the nervous system [56]. It maintains the pluripotency of early embryonic stem cells (ESCs) and the multipotency of NSCs [57].

The decision of a pluripotent stem cell to remain pluripotent or to differentiate into one of the three germ layers is controlled by cell-intrinsic factors, which are commonly transcription factors. The main responsible factors are octamer-binding transcription factor 4 (Oct4), homeobox protein Nanog, and Sox2. They can also be described as lineage specifiers. Specifically, Sox2 regulates the differentiation into neuroectoderm and marks the formation of the nervous system [58]. Furthermore, Sox2 has been determined as one of the four factors known as Yamanaka factors that can reprogram differentiated somatic cells to a pluripotent state. The expression of these factors, Sox2, Oct4, Myc, and Krüppel-like factor 4 (Klf4), are used to generate induced pluripotent stem cells (iPSCs) [59]. After this reprogramming process, it is possible to transition the iPSCs into neural stem cells and differentiate them into neurons.

The crucial role of Sox2 during development can be understood from the fact that the homozygous null mutation of Sox2 causes embryonic arrest [56]. On the other hand, its heterozygous loss of function mutation results in rare eye disorders such as anophthalmia (absence of one or both eyes) or microphthalmia (abnormally small eyes). These disorders can cause vision loss or blindness [59]. Additionally, mutations in the gene can impact brain function in various different ways, causing underdevelopment of the hippocampus, seizures, cognitive defects, and motor function impairments [60]. The incomplete development of the hippocampus in Sox2-mutants is thought to be due to stem cell pool depletion since it causes a decrease in neural stem cells and granule neurons. Further electrophysiological studies show alteration in the excitatory activity in CA1 and CA3 of the hippocampus [61].

1.3.1 Sox2 Expression in the Adult Brain

In the adult central nervous system, Sox2 is needed for stem-cell maintenance to sustain self-renewal ability [58]. Sox2 is expressed at a high degree in neural stem and progenitor cells, and it promotes the expression of self-renewal genes, inducing maintenance and proliferation of the cells. At the start of their differentiation into post-mitotic neurons and glial cells, the Sox2 expression is downregulated [55] (Figure 1.3). Therefore, Sox2 protein has been used to identify the neural stem cells and progenitors in adult brains of vertebrates, including zebrafish.

In the adult mammalian central nervous system (CNS), Sox2 is most commonly found in two regions. In the dentate gyrus, neural progenitors (type 1 cells) that act as NSCs and type 2a progenitors show high Sox2 levels, while type 2b progenitors express Sox2 at a lower amount. In SVZ, radial glia-like cells (type B) are the NSCs and show high Sox2 levels. Sox2 is also found in the progenitor cells of the enteric nervous system (ENS) and in sensory progenitors of dorsal root ganglia (DRG) in adults [62]. Recently, its expression has also been detected in differentiated neurons in different brain regions. Pyramidal and GABAergic interneurons in the cerebral cortex and thalamic neurons are shown to express Sox2. This observation suggests that Sox2 has an important function in the development of neuronal connections, ensuring the proper establishment of neuronal networks [63].

The studies investigating the distribution of Sox2 protein in the zebrafish brain showed that Sox2-positive cells are dispersed throughout the telencephalon, diencephalon, mesencephalon, and rhombencephalon at 15 dpf. Sox2 presence progressively declined until the brain was developed, then remained stable at 180 dpf. Sox2-expressing cells were found in the telencephalic ventricle walls and olfactory bulb. In terms of the expression of Sox2 at mRNA and protein levels, the highest levels were observed at 10 dpf. It decreased gradually and remained constant at around 50 dpf [64].

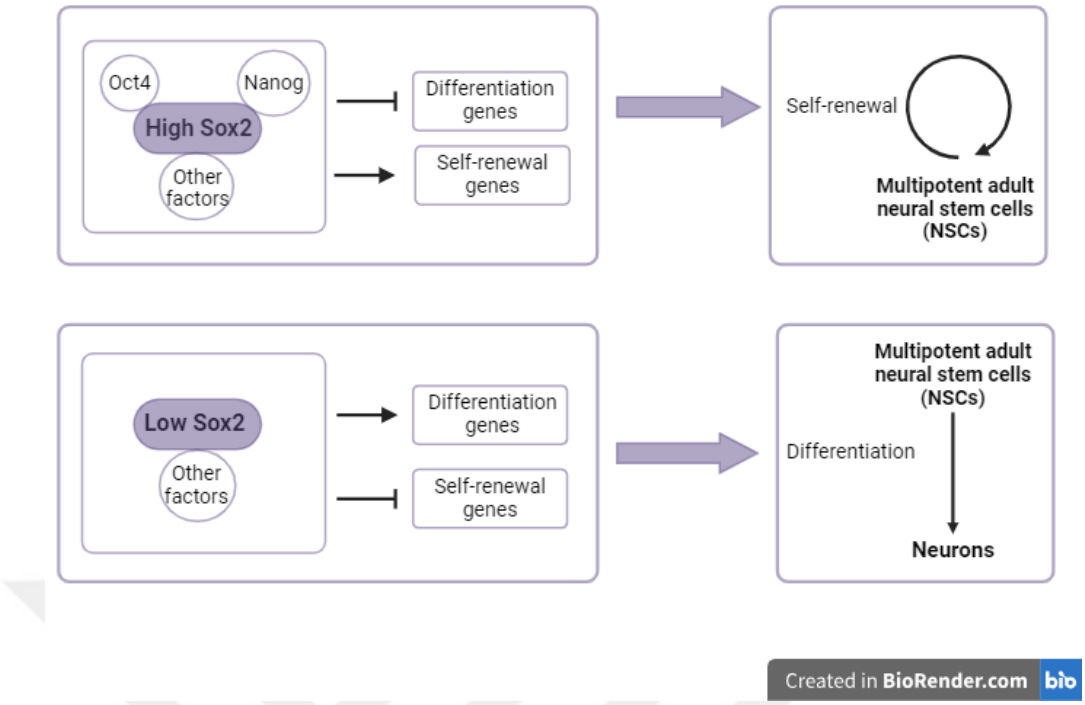

Figure 1.3 Scheme showing Sox2 and other factors regulate the genes that lead to self-renewal or differentiation of adult neural stem cells (NSCs). Created with BioRender.

1.3.2 The Regulation of Sox2 Activity

The Sox2 activity is controlled by various intrinsic and extrinsic mechanisms. The activity of some extracellular signaling factors such as WNT/β-catenin, Akt, Notch, FGF, BMP, and leukemia inhibitory factor (LIF) is found to be highly important for the regulation of Sox2 through their importance in the neurogenic niche [65]. Furthermore, cell cycle regulator E2f3a inhibits Sox2 expression, decreasing the self-renewal of progenitor cells and facilitating their differentiation. On the other hand, another cell cycle regulator, E2f3b, activates Sox2, increasing the self-renewal of progenitor cells and the growth of the neural stem cell pool. On top of this, cyclin-dependent kinase inhibitor P21 directly interacts with the enhancer of Sox2, thereby inhibiting the expression of Sox2 in neural progenitor cells [66]. Sox2 expression can also be controlled through epigenetic mechanisms such as microRNA regulation, methylation, acetylation, and phosphorylation [67].

1.3.3 Sox2 Expression During Aging

Sox2 expression can be observed in adult epithelial tissues of many organs, such as the brain, testes, lungs, spleen, liver, eyes, kidneys, and glands. As revealed by a study on mice and human samples, its expression in these tissues decreases with older age. In the brains of the old group, the levels of Sox2 were found to be diminished compared to the young. The situation was similar for different brain regions: the hippocampus and cortex. Moreover, this finding was negatively correlated with the expression levels of cyclin-dependent kinase inhibitor $p16^{INK4a}$, which is one of the most important biomarkers of aging. This suggests that the decrease in Sox2 expression can be considered a biomarker of aging [68]

1.4 Dietary Interventions

The age-associated molecular, structural, and physiological changes observed in the brain are affected by different environmental conditions, such as the regulation of nutrient intake. Different types of dietary restriction (DR) methods can be applied to improve age-related deficits. One form is the intermittent fasting (IF) regime, which can be achieved by alternate-day fasting. The second type is caloric restriction (CR), which lowers the overall intake of calories without changing the percentage of any nutritional components. CR is achieved by reducing the daily food intake compared to an ad libitum diet without causing any nutritional deficit. The third approach is lowering the intake of a specific nutritional component [69].

Applying a CR diet starting from young adulthood resulted in a significant increase in the lifespan of rodents. In addition to the lifespan-extending effect, it reduced the occurrence of age-associated diseases such as diabetes, cardiovascular diseases, and cancer [70]. In studies with monkeys and rodents, CR and IF diets are found to be protective against cognitive and structural deterioration due to aging. In disease models of AD, PD, stroke, and epilepsy, the IF diet had a preventive role against neuronal dysfunction [18]. Furthermore, a 30% calorie reduction protected grey matter volume from shrinking. Conversely, high food intake levels impair cognitive functions such as learning and memory and cause heightened oxidative stress and inflammation [71], [72]. In rats, a high-fat, high-sugar diet negatively affected their score on place recognition and delayed matching to position tasks [73].

When it comes to the regulation of neurogenesis, NSCs are responsive to the changes in their niche. The functioning of the neurogenic niche can be altered through outside factors such as diet [74]. It was evidenced that the CR diet increased hippocampal neurogenesis in rodents. A study done with young adult mice showed that calorie intake reduced by 40% led to the prevention of age-related decrease in neurogenesis in the SVZ and improved performance in olfactory memory [37]. Moreover, alternate-day feeding in rodents for 3 months increased newborn granule neurons of the DG. Diet caused an increase in the survival rate of new neurons, but it did not affect the proliferation of NSCs. According to another finding, a 40% reduction in calorie intake did not change neurogenesis but increased gliogenesis in the dentate gyrus [75]. On the other hand, diet-induced obesity impaired the synaptic spine density of granule neurons and decreased NSC activity and neurogenesis in the rodent hippocampus [76].

The exact mechanisms displaying how dietary interventions modulate adult neurogenesis are not entirely known, but there are some pathways and factors that are considered to play an important role. One proposed mechanism is through the increase in BDNF [77]. A decrease in the secretion of pro-inflammatory cytokines in the neurogenic niche is also thought to play an important role [78]. Nutrient sensing pathways such as insulin/IGF-1, mTOR, AMPK, and sirtuins are considered to play a crucial role in response to the changing dietary intake. They detect the changes in the levels of nutrients or energy and regulate cellular mechanisms accordingly. Insulin/IGF1 receptors are highly expressed in neurogenic niches. IGF-1 signaling is decreased due to dietary restriction, inhibiting the PI3K/Akt pathway. This also inhibits the phosphorylation of FoxO transcription factors, thereby increasing the expression of FoxO and FoxO-regulated genes. This improves the self-renewal activity of NSCs and slows down brain aging [79]. Suppressing IGF-1 expression in NSCs prevented the age-related decrease in neurogenesis in the olfactory bulb. Moreover, mTOR signaling facilitated the transition from quiescent to activated state, leading to the increased proliferation of NSCs. Lastly, levels of SIRT1, which is one of the sirtuins, are increased in response to decreased nutrient intake. It is highly expressed in neurons and acts to

reduce Notch1 activity, which is important for self-renewal of NSCs. Reduced Notch1 activity leads to the differentiation of NSCs [80].

1.5. Zebrafish as a Model Organism

Zebrafish (*Danio rerio*) is a small teleost fish living in freshwater, and it has been a popular vertebrate model organism used in biological research since it was introduced in 1972 by George Streisinger [81]. It became an especially important organism for studying development due to its numerous advantages. They have a small size and a lifespan of 3-5 years. One female can produce several hundred eggs in one mating. The embryos are transparent and develop externally, which makes them easily screened for organ development and morphological abnormalities and easily manipulated for pharmacological and molecular interventions [82]. The embryos are able to transform into free-swimming larvae in approximately five days [4]. Compared to mammalian models, the cost of maintenance and breeding is cheaper and they have a shorter generation time, around 3-4 months. Similar to invertebrates such as *D. melanogaster* and *C. elegans*, they are a convenient model for mutational analysis, and genetic manipulations are easily applied [82]. Furthermore, the majority of its genome is sequenced, and a variety of antibodies and cDNA libraries specific to zebrafish can be found [83]. Although they are not mammals, they share important common features with humans. For instance, they are diurnal vertebrates that sleep during the night [84]. Moreover, 70% of human genes and 76–82% of genes associated with human diseases have zebrafish orthologs [85].

1.5.1 Using Zebrafish to Study Aging

The aging process in organisms can be categorized into rapid, gradual, and negligible. Invertebrates generally exhibit rapid aging with a short life span. Thus, they would not be suitable models for characterizing the gradual decline of molecular and cellular events. In contrast, humans, as well as most vertebrates, show gradual aging with a longer lifespan. Zebrafish are also one of these organisms, which makes them a relevant model for studying aging [86]. They continue to grow throughout their lives. However, they show age-related

physiological and molecular changes such as skeletal and retinal degeneration, spinal curvature, decreased swimming ability, altered cardiovascular system, increased oxidative stress, and decline in neurogenesis [83]. They are detected to express an important biomarker of aging, which is SA-β-gal. Age-related changes in behavioral aspects can also be observed, such as impairment in associative learning skills and spatial memory [87]. Furthermore, they can be used to study important molecular pathways in aging, such as the insulin pathway. The main proteins in this signaling pathway are similar to mammals in terms of their structure and function [88]. Overall, they exhibit all the hallmarks of brain aging previously mentioned.

Important age-related human disorders, such as AD, PD, diabetes, and cancer, can be modeled in zebrafish. Foliate-deficient zebrafish can be used as a model to observe the common neurodegenerative pathology of AD in humans since they produce amyloid beta plaques and Tau protein is accumulated. Moreover, zebrafish express amyloid precursor protein and apoliprotein E, which are involved in familial AD [89].

1.5.2 Using Zebrafish to Study Brain and Adult Neurogenesis

When it comes to the similarities of the central nervous system, main regions such as the forebrain, midbrain, hindbrain, and spinal cord and the genes specifically expressed in these regions are conserved amongst vertebrate species. Zebrafish brain anatomy is structurally and functionally similar to mammals. On top of this, brain chemistry is also highly similar, including the presence of neurotransmitters and their receptors, major signal transduction pathways, and enzymes [90], [91]

16 distinct cell proliferation regions were revealed in the brain of adult zebrafish, primarily found along the ventricular zone but also in the parenchyma. Overall, zebrafish display a greater and more widespread neurogenic activity compared with mammals, which makes it a good model for studying adult neurogenesis [92]. Zebrafish having a higher proliferative capacity than humans might allow advancements in treating neurodegenerative disorders and other age-related diseases [84]. In addition to that, they have an excellent regenerative capacity, which makes them a good model for understanding the mechanism of tissue

regeneration and developing therapeutic interventions [93]. Neurogenic niches of the mammalian brain are located in the inner parts of the brain, not allowing for direct observation. On the other hand, in zebrafish, niches are located at the outer surface of the brain, making them accessible for live imaging. Similar to mammals, age-dependent reduction in neurogenesis is also detected in zebrafish. Proliferating neural stem cells decrease with age, resulting in a gradual depletion of the neural stem cell pool [94].

1.6. Aim of This Thesis

As mentioned earlier, the aging of the population is becoming more evident as the years pass, and age-related health problems emerge as a crucial issue. Accordingly, understanding the changes in the body that lead to the occurrence of diseases is highly important. Nine hallmarks are defined as explaining the molecular and cellular changes with age. Focusing on the aging brain, these nine hallmarks were seen to be leading to structural and cognitive deficiencies that disrupt the functioning of the nervous system.

Neurogenesis was recognized as a complex process that has a vital role in brain function during adulthood. The maintenance of neural stem cells, their proliferative ability, and differentiation into new neurons are all tightly regulated stages that ultimately lead to the integration of new neurons into the brain circuitry. Age-related alterations in the brain cause a decline in the proper functioning of these stages, resulting in decreased neurogenesis.

One of the hallmarks of aging is stem cell exhaustion, which leads to the loss of stem cells and their self-renewal and differentiation into new neurons. Sox2 is a transcription factor crucial for regulating the maintenance of stem cells in the brain, promoting their self-renewal. Thus, age-related changes in stem cells are thought to be associated with the activity of Sox2, which ultimately leads to the impairment of neurogenesis.

Dietary intervention methods were described as an effective regulator to reverse the age-related decline in the brain. Specifically, the reduction in daily CR was found to rejuvenate the biological pathways damaged due to aging and decrease the risk

of developing age-related diseases, whereas high-calorie intake had an inverse effect. Moreover, the calorie restriction diet improved the age-related decline in NSC maintenance and adult neurogenesis. Further research is needed to understand the underlying mechanisms regulating neurogenesis in the aging brain and which aspects of this complex process are targeted by applying different dietary regimes.

Consequently, this thesis work is focused on several objectives. The first objective is to investigate the impact of different dietary regimens on Sox2 protein levels and adult neurogenesis in the aging brain using zebrafish as a model organism. For this aim, Sox2 was used as a marker for neural stem cells to detect the changes in the neural stem cell pool due to age, gender, and dietary intake. Changes in the protein levels of this marker were determined by investigating whole brain tissues. The results were compared with the changes in the marker of proliferating cells and neuronal lineage markers to better illustrate how neurogenesis might be affected overall. The second objective is effectively expressing and purifying the His-tagged Sox2 protein from *E. coli* for further use as a biomarker for detecting stem cells in a newly developed biosensor.

CHAPTER 2

METHODS

2.1 Animal Subjects

Zebrafish raising, maintenance, and experimental procedures were carried out in a zebrafish facility at Bilkent University Molecular Biology and Genetics Department, Ankara, Turkey, for a previous study done in our lab, as described [95]. The feeding intervention experiment was performed on wild-type male and female zebrafish. Animal euthanization, brain tissue dissection, and protein isolation protocols were all performed previously [95]. The experimental design is illustrated in Figure 2.1, and the detailed feeding schedule is explained in Table 2.2 Both dry flakes and newly hatched artemia were used for feeding the animals. Commercial dry flakes (TetraMin Flakes, Melle, Germany) were composed of 46% protein, 11% oils, and 3% fiber [95]. Artemia is a live food also known as brine shrimp and is often provided as nutritional enrichment for zebrafish [96]. The protocol of the study was approved by the Bilkent University Local Animal Ethics Committee (HADYEK) with the approval date of Sept 6, 2017, no: 2017/12.

For this thesis work, previously obtained brain homogenate samples were utilized for the Western Blot experiments. In total, 36 animals were included: 18 of them were from the young animal group, and 18 were from the old animal group. In both young and old animal groups, three different dietary regimen groups (*ad libitum*, dietary restriction, and overfeeding) were included, each consisting of 6 animals. In each diet group, there were 3 females and 3 males.

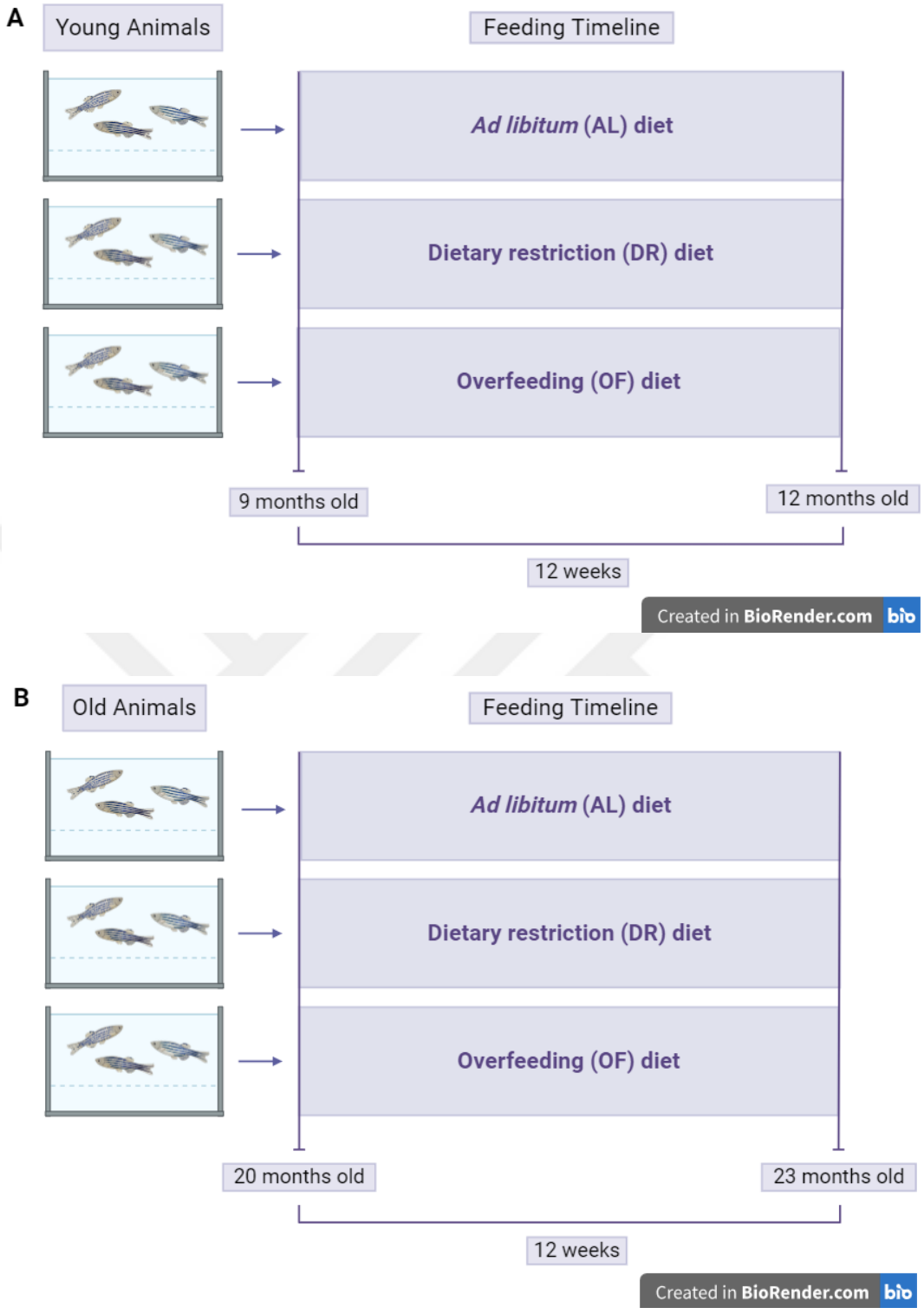

Figure 2.1 The illustration of experimental design A) For young animals B) For old animals AL: *ad libitum* group (control), OF: over-feeding group, DR: dietary restriction group. The figure is created with BioRender.

Table 2.1 Feeding schedule and amounts for the animals.

Diet Group	Dry flakes	Artemia
AL (n=16 for young animals, n=13 for old animals)	180 mg every day	3 times every week
DR (n=16 for young animals, n=13 for old animals)	90 mg every two days	Once every week
OF (n=15 for young animals, n=13 for old animals)	360 mg every day	Twice every day

2.2 Whole Protein Isolation from Brain Tissues

For the optimization of Sox2 protein for western blot experiments, wild-type young zebrafish brain tissues that were not subjected to feeding interventions were used. The homogenization of the brain tissues was done using Radioimmunoprecipitation assay (RIPA) buffer. The RIPA buffer was prepared according to the recipe in Table 2.2, and a 2X protease inhibitor (05 892 970 001, cOmplete ULTRA Tablets, Roche, Basel, Switzerland) was freshly added. The brain tissues stored at -80°C were placed on ice for thawing. After waiting for approximately 30 minutes, RIPA buffer was added. For every 1 mg of tissue, 60 µl of buffer was used. The whole protein isolation procedure was done on ice to avoid degradation. The tissues were homogenized inside the buffer by passing through the solution in a 1ml insulin syringe around 15-20 times. The homogenized tissues were incubated on ice for half an hour. After every 10 minutes, they were mixed by tapping the tubes approximately 20 times. After the incubation, the samples were centrifuged at 13.000 rpm for 20 minutes at 4°C. The supernatants were collected slowly and carefully without disturbing the pellet and aliquoted. The aliquots were temporarily stored at 4°C until the Bradford Assay was performed. After that, they were stored at -80°C.

Table 2.2 Recipe for RIPA Buffer (for 1 ml)

Materials	**Amount**
2M Sodium Chloride (NaCl)	75 µl
1M Tris-HCl (pH 8.0)	50 µl
100% nonyl phenoxypolyethoxylethanol (NP-40)	10 µl
10% Sodium Dodecyl Sulfate (SDS)	10 µl
dH_2O	355 µl
2x Protease Inhibitor	500 µl

2.3 Bradford Assay

For the homogenates isolated for the optimization experiments, Bradford Assay was performed. This assay was used to determine the protein concentrations of the homogenates. In a 96-well plate, 1 mg/ml Bovine Serum Albumin (BSA) (A7906, Sigma-Aldrich, St. Louis, MO, USA) used as a standard control protein was added first. The design used for adding the BSA standards is shown in Table 2.3. Then, samples were prepared in a 1:10 dilution by adding 4.5 µl dH_2O and 0.5 µl homogenate. All samples, including BSA standards, were added as duplicates. 250 µl of Bradford Reagent (B6916, Sigma, St. Louis, MO, USA) was added into each well carefully drop by drop. The 96-well plate was put on an orbital shaker at 240 rpm at room temperature for 45 seconds, allowing the solutions to mix. Then, the plate was incubated for 10 min at room temperature without shaking. It was covered with tin foil at this stage since the Bradford reagent is light-sensitive. After incubation, any air bubbles remaining were removed using a syringe needle. Next, the plate was put inside a multi-plate reader (Synergy HT Multi-Mode Microplate Reader). The absorbance values were read at 595 nm to measure the protein concentrations. A standard curve was generated using the BSA standards with known concentrations, the x-axis showing BSA standards (mg/ml), and the y-axis showing absorbance values at 595 nm. From this standard curve, the concentrations of isolated proteins can be calculated according to their absorbance values.

Table 2.3 BSA Standard for Bradford Assay

Standards	**dH_2O**	**BSA (from 1 mg/ml stock)**
1	5 µl	0 µl
2	4.5 µl	0.5 µl
3	4 µl	1 µl
4	3 µl	2 µl
5	2 µl	3 µl
6	1 µl	4 µl
7	0 µl	5 µl

2.4 Western Blot

2.4.1 Sample Preparation

According to the concentration results of the tissue homogenate from The Bradford Protein Assay, the volume of homogenate that should be used for different loading amounts is calculated. To determine the optimal amount of homogenate that will be used for sample preparation to avoid saturation, optimization experiments were carried out for the proteins Sox2 and the housekeeping protein tubulin. The western blot images and the saturation curve graphs for the optimizations can be found in the Results part. According to the graph, using 15 µg of homogenate prevented the saturation of Sox2 and tubulin proteins.

The aliquots of tissue homogenates stored at -80°C were taken out for thawing on ice before the sample preparation. The next steps in the procedure were performed on ice. 15 µg of homogenate was mixed with an appropriate volume of dH_2O in 1.5 ml. Eppendorf tube via pipetting to achieve a volume of 10 µl. Then, 10 µl of 2X loading buffer was added and mixed with pipetting to the sample to achieve a final

volume of 20 ul. The 2X loading buffer was prepared by freshly adding 10% dithiothreitol (DTT) to the 2X loading dye. The 2X loading dye was prepared beforehand according to the recipe shown in Table 2.4. and stored at room temperature to use when needed. The samples were then heated by boiling at 95°C for 10 minutes. Denaturation of proteins via heating is needed for their proper detection. After this step, the samples are vortexed and subjected to spinning using QuickSpin Mini Centrifuge, then stored at -20°C until use.

Table 2.4 Recipe for 2X Loading Dye

Materials	**Amount (for 20 ml)**
10% Sodium Dodecyl Sulfate (SDS)	8 ml
100% Glycerol	4 ml
4% Bromophenol blue	250 µl
1 M Tris-HCl (pH 6.8)	2 ml
dH_2O	up to 20 ml

2.4.2 SDS-PAGE Gel Preparation

A mini-PROTEAN Tetra Vertical Cell Electrophoresis system (Bio-Rad, USA) was used for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For casting the SDS-PAGE gel, glass gel casting plates were clamped together via a casting plate and then placed into a casting stand. A glass spacer plate with a 1.5 mm integrated spacer was used, and a shorter glass casting plate was aligned in front of it to form a glass plate sandwich. This sandwich was used to cast a gel with a 1.5 mm thickness. The gel was prepared as two different layers: the bottom layer is the resolving gel, and the top layer is the stacking gel. 5% stacking gel lines up all the samples loaded on the gel to ensure they reach the resolving gel layer simultaneously. 15% resolving gel was used to separate proteins based on their

molecular weight, and its percentage is determined based on the molecular weight of the protein of interest. 15% resolving gel was appropriate for the best separation of proteins of interest.

Stacking and resolving gels were prepared based on the recipes shown in Tables 2.5-6. The resolving gel was prepared first and poured between the glass plates using a Pasteur pipette. The top of the gel was layered with isopropanol to avoid air bubbles and ensure an even layer. After waiting for the gel to polymerize, which took approximately 15 minutes, isopropanol was removed and washed out with dH_2O. The stacking gel was prepared and poured with a Pasteur pipette on top of the resolving gel layer. Immediately after pouring the gel, a 15-well 1.5 mm comb was placed carefully with an angle. The stacking gel polymerized in approximately 15 minutes, and wells were formed.

Table 2.5 5% Stacking Gel (for one gel)

Materials	**Amount (for 3.75 ml)**
dH_2O	2.83 ml
1 M Tris-HCl (pH 6.8)	0.47 ml
10% Sodium Dodecyl Sulfate (SDS)	37.5 µl
40% Acrylamide	375 µl
10% Ammonium persulfate (APS)	37.5 µl
N,N,N'N'-tetramethylethane-1,2-diamine (TEMED)	3.75 µl

Table 2.6 15% Stacking Gel (for one gel)

Materials	**Amount (for 10 ml)**
dH_2O	2.4 ml
1 M Tris-HCl (pH 8.8)	3.75 ml
10% Sodium Dodecyl Sulfate (SDS)	100 µl
40% Acrylamide	3.75 µl
10% Ammonium persulfate (APS)	100 µl
N,N,N'N'-tetramethylethane-1,2-diamine (TEMED)	10 µl

2.4.3 Loading of Samples and Gel Electrophoresis

The glass plate sandwich containing the polymerized gel is removed from the casting plate and placed into the slot on one side of the electrode assembly. A second glass plate sandwich was placed into the slot on the opposite side of the assembly. They were placed so that the shorter casting plates were facing inward. This assembly was then put inside the buffer tank and filled with 1X Running Buffer, which was prepared by diluting the 10X Running Buffer with dH2O. 10X Running Buffer was prepared beforehand according to the recipe in Table 2.7. and stored at room temperature. Running Buffer was poured into the until the 4-gel line. 15-well combs were removed slowly and carefully so as not to damage the wells. The inside of the wells were washed with 1X Running Buffer using a Pasteur Pipette to get rid of any residue. The prepared samples were taken out to room temperature. PageRuler Prestained Protein Ladder (26619, Thermo Fisher Scientific, Rockford, IL, USA) was used as a marker for detecting the molecular weight of the proteins. 1 µl of the protein ladder was loaded into the first well and last two wells. After

that, 18 µl of sample protein were loaded into each remaining well. The buffer tank lid with cables connected to a power supply was covered. The samples were initially run at 80 V for 45 minutes at room temperature until all the samples aligned and left the stacking gel. After that, the voltage was increased to 100 V, and the samples were run for approximately 90 minutes.

Table 2.7 Recipe for 10X Running Buffer

Materials	**Amount (for 1 L)**
Tris base	30.285 g
Glycine	144.134 g
10% Sodium Dodecyl Sulfate (SDS)	100 ml
dH_2O	up to 1 L

2.4.4 Transfer

Before the time gel electrophoresis ended, the apparatus needed for the wet transfer method was prepared. Two transfer sandwiches were assembled on an assembly tray, according to the visualization in Figure 2.2. 20% methanol (MeOH) (vol/vol) was added freshly into the 1X Transfer Buffer that was prepared beforehand according to the recipe shown in Table 2.8 and stored at 4°C. The assembly tray was filled with Transfer Buffer for Whatman paper and sponge pads to soak into the buffer.

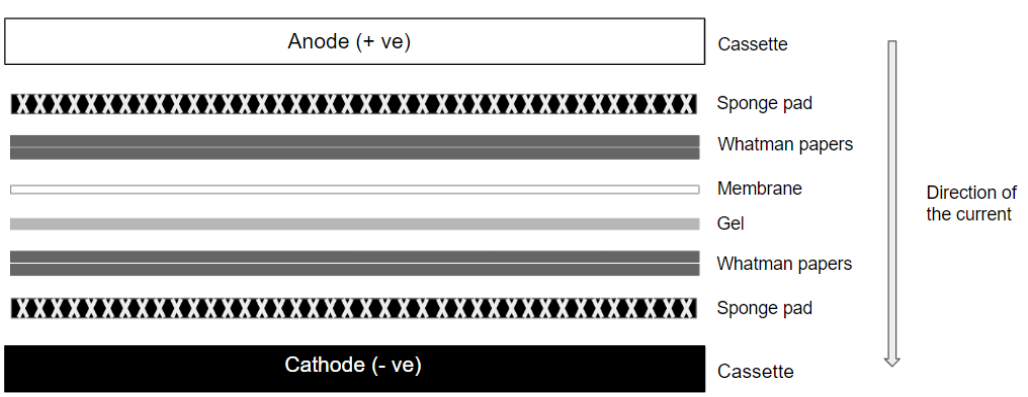

Figure 2.2 The visualization of the transfer sandwich system

Table 2.8 Recipe for 1X Transfer Buffer

Materials	**Amount (for 1 L)**
Tris base	3.02 g
Glycine	14.4 g
dH_2O	up to 800 ml
100 % Methanol (freshly added)	200 ml

After the running, the glass plates were removed from the tank, and then the plates were separated, exposing the gel itself. The stacking gel layer was discarded, and the resolving gel was carefully placed on top of the Whatman paper in the transfer sandwich system. The gel was kept wet by submerging it into the Transfer Buffer. In the meantime, the Thermo Scientific™ PVDF (polyvinylidene difluoride) Transfer Membrane with a pore size of 0.45 μm was soaked into 100% MeOH, keeping it there for at least one minute to activate it. After activation, the membrane was taken out and placed on top of the gel. A roller was used to remove air bubbles between the gel and the membrane, allowing a proper transfer. The membrane was soaked up in the Transfer Buffer as well.

After placing the membrane, the gel holder cassette was closed, and the transfer sandwich assembly was completed. The transfer sandwich was then placed into the electrode assembly inside the buffer tank. The black side of the gel holder cassette should be facing the black side of the electrode assembly to ensure the proteins are transferred from the gel to the membrane. Transfer Buffer was poured into the tank until the 4-gel line and a cooling unit were also put inside to keep the buffer cold during the transfer. The tank was covered with a lid and connected to a power supply. The transfer was conducted at 100 V for 90 minutes at 4°C.

2.4.5 Blocking and Primary Antibody Incubation

Before the end of the transfer process, washing and blocking solutions were prepared. Firstly, 10X Tris-buffered saline (TBS) solution was prepared according to the recipe in Table 2.8 and diluted to 1X concentration using dH2O. After that, Tween-20 was added to the buffer to prepare 1X TBS-Tween-20 (TBS-T) (Table 2.10). Two different blocking solutions were used: 5% non-fat dry milk in 1X TBS-T and 5% BSA in 1X TBS-T. They were prepared in falcons and placed on a shaker until the milk powder and BSA fully dissolved. After the transfer, membranes were taken out from the gel holder cassette and placed inside a clear plastic box with a lid. The membrane was covered with the blocking solution and then placed on a shaker in RT for 1 hour for incubation with gentle continuous agitation. Incubation with the blocking solution was crucial for preventing the non-specific binding of

primary antibodies to the membrane.

Table 2.9 Recipe for 10X Tris-Buffered Saline (pH was adjusted to 7.6 before completing the buffer to 1 L)

Materials	**Amount (for 1 L)**
Tris base	24 g
Sodium Chloride (NaCl)	80 g
dH_2O	up to 1 L

Table 2.10 Recipe for 1X TBS-Tween-20

Materials	**Amount (for 1 L)**
10X TBS	100 ml
Tween-20	3 ml
dH_2O	up to 1 L

Before the end of the blocking part, the primary antibodies were prepared for the primary incubation. Two different primary antibodies were used: Sox2 Antibody (MAB2018, R&D Systems, Minneapolis, MN, USA, 1:1000 dilution) and β-Tubulin Antibody (#2146, Cell Signaling Technology, Danvers, AM, USA, 1:5000 dilution) as a loading control. Sox2 and β-tubulin antibodies were both diluted using 5% non-fat dry milk in 1X TBS-T. As explained in Table 2.11, blocking was made with 5% BSA in TBS-T for the Sox2 antibody. Accordingly, the membrane was washed with 1X TBS-T two times for 5+5 minutes on a shaker before adding the primary antibody solution. This was done to get rid of any remaining BSA solution. After this short washing step, the primary antibody solutions were added into the plastic box, and the membranes inside were incubated with primary antibodies on a

shaker with continuous gentle agitation overnight (~16 hours) in a cold room (4°C).

Table 2.11 List of the primary antibodies used in the Western Blot experiments

Antibody Name	**Company, Catalog No.**	**Dilution Ratio**	**Dilution Solution**	**Blocking**	**Secondary Antibody**
Sox2	R&D Systems, MAB2018	1:1000	5% milk in TBS-T	5% BSA in TBS-T	Mouse HRP
β-Tubulin	Cell Signaling Technology, #2146	1:5000	5% milk in TBS-T	5% milk in TBS-T	Rabbit HRP

Primary antibody solutions were collected the next morning using a 100-1000 µl adjustable volume micropipette. The antibody solutions were collected inside a falcon and stored at -20°C for further use. Membranes were then washed with 1X TBS-T 5 times for a total of 30 minutes on a shaker at RT. Washing durations were 5, 5, 10, 5, and 5 minutes respectively.

2.4.6 Secondary Antibody Incubation

While the washing step was continuing, secondary antibodies were prepared. Goat Anti-Mouse IgG H&L, Horseradish Peroxidase(HRP)-conjugated antibody (ab97023, Abcam, Cambridge, UK, 1:5000 dilution) was used for Sox2 while anti-rabbit IgG, HRP-linked antibody (#7074, Cell Signaling, Danvers, AM, USA, 1:5000 dilution) was used for β-tubulin. Anti-mouse and anti-rabbit secondary antibodies were prepared in 5% non-fat dry milk in 1X TBS-T (Table 2.12). After the washings, the secondary antibodies were added, and membranes were incubated for 1 hour at RT on a shaker with gentle, continuous agitation.

Table 2.12 List of the secondary antibodies used in the Western Blot experiments

Antibody Name	**Company,** **Catalog No.**	**Dilution Ratio**	**Dilution Solution**
Mouse HRP	Abcam, ab97023	1:5000	5% milk in TBS-T
Rabbit HRP	Cell Signaling Technology, #7074	1:5000	5% milk in TBS-T

After the secondary antibody incubation, secondary antibody solutions were collected inside a falcon and stored at -20°C for further use. Membranes were washed with 1X TBS-T 5 times for 30 minutes on a shaker at RT. Washing durations were the same as the washings after the primary incubation.

2.4.7 Chemiluminescent Detection

After the washing step, membranes were prepared for image detection. The chemiluminescent detection method was used to acquire images. SuperSignal™ West Femto Maximum Sensitivity Substrate (34095, Thermo Scientific, Rockford, IL, USA) was used as a chemiluminescent substrate. This kit included two solutions: Luminol/Enhancer and Stable Peroxide Buffer. Using a micropipette, these solutions were mixed inside a microcentrifuge tube in a 1:1 ratio. Since the Luminol/Enhancer solution is sensitive to light exposure, the tube was covered with tin foil. The membranes were carefully removed from the box using tweezers and placed on a smooth surface. The Luminol/Enhancer-Stable Peroxide Buffer mixture was then added to the surface of the membrane carefully drop by drop using a micropipette. It was ensured that the whole surface was covered with the solution to avoid membrane drying. Immediately after that, the membrane was incubated for 5 minutes in the dark. After the incubation, the membrane was placed inside The ChemiDoc XRS+ Gel Imaging System (Bio-Rad, CA, USA) and imaged using the ImageLab software (Bio-Rad, CA, USA).

2.4.8 Image Quantification

The images were exported from the ImageLab software and opened with ImageJ software (NIH, Bethesda, MD, USA). The obtained images were quantified by Büşranur Şeker, who did not have information on the order of the protein samples loaded into the gel to avoid any possible bias during quantification. First, using the rectangle selection tool, observed bands corresponding to the experimental samples were selected one by one. Each sample in all three cohorts was selected in this manner, and then the rectangular areas were plotted using the plot profile function. This function created a column average plot for each area, where the x-axis shows the horizontal distance through the selection and the y-axis shows the average vertical pixel intensity. For each plot, the area under the peak was selected using the line selection tool, and then the selected areas were measured using the wand (tracing) tool.

After obtaining the measurements for band intensities, two different normalizations were performed: within gel normalization and the loading control protein normalization. In each gel, one cohort containing 12 samples was represented. The experiment had three cohorts, meaning three biological replicates, and each cohort had two technical replicates. Thus, within-gel normalization was required to eliminate exposure differences during imaging between the two technical replicates. To achieve this, the band intensity values for each sample on the gel were divided by the average value of all band intensity values within one gel. This normalization was performed for all proteins used in this experiment, which are Sox2 and the housekeeping protein tubulin. After that, loading control protein normalization was performed, which was necessary to eliminate any error that might happen while loading the samples into the gel. For this experiment, the housekeeping protein β-tubulin was used as the loading control. Thus, the within-gel normalized value of the protein of interest (Sox2) for each sample was divided by the within-gel normalized value of β-tubulin for that sample. After normalizing all data, the average values of two technical replicates were calculated, and these values were used for the statistical analysis.

2.4.9 Statistical Analysis

All statistical analyses were done with IBM SPSS Statistics 26.0 (Armonk, NY, USA) and GraphPad Prism 10 (Boston, USA). Before conducting any parametric test, the assumptions of normality were tested. The normal distribution was checked using the Shapiro-Wilk test, and Levene's test was conducted to check the homogeneity of variance. The assumptions of normality were met in the case of age and gender. However, the assumption of homogeneity of variance was violated in the case of diet. Thus, non-parametric tests were conducted for all three groups (age, gender, and diet). Mann-Whitney U test was used to analyze the effects of gender with two levels (male and female) and age with two levels (young and old) in the protein expression levels. Kruskal-Wallis test was conducted for the effect of diet with three levels (ad libitum, overfeeding, dietary restriction) to analyze the protein expression differences. If a significant effect of diet was observed, pairwise comparisons were made using the Mann-Whitney U test, and p-value adjustments were made accordingly.

Furthermore, the Pearson correlation coefficient was used to measure linear correlations. After that, principal component analysis (PCA) was performed to analyze different neuronal and cellular proteins together. Varimax was chosen as a rotation method. To determine the number of principal components to be extracted, the Kaiser criterion and Scree plot method were taken into account. According to the Kaiser criterion, the eigenvalues of the principal components were determined, and the values equal to or higher than 1 were chosen to be extracted. Accordingly, the first two principal components were extracted since only these two met the rule. Overall, $p<0.05$ was accepted as the significance level for all tests other than the cases in which p-value adjustments were done.

2.5 Expression and Purification of 6xHis-Tagged Proteins

For this experiment, a high-level expression of Sox2 protein is achieved via using bacterial cloning vectors. Later, the expressed Sox2 protein was purified using the immobilized-metal affinity chromatography (IMAC) technique. For this, the open reading frame (ORF) of human SOX2 mRNA (NM_003106.2) was first cloned into a vector that carries a polyhistidine tag consisting of six histidine residues. pREP4

and pQE cloning vectors and a host bacteria, E. coli, were used for this aim. Adding a His tag into the N-terminus of SOX2 allows metal ions such as Ni^{+2} to bind so that the protein can be efficiently purified using Ni^{+2}-affinity chromatography.

2.5.1 Expression of 6xHis-tagged Sox2 Protein using *E. coli*

First, 15 ml Luria Bertani (LB) Broth was prepared according to the recipe in Table 2.13. Then, 1000X Kanamycin and Carbenicillin stocks, which were kept at -20 ºC, were taken out to room temperature. 3 µl from each was added to the broth. At the -80 ºC freezer, E. coli glycerol stock was stored. Bacteria from the stock were also added to the broth using a pipette tip. The bacteria in the media were grown with overnight incubation using a shaker at 225 rpm, 37 ℃. The next day, the broth becomes foggy, indicating that the bacteria has been successfully grown. This time, a 250 ml LB Broth was prepared in a flask by adding 250 µl Kanamycin and Carbenicillin. 3 ml of the overnight grown medium was taken out using a micropipette and transferred to the flask. The medium was placed on a shaker at 225 rpm, 37 ℃. During this incubation, the medium was checked regularly for the optical density (OD) value measurement. This process was repeated until the OD_{600} value reached 0,6. After the OD value measurement, isopropyl β-D-1-thiogalactopyranoside (IPTG) stock kept at -20 ℃ was taken to room temperature, and 250 µl of IPTG was added into the medium. This reagent functions as an inducer for the Sox2 expression in bacteria. The medium was incubated again for 4 hours at the same conditions. Later, it was placed on ice for 5 minutes. The culture was then transferred to a 500 ml centrifugation bottle to centrifuge at 4 ℃, 3500 rpm for 20 min. The supernatant on top was discarded, and the pellet at the bottom containing the Sox2-expressing bacteria was stored at -80 ℃ until use.

Table 2.13 Recipe for LB Broth (for 500 ml)

Materials	**Amounts**
Tryptone	5 g
NaCl	2,5 g

Yeast Extract	2,5 g
2 N NaOH	250 µl
dH_2O	up to 500 ml

2.5.2 Purification of 6xHis-tagged Sox2 Protein

After acquiring the bacteria pellet expressing His-tagged Sox2 protein, purification by Ni^{+2}-affinity chromatography was performed. The QIAexpressionistTM handbook for high-level expression and purification of 6xHis-tagged proteins was used as a guide for this procedure. 10 ml of the Lysis Buffer A (Table 2.14) was added to the bacteria pellet. Then, the pellet was resuspended inside the buffer by doing up and down movements with a pipette. The resuspended solution was centrifuged at 10.000 rpm for 20 min (4 °C). The supernatant that contains the lysate was transferred to a falcon tube. Ni^{+2}-NTA (Nitrilotriacetic acid) Agarose Resin was used to mix with the lysate. Ni^{+2}-NTA allows the nickel ions to interact with the 6xHis tag, immobilizing the lysate. 20 ml lysate was added to the 5 ml of agarose resin and then put on a shaker for an hour for them to fully mix.

Table 2.14 Recipe for Lysis Buffer A (pH was adjusted to 8.0 before completing the buffer to 50 ml)

Materials	**Amounts (for 50 ml)**
Tris base	0.06 g
Guanidinium chloride (Gu-HCl)	28.66 g
$NaH_2PO_4.H_2O$	0.69
dH_2O	up to 50 ml

The setup for the chromatography Ni^{+2}-affinity chromatography was prepared by

setting up a column and connecting a peristaltic pump (Ismatec™ MS-4/6 Reglo Digital Pump) to it. This pump was used to ensure the flow rate was stable at 1 drop/3 seconds. Before moving on to the purification part, the setup was cleaned by passing dH_2O through the column and peristaltic pump tubing. After that, Ni^{+2}-NTA resin and lysate mix were added into the column, and 25 ml of Lysis Buffer A was flowed through the column containing resin+lysate by keeping the rate at 1 drop/3 seconds with the help of the pump. After flowing it through, Lysis Buffer A was discarded. This process was repeated twice. Later, the same process was repeated with Lysis Buffer B, Wash Buffer C, and Elution Buffer D (recipes shown in Table 2.15). Lastly, Elution Buffer E was added and transferred through the column. This time, the protein of interest was eluted along with the buffer. Thus, the buffer that flowed through the peristaltic pump was collected in 2 ml tubes. During this stage, it was checked that the drop rate remained at 1 drop/3 seconds. The collected buffer contained the purified protein. The concentration of the purified Sox2 protein was detected by measuring the OD_{280} value using Invitrogen Qubit Fluorometer (Thermo Fisher Scientific, Rockford, IL, USA).

Table 2.15 Recipe for Lysis Buffer B (pH adjusted to 8.0), Wash Buffer C (pH adjusted to 6.3), Elution Buffer D (pH adjusted to 5.9) and Elution Buffer E (pH adjusted to 4.5)

Materials	**Amounts (for 100 ml)**
Tris base	0.12 g
8 M Urea	48.0 g
$NaH_2PO_4.H_2O$	1.38 g
dH_2O	up to 100 ml

CHAPTER 3

RESULTS

3.1 Western Blot Results

3.1.1 Optimizations for Sox2 Protein

Western blot experiments were conducted to optimize the detection of Sox2 protein. The observed band images for zebrafish brain homogenate and mouse ESC samples are shown in Figure 3.1. mESCs were used as a positive control to confirm the bands observed in zebrafish samples were correct. In the western blot image, above the band at 36 kDa, a faint band was also observed for the control mouse sample. The amino acid sequence of mouse Sox2 shows a high, 85.62%, similarity with zebrafish Sox2, according to BLAST results. However, various types of post-transcriptional modifications have previously been observed for mouse Sox2. In mouse embryonic stem cells, modification of threonine residue was observed through a form of glycosylation called O-GlcNAcylation [97]. Accordingly, the upper band observed might be the glycosylated form of the protein. Zebrafish samples were loaded to the gel as 10, 15, and 20 µg, respectively, to calculate the change in band intensity with increasing sample amount. Saturation graphs shown in Figure 3.2 were generated according to the band intensity values obtained from the western blot images. According to the observed band images and the saturation curve graphs, using 15 µg of sample prevented the saturation of Sox2 and β-tubulin proteins.

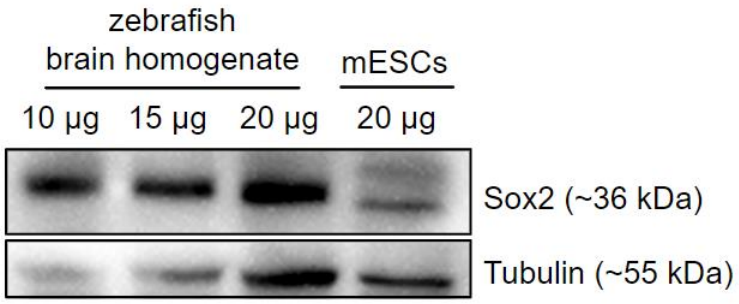

Figure 3.1 Western blot images of optimizations for Sox2 protein using wild-type zebrafish brain homogenates. β-tubulin was used as a loading control. mESCs: mouse embryonic stem cells were used as a positive control for Sox2 protein.

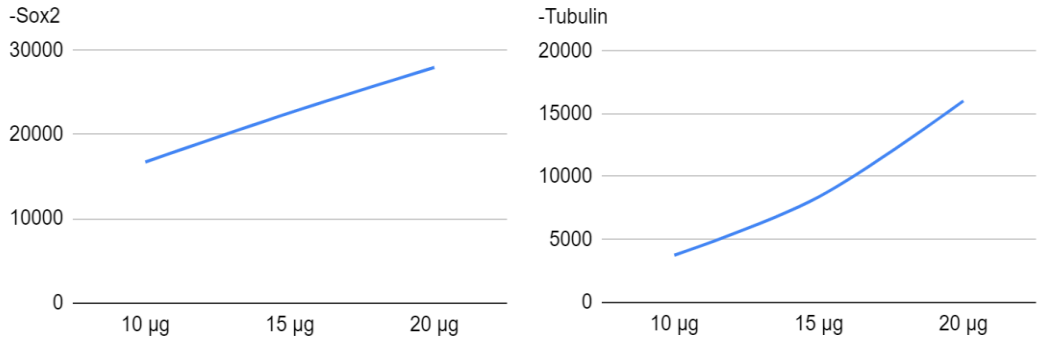

Figure 3.2 Saturation curve graphs for Sox2 and β-tubulin proteins.

3.1.2 Changes in Sox2 Protein Levels

The band intensities were quantified from the western blot image (Figure 3.3), as described in Chapter 2. The normality of the data was checked using the Shapiro-Wilk test, and the homogeneity was checked using the Levene Test. Mann-Whitney U test has revealed that neither gender (U(x) = 155.000, p = 0.839) nor age (U(x) = 146.000, p = 0.613) has a significant effect on Sox2 expression, Figure 3.4. As for the age-dependent changes in gender, neither female (U(x) = 31.000, p = 0.436) nor male animals (U(x) = 39.000, p = 0.931) show a significant change according to age. As for the gender-dependent changes in age, neither young (U(x) = 30.000, p = 0.387) nor old animals (U(x) = 36.000, p = 0.730) show significant changes between males and females.

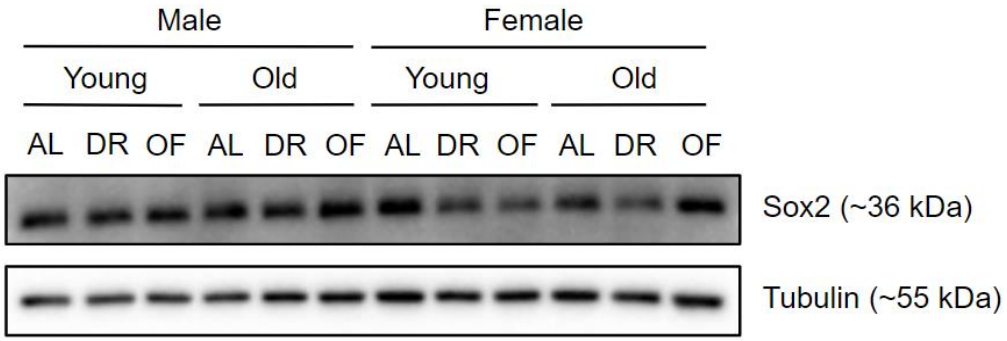

Figure 3.3 Representative western blot images of observed bands from one cohort showing Sox2 and housekeeping protein β-tubulin. AL: Ad-libitum; OF: overfeeding; DR: Caloric restriction; F: Female; M: Male.

The assumption of normality was violated for the diet group. Thus, the Kruskal-Wallis test for the effect of diet was applied. The results revealed a significant overall effect of diet ($H(2) = 8.083$, $p = 0.018$), Figure 3.5. Moreover, the Mann-Whitney U test with adjusted p values was used for pairwise comparison, revealing a significant change between DR and AL groups, Figure 3.5. Animals fed with the DR diet had significantly lower levels of Sox2 than the AL animals ($U(12) = 23.000$, $p = 0.004$), Figure 3.5. However, there was no significant effect of diet both in the young group (H(x) = 3.556, p = 0.169) and in the old group (H(x) = 4.667, p = 0.097), Figure 3.4.

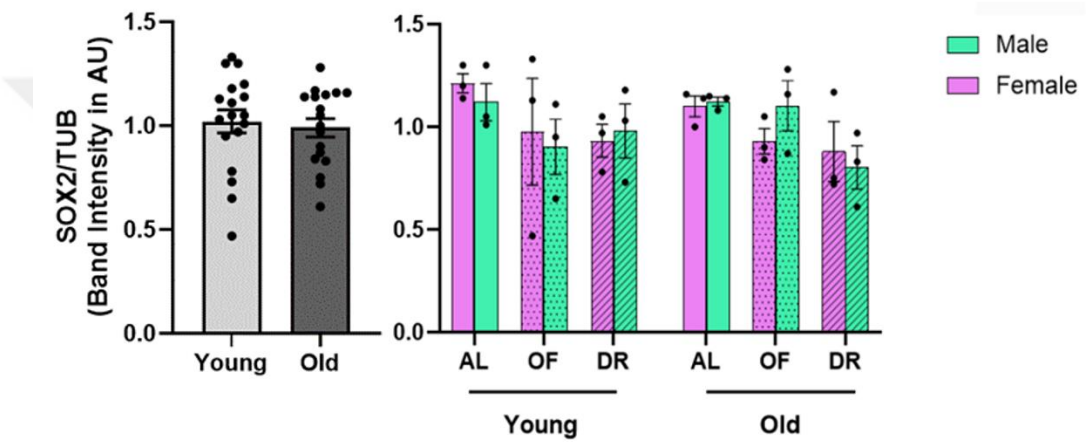

Figure 3.4 Sox2 expression across age, diet, and gender groups. Within gel and tubulin normalized data. AL: Ad-libitum; OF: overfeeding; DR: caloric restriction. The error bars represent mean ±1 SEM. n=3 for all groups.

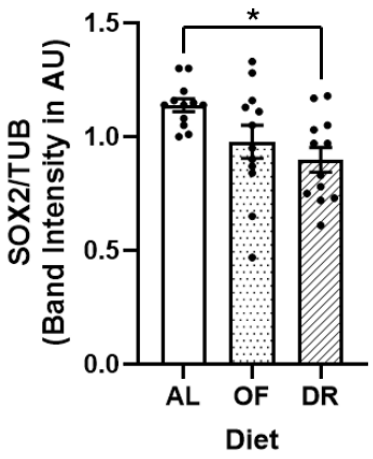

Figure 5 The overall effect of diet on Sox2 expression. Within gel and tubulin normalized data. A significant effect of diet was demonstrated on Sox2 levels. *: $p < 0.05$. AL: ad libitum; OF: overfeeding; DR: caloric restriction.

3.1.3 Further Correlational and Multivariate Analyses

To have an understanding of how neurogenesis is affected by the factors of age and diet, further analyses were conducted incorporating the previously obtained data of different neuronal markers DCAMKL1 and HuC and a proliferating cell marker PCNA with the data of the neural stem cell marker Sox2 from this thesis study. The Western blot experiments were performed, and analyses revealing the expression levels of the proteins were done in a previous study in our lab [95]**.**

To observe the overall change in neurogenesis, markers of neural stem cells (Sox2), cell proliferation (PCNA), neural progenitors (DCAMKL1), and post-mitotic neurons (HuC) were analyzed together to understand the correlations and clustering profiles for age and dietary status. An overall correlation was analyzed using Pearson correlation coefficient *r*, and significant correlations among the proteins were revealed. All age, diet, and gender groups were collapsed together to generate the correlation matrix, as shown in Figure 3.6.

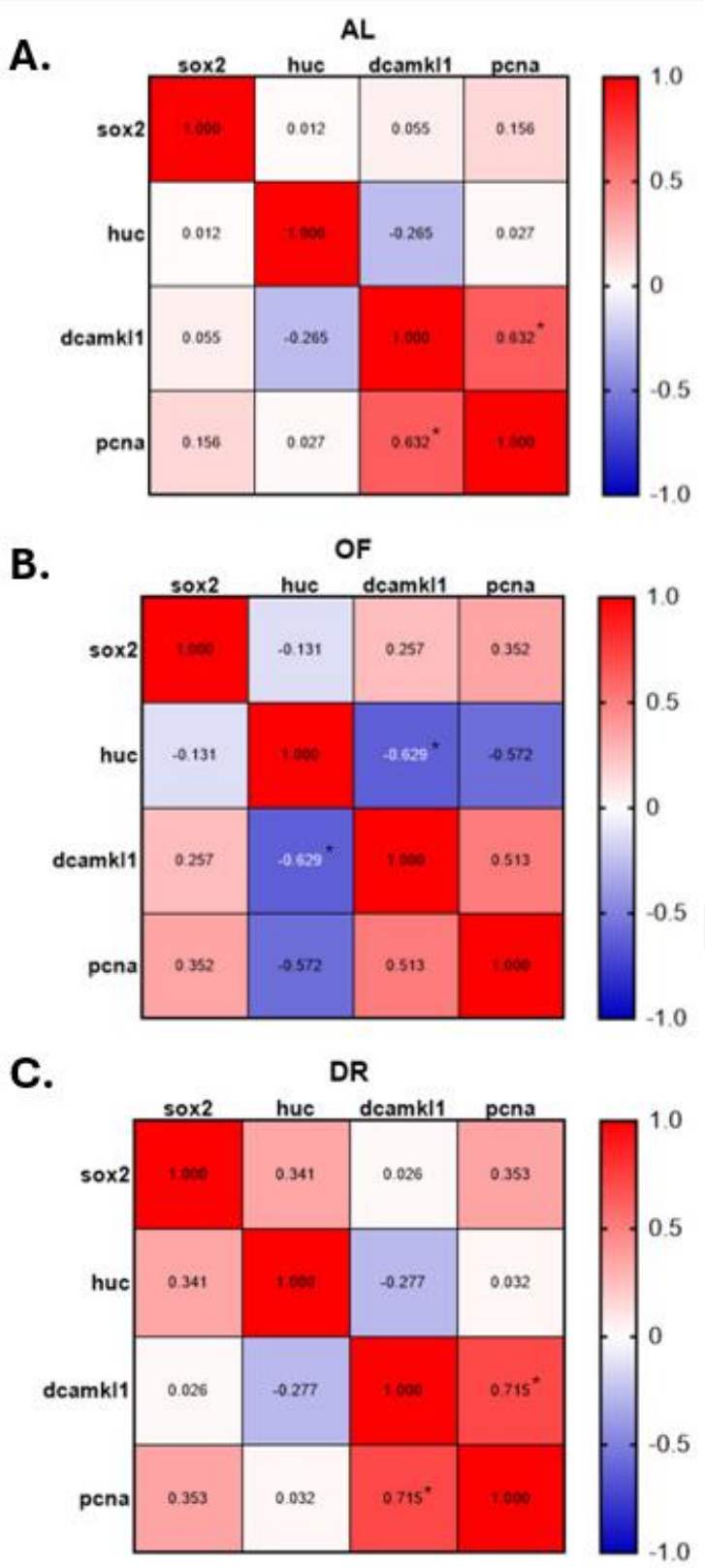

Figure 6 Analyses revealed significant correlations among the neuronal and cellular proteins. Correlational matrices were shown for A) AL: ad libitum, B) OF: overfeeding, and C) DR: dietary restriction animals. *: $p<0.05$.

DCAMKL, a neural progenitor marker, was significantly and positively correlated with PCNA, a global cell proliferation marker for AL ($r(10) = 0.632$, $p = 0.028$) and DR ($r(10) = 0.715$, $p = 0.009$) diet groups, Figure 3.6. According to the results for expression levels in the previous study, although there was no overall effect of diet in the expression of PCNA and DCAMKL1, the levels are numerically decreasing in the DR group compared to AL at a young age [95]. Thus, the positive correlation between these three proteins might be related to this similar pattern. On the other hand, DCAMKL1 was significantly and negatively correlated with a marker of post-mitotic neurons, HuC for OF ($r(10) = -0.629$, $p = 0.028$), Figure 3.6. HuC levels were reported to be decreased with OF diet in the aged brain. In contrast, there was a significant increase in the DCAMKL1 levels [95]. This might explain the negative correlation between the two proteins.

A further multivariate analysis incorporating all neuronal and cellular proteins was performed using PCA. To determine the number of principal components to be extracted, the Kaiser criterion and Scree plot method were taken into account. Accordingly, two principal components were extracted. The first principal component (PC1) accounted for 49.35% of the total variance, Table 3.1. It was contributed largely by PCNA and DCAMKL1. The negative contribution by Sox2 was less prominent. Therefore, PC1 can be defined as being driven by the proliferating adult neural stem and progenitor cells. The second principal component (PC2) explained 27,22% of the variance, Table 3.1. It was mainly driven by HuC positively. Sox2 also had a positive contribution but was slightly weaker. Together, these two factors explained the 76.57% of the variance in the data.

Table 3.16 Factor loading scores for two principal components for neuronal and cellular proteins. In the second column, scores for PC1 were shown. PC1 was altered negatively by DCAMKL1, PCNA, and Sox2. In the third column, scores for PC2 were shown. PC2 was contributed by HuC and Sox2 positively.

Markers	PC1 (49.35%)	PC2 (27.22%)
Sox2	-0.555	0.614
HuC	0.398	0.803
DCAMKL1	-0.867	-0.195
PCNA	-0.869	0.171

Both PC1 and PC2 were visualized for each animal separately by indicating the age and diet groups, as shown in Figure 3.7. Age-specific changes in the PC2 scores of OF and DR were observed in the clustering profiles. The old group had lower PC2 scores than the young group, Figure 3.7. Similarly, HuC levels of the OF and DR groups significantly declined with older age [95]. Furthermore, age-specific changes in the PC1 scores of OF were observed. In the aged brain, PC1 scores decreased in the OF group, Figure 3.7. Related to that, it was shown that the OF group is showing a significant increase in DCAMKL1 levels in the aged brain [95].

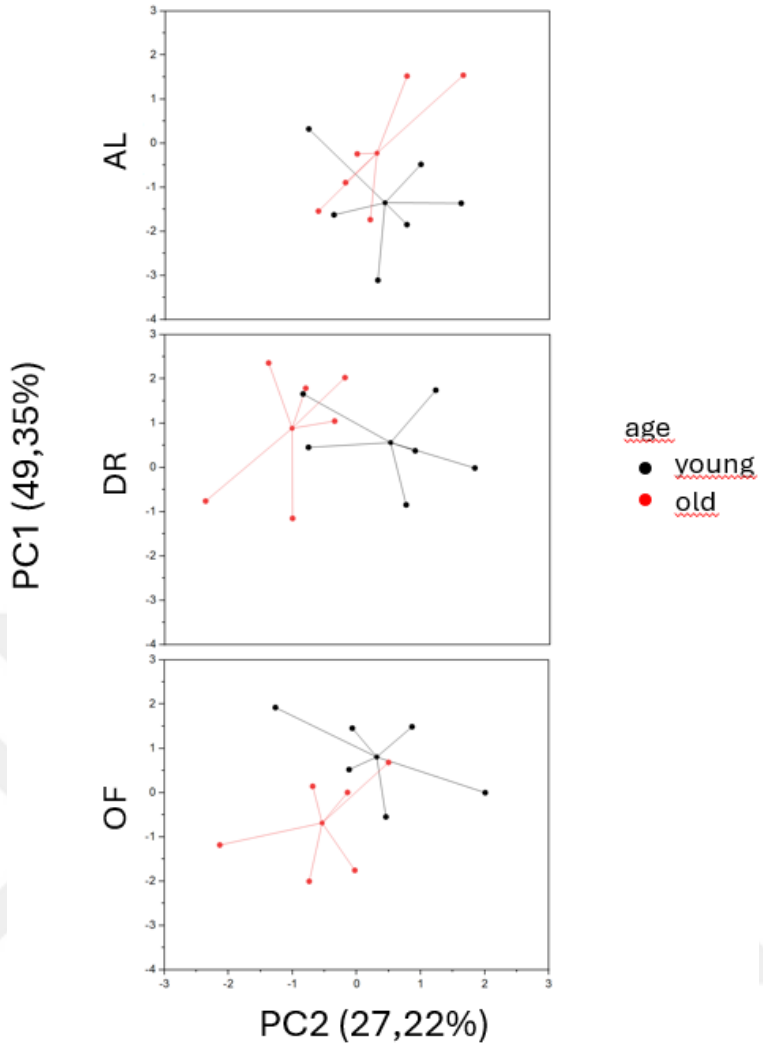

Figure 3.7 Clustering profiles of components extracted from protein expression data of neuronal and cellular proteins. Diet groups were shown in separate panels, while age groups were shown with different colored dots. Young: Black, Old:Red

3.2 Purified His-tagged Sox2 Protein Concentration Results

His-tagged Sox2 protein that was expressed in *E. coli* was purified using IMAC. Using a Ni-NTA agarose resin column, the protein was collected in an elution buffer containing urea, with a pH value adjusted accordingly. 25 ml elution buffer was transferred through the column in total and the buffer containing the purified protein was collected in 2 ml Eppendorf tubes. There were 10 tubes in total. The measurements were obtained for each tube respectively using a fluorometer, and OD values at 280 nm were measured, indicating the presence of protein (Table 3.2).

Table 3.17 Concentration results for purified His-tagged Sox2 protein

Tube Numbers	**OD_{280} values (µg/ml)**
1	37.3
2	47.3
3	21.6
4	19.7
5	13.9
6	2.6
7	3.7
8	11.2
9	3.6
10	2.4

CHAPTER 4

DISCUSSION

4.1 The Effects of Dietary Treatments on Sox2 and Other Markers of Neurogenesis in the Aging Brain

Due to molecular and cellular deterioration over time, aging can lead to detrimental effects on biological functions. The decline in nervous system-related functions is a risk factor for many age-related diseases. Adult neurogenesis was accepted to be present throughout life in mammals, but it gradually diminishes over time due to age-related impairments in brain function. Some argue that the proliferation of neural stem cells decreases with age. The exact mechanism of how it is affected is not solved since various intrinsic and extrinsic factors regulate the neurogenic niche. Furthermore, these factors can also be affected by changing environmental conditions. Nutrient intake plays a crucial role in the lifespan and healthy aging of an organism. Moreover, it has a rejuvenating effect on age-related cognitive decline. It has been observed that the changing nutrient levels also impact the modulation of neurogenesis.

Thus, the first objective of this thesis work was to observe the neural stem cell and neurogenesis levels due to dietary intake in the aging brain. For this objective, the zebrafish brain was used as a model to observe age-related neurogenesis alterations. The study investigated whether or not different feeding regimes affected neurogenesis during aging. To understand this, AL, DR, and OF diets were applied for a short-term 12-week period to both young and old animal groups. The effect of gender was also considered by including both females and males in these groups. At the end of the feeding experiment, whole brain tissues were acquired, and the western blot analysis was conducted on tissue homogenates. The main protein of interest was Sox2, a cellular marker for detecting neural stem cell identity. First, alterations of the Sox2 expression levels were investigated due to gender, age, and diet factors. Secondly, the dynamics between Sox2 levels and other neuronal and cellular markers were investigated by performing a correlational analysis. PCNA, a marker for proliferating cells, was included in this correlation to understand the changes in proliferation along with the changes observed in the NSCs. DCAMKL1,

a marker for migrating neuroblasts, was included to observe the changes in neuronal commitment. Lastly, HuC, a marker for early post-mitotic neurons, was added to see the changes in the newborn neuron production. Next, a multivariate analysis was performed, gathering together all these markers that are important for detecting the changes in different stages of neurogenesis. It allowed for the detection of the changing dynamics of neurogenesis, starting from the NSCs to the generation of new neurons.

According to the results, Sox2 protein levels did not change significantly due to gender. A study demonstrated that females and males have differing proliferating cell levels depending on the brain area [98]**.** From this, it can be inferred that different subregions can have opposing sex-dependent differences in NSC activity. In our experiment, protein samples were acquired from the whole brains of the adult zebrafish. Thus, the reason that there were no sex-specific changes in our results might be due to observing the brain as a whole, which might not be suitable for detecting these differences.

Secondly, Sox2 protein levels did not show any significant change due to age in our study. Due to old age, a decline in the neural stem cell marker Sox2 was observed in many tissues, including the brain, according to previous studies in humans and rodents [68]**.** In the telencephalon of zebrafish, neurogenesis decreases with old age [99]. Nevertheless, zebrafish display a neurogenic ability that continues throughout life, so age-related changes might not be as easily detected as those of mammals. Moreover, even if there are changes in the Sox2 protein levels with age, investigating the changes in the protein levels by examining all brain regions together can result in a failure to identify the prominent age-related changes in the specific neurogenic regions in the brain. Furthermore, in some cases, aging-associated pathologies in the brain might show a compensatory mechanism that results in higher levels of NSC activity, as demonstrated by a study with non-demented Alzheimer's pathology patients. They showed a higher number of Sox2+ cells in their brain than the healthy individuals [100]**.** Thus, it can be interpreted that age-related decline in the Sox2 expression might not always observed.

Another explanation for the lack of decrease in Sox2 in our results can be the age-related decline in active NSCs rather than the decline in the overall NSC pool. According to some studies, age-related changes cause a depletion in the NSC pool by elevating the quiescence. An immunostaining study on aged zebrafish showed that diminished neurogenesis is due to the increased quiescence of RGL cells. The proportion of RGL cells that get activated and start dividing decreases, thereby reducing the overall number of neurons produced [101]. Sox2 is expressed in both quiescent and activated NSCs. So, the Sox2 expression alone makes it impossible to conclude this. According to a previous study, the number of Sox2+ positive stem cells remained stable during aging in rodents, but quiescence increased, leading to a reduction in proliferation [80]**.** Thus, having an increased quiescence in NSCs might explain the lack of any significant change in the Sox2 levels in our experiment.

Several studies argued that the survival of newborn neurons is reduced with age, as opposed to a change in NSC proliferation [102], [103]. In agreement with this, lower HuC expression levels were previously reported, while no significant change in the DCAMKL1 levels was observed for the old animal group of this study [95]. In addition, correlation analyses performed in this thesis study showed a negative relationship between DCAMKL1 and HuC for OF, which tells us that although there is no alteration in the neuronal fate commitment, newborn neurons decrease. This indicates that the survival rate of newborn neurons is affected by aging.

This thesis study also showed that a short-term diet significantly affects Sox2 expression. According to previous studies, a short-term dietary intervention was enough to alter hippocampal neurogenesis and cell proliferation in rodents [104], [105]. Thus, this result was consistent with the findings. DR was observed to cause a significant decline in Sox2 levels compared to AL, indicating that low-calorie intake resulted in a decrease in neural stem cell identity. While high Sox2 levels are correlated with the self-renewal of NSCs, low Sox2 levels are correlated with the differentiation of NSCs to neurons. Sox2 decrease leads to cell cycle exit, and cells start to express early neuronal differentiation markers [106]. Research findings show that caloric restriction is followed by the activated expression of genes associated with neurogenesis [107]. Thus, lower levels of Sox2 might mean that the

expression of differentiation-associated genes is increased and differentiation is promoted. In addition to that, the enhancement of neurogenesis can be achieved through altering various stages that cannot be explained by the levels of Sox2. For instance, it can lead to the transition of NSCs from quiescent to activated state, an increase in the proliferation of neural progenitors, an increase in migration rates, or an increase in the survival rate of newborn neurons. In support of this, the DR diet was shown to affect neurogenesis by regulating the survival rate of new neurons [108]. Furthermore, investigating the Sox2 levels in the brain helps to identify the NSC pool, but it does not give information about the quiescence or dividing activity of these cells. Thus, calorie restriction is thought to promote quiescence to inhibit stem cell exhaustion through the regulation of nutrient-sensing pathways [80]**.** The previous results for the animals used in this thesis study showed that diet did not cause any overall significant changes in the PCNA, DCAMKL1, or HuC levels**.** This tells us that proliferative activity and new neuron formation did not significantly change. From this, it can be argued that the balance between self-renewal and differentiation is disrupted, as shown by the decreased Sox2 levels with the DR diet. However, this is not a definitive result for concluding that neurogenesis is significantly affected by DR.

On top of all these, brain and body growth continues throughout the life of zebrafish, unlike mammals. The amount of food intake becomes crucial in this case, and changes that might restrict the organism's growth might also affect brain growth by negatively affecting the production of new neurons [41] In relation to this, changes in the body parameters of the animals used in this thesis study were reported previously. Diet was shown to have an overall significant effect on BMI values. Moreover, DR animals had lower BMI compared to AL animals [95]. This result was consistent with other studies showing a decreased body weight due to DR diet [109], [110]. These can explain the decline in the Sox2 expression resulting from DR.

The next finding of this thesis study is that the OF diet did not cause any significant change in the Sox2 expression compared to AL. This is an interesting result since, in an HFD-induced obesity mice model, Sox2 was observed to be reduced [111]. It should also be considered that different neural stem cell populations can react

differently to external interventions due to their unique niche. A study demonstrated that a high-fat diet prevented neurogenesis in hypothalamic parenchyma, whereas a second study stated neurogenesis is elevated in the median eminence [112], [113]. This might show that specific areas in the brain can be more vulnerable to changes in nutritional intake. Due to this, our results might not show any significant change due to high-calorie intake. Moreover, previous results for the animals of this thesis study displayed opposing effects of the OF diet in old animals. DCAMKL1 levels were enhanced while HuC levels were dropped [95]. The correlational analysis also showed a negative relationship between these two proteins. This result suggests that a high nutrient intake somehow elevated the neuronal fate committed progenitors in the brain but negatively affected the survival of newborn neurons.

As a final analysis, the multivariate analysis combining all four markers, Sox2, PCNA, DCAMKL1, and HuC, was conducted using PCA. Two principal components were identified, explaining 76,57% of the variance in the data. Negative contributions from PCNA and DCAKML1 were most apparent for PC1. A decrease was observed with age in the OF group, which correlates with the previous findings showing the increase in DCAMKL1 with age in the OF group. This could mean that the OF diet had a more beneficial effect on neuronal commitment at older ages. For PC2, the main positive contribution is observed to be coming from HuC. The old group showed decreased PC2 scores than the young group for OF and DR diets. HuC was previously displayed to have decreased levels with age for diet groups OF and DR. From this, it can be commented that dietary interventions have age-specific effects on the formation of new neurons. DR and OF were less impactful in the old group for the improvement of neurogenesis.

4.2 His-tagged Sox2 Protein Concentration Results

In the nervous system, Sox2 is essential for preserving the stem cell identity. Its role also can be observed in various organs in the body, maintaining the self-renewal of tissue-specific adult stem cells [114]. In addition to that, it also functions as an important factor for maintaining the stem cell-like properties of cancer stem cells, promoting their migration and growth [115]. Thus, in many cancer types, Sox2 overexpression is observed, which makes it suitable to be used as a cancer

biomarker for detecting cancer stem cells and tumor progression in cancer patients [116].

Accordingly, as the second aim of this thesis, His-tagged Sox2 protein was overexpressed in E. coli and purified using Ni^{+2}-affinity chromatography to be used in the project involving the development of a biosensor for the detection of lung carcinoma. In cancer patients, elevated antibody responses were observed for Sox2, which allows for using a biosensor coated with Sox2 protein to detect Sox2 antibodies in a serum solution [117]. Recombinant proteins can be purified due to the high affinity of its Histidine-tag with Ni-NTA agarose beads. The presence of a His-tag does not inhibit the function of the protein. Then, the protein bound to the Ni-NTA agarose can be easily eluted using an elution buffer [118]**.** The concentration of the purified protein was then measured using a fluorometer. This method makes producing a high yield of protein with high purity possible by following a simple and low-cost procedure.

Contrasting to the heightened stem-cell activity and Sox2 expression in cancer, a significant decline is observed in the number and function of stem cells and the Sox2 levels during aging caused by the impairments in tissue function [119]. The loss of stem cells is considered to be one of the hallmarks of aging playing a vital role in the cellular damage accumulating in the body with increasing age [120]. Moreover, during aging, reduced levels of Sox2 expression were detected in the blood samples acquired from older individuals. Through this finding, it is proposed that Sox2 can also be used as a biomarker for aging [57]. Thus, using the recombinant Sox2 protein purified for the cancer biosensor can also potentially be utilized for the detection of aging and the risk of developing age-related diseases. In our study, it was demonstrated that aging did not cause a significant change in the Sox2 expression levels in the brain. Thus, this result challenges the assumption that Sox2 would be an accurate and reliable biomarker for aging and aging-associated deficits. Alternative biomarkers should be studied that might give a better insight into detecting age-related changes in the brain.

CHAPTER 5

CONCLUSION AND FUTURE PROSPECTS

In the first part of the study, the changing Sox2 protein levels marker with increasing age was shown. It was reported that old age did not alter the Sox2, which indicates there was no change in neural stem cell maintenance. Furthermore, nutrient intake was utilized as an environmental intervention strategy to alter the cellular processes in the aging brain. The differing effects of high and low-calorie intake on Sox2 levels were observed. A short-term low-calorie diet negatively altered the Sox2 levels, while a short-term high-calorie diet had no significant impact. The significant decline in the Sox2 levels was thought to be associated with a decrease in the self-renewal capability of NSCs and the promotion of differentiation into neurons.

The correlational and multivariate analyses with cellular proliferation and neuronal lineage markers revealed the dynamic between the changing neural stem cell behavior and neurogenesis. According to correlational analysis, DCAMKL1, a marker for neuroblasts committed to a neuronal fate, had a positive correlation with PCNA for the diet groups AL and DR. However, DCAMKL1 was negatively correlated with HuC, a newborn neuron marker for OF animals. These results revealed that changes occurring at different stages of neuronal lineage are in close association and affected similarly by the regulations due to aging and diet. However, the negative correlation between DCAMKL1 and HuC indicates that even though the commitment to a neuronal fate increases, it does not necessarily lead to an increase in newborn neurons. Moreover, PC analysis revealed two components. PC1 is negatively driven by PCNA and DCAMKL1 and, to a lesser extent, by Sox2. PC2 is positively driven by HuC and slightly weaker by Sox2. These components were shown to have been affected in an age-dependent manner by OF and DR diet.

In this study, western blot experiments were carried out, allowing for the observation of protein expression changes. However, it is not a highly accurate method for quantification of the protein levels in the brain. Also, it does not provide

a clear image of the presence of specific cell types and their changing dynamics in the context of neurogenesis. Methods that can more accurately identify NSCs and their differentiation can be utilized in the future. Techniques that allow detection at the single-cell level and observation of neuronal differentiation in real-time, such as cell-based biosensors, can be used [121]. Voxel-based morphometry and 3d immunohistochemistry techniques such as CLARITY can provide high-resolution visualization of the brain so that changes in neurogenesis between different regions can be compared [122]. Also, with single-cell RNA sequencing (snRNA-seq) and fluorescence-activated cell sorting (FACS), cell populations can be identified, and age-related changes in the production of new neurons can be quantified [123], [124]. To investigate whether the quiescence of NSCs is affected by age, markers that specify the quiescent state can be used [125]. For instance, c-Myc is not expressed by quiescent NSCs but is found in activated NSCs, so it might be a valuable marker for future studies [126]. Since Sox2 is not strictly found in NSCs but also in some mature neurons and glia, performing immunohistochemistry and double staining with Sox2 together with some other stem cell markers, such as Nestin, can be appropriate for accurately detecting NSCs [111]

This study provided important information on the specific alterations in the neuronal lineage during neurogenesis with respect to diet. Future studies investigating how the duration of the diet impacts neurogenesis can also be useful. A previous study shows significant changes in the miRNA profile of mice fed with CR diet from 10 weeks to 82 weeks old [127]. Therefore, adding a long-term diet group or a life-long diet group can allow us to understand the long-term alterations in the mechanisms related to age-associated neurogenesis. However, it is not sufficient to understand the exact mechanisms of how nutrition affects neurogenesis. In the future, specific nutrient-sensing pathways in the brain can be targeted, and their activation/inhibition can be analyzed. Furthermore, an experimental design can be developed by combining the effect of diet on markers of neurogenesis in the aging brain with behavioral studies that test cognitive skills such as learning and memory. In this way, we can draw a relation between cognitive decline and age-related changes in neurogenesis and observe the improvements in cognitive performance achieved by the feeding regime. Moreover,

a study presents that DR leads to the inhibition of astrocyte formation [128], and another study demonstrates that a high-fat, high-sugar diet causes the shortening of the astroglial processes [129]. These results might indicate a changing dynamics in the differentiation of NSCs into glial cells, and future studies focusing on gliogenesis can shed light on this. On top of this, according to a finding, applying a high-fat choline-deficient diet on mice showed an alteration in gut microbiome production of short-chain fatty acids that play a role in fatty acid and glucose metabolism [130]. Thus, future studies focusing on the effect of diet on the gut microbiome-brain axis might provide insight into understanding the mechanism by which nutrition affects brain function.

In the second part of the study, the *E. coli* expression system was utilized using plasmid vectors to express His-tagged Sox2 protein, followed by protein purification using metal ion affinity chromatography. However, the efficiency of the method and the resulting protein concentrations can be increased through some improvements. Using Ni-Sepharose High-Performance resin can provide better binding of Ni^{+2} ions to the His-tag [131]. Moreover, the nonspecific bindings from the residual host proteins can prevent the target protein from efficiently binding to the metal ion. For this, increasing the centrifugation to get rid of the unwanted bacterial substances or changing the pH level or concentration of salts in the washing buffer can be helpful. In addition to that, there can be some limitations of this purification method. For instance, the low-pH level of the elution buffer may create an issue by altering the function of the target protein or causing denaturation [132] The presence of the target protein in the buffer can be further confirmed with SDS-PAGE analysis. Aside from this, there is a possibility that a small number of metal ions might also be eluted with the target protein, which causes a risk since Ni^{+2} used in the chromatography can be a carcinogen. Additional steps are often performed to increase the purity of the protein, such as ion-exchange chromatography [132]. As a future direction, his-tagged protein expression using zebrafish as a host might be investigated. For this aim, the microinjection method utilizing glass capillary needles can be used. In this way, target DNA, RNA or antibodies can be introduced into the cytoplasm of zebrafish embryos [133]. mRNA encoding the his-tagged protein can be used to inject into the embryo then the his-

taged Sox2 protein can be overexpressed in zebrafish. Optimizing this method might allow us to better understand the role of Sox2 protein during age-related changes in zebrafish. Protein can be detected through its his-tag and it can later be manipulated or altered to observe the effects.

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