IMPACT OF THE INFLAMMATORY PROCESS IN THE AGING BRAIN: EVIDENCE FROM *IN VITRO* AND *EX VIVO* MODELS

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Serena Sevdiye Aktürk

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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.

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ABSTRACT

IMPACT OF THE INFLAMMATORY PROCESS IN THE AGING BRAIN: EVIDENCE FROM *IN VITRO* AND *EX VIVO* MODELS

Serena Sevdiye Aktürk

M.Sc. in Neuroscience

Advisor: Michelle Marie Adams

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Aging is a complex and dynamic process that is characterized by a gradual decline over time in the physiological integrity of organisms. Several cellular mechanisms contribute to aging, including telomere shortening, damage accumulation in DNA, disabled macroautophagy, mitochondrial dysfunction, and cellular senescence. These processes, consecutively, lead to impaired cellular function, declined tissue repair, and stem cell exhaustion and are seen in the development of neurodegenerative disorders and healthy aging. One of the hallmarks of brain aging is the altered chronic inflammatory status of the brain. The over-activation and polarization of microglia, increased secretion of pro-inflammatory cytokines and reactive oxygen species, inflammasome activation, and the upregulation of the NF- κB signaling pathway are among the markers of neuroinflammation. This mechanism's anticipated effects include dysregulated nutrition sensing via the mTOR (mammalian target of rapamycin) pathway, decreased neurogenesis, and synaptic integrity over time. Another element that contributes to vulnerability to inflammation is genetic predisposition. Hence, additional research endeavors are required to investigate the influence of dietary interventions and therapeutic modalities targeting inflammation on genetic pathways.

Thus, this study aimed to understand how inflammation can be triggered on different models, investigate potential inflammation-related biomarkers with meta-analysis and observe the effect of inflammation for both zebrafish primary brain cells and murine microglial cells. We conducted short-term copper sulfate treatments on both models for this objective. Moreover, to examine the effects of intermittent fasting, an mTOR downregulator, and high-fat diet, an inflammation inducer, on the brain of zebrafish at the molecular level by primary cell culture method. Finally, we applied rapamycin+DMSO treatment to primary cells to assess the possibility of reversing the progression of inflammation. The results showed that copper sulfate is an efficient oxidative stress-induced inflammatory reagent for zebrafish; however, it did not cause a direct inflammatory response in murine microglial cells. For zebrafish, in the copper sulfate+DMSO treated group, age affected Nrf2a mRNA, altering oxidative stress in old animals. Regardless of diet and treatment group, inflammation markers were higher in old animals, which underscores the association between aging and chronic inflammation. Elevated Lc3b levels in young and old animals captured that high copper concentrations can trigger autophagy. Results for neurogenesis markers revealed that overfeeding or acute inflammation could contribute to compromised neurogenesis in advanced stages of life. On the contrary, the enhanced neurogenesis potential of intermittent fasting in old animals was revealed.

In conclusion, this study has demonstrated that the modulation of neuroinflammatory responses, as well as oxidative stress, neurogenesis, and autophagy, occurs in an age-related manner. Moreover, dietary or pharmaceutical interventions could yield comprehensive outcomes in perceiving the brain's neuroinflammatory profile during aging.

Keywords: zebrafish, neuroinflammation, oxidative stress, neural stem cells, mTOR signaling

ÖZET

YAŞLANAN BEYİNDE İNFLAMASYON SÜRECİNİN ETKİSİ: *IN VITRO* VE *EX VIVO* MODELLERİNDEN ELDE EDİLEN KANITLAR

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Yaşlanma, organizmaların fizyolojik bütünlüğünde zamana bağlı ve kademeli bir düşüş ile karakterize edilen karmaşık ve dinamik bir süreçtir. Telomer yıpranması, DNA'da hasar birikmesi, bozulmuş makrootofaji, mitokondriyal işlev bozukluğu ve hücresel yaşlanma dahil olmak üzere çeşitli hücresel mekanizmalar yaşlanmanın ana bileşenleridir. Bu süreçler sırasıyla hücresel fonksiyonun bozulmasına, doku onarımının azalmasına ve kök hücre tükenmesine yol açar ve hem nörodejeneratif bozuklukların gelişiminde hem de sağlıklı yaşlanmada görülür. Beyin yaşlanmasının ayırt edici özelliklerinden biri, beynin değişen kronik inflamatuar durumudur. Mikroglia'nın aşırı aktivasyonu ve polarizasyonu, proinflamatuar sitokinlerin ve reaktif oksijen türlerinin artan salgılanması ve NF-κB sinyal yolunun yukarı regülasyonu nöroinflamasyonun belirteçleri arasındadır. Bu mekanizmanın beklenen etkileri arasında mTOR (Rapamisin protein kompleksinin memeli hedefi) yoluyla düzensiz besin algılaması, azalmış nörogenez ve sinaptik bütünlüğün zamanla azalması yer alır. İnflamasyona karşı savunmasızlığa katkıda bulunan bir başka unsur da genetik yatkınlıktır. Bu nedenle, inflamasyonu hedefleyen diyet müdahalelerinin ve terapötik modalitelerin genetik yollar üzerindeki etkisini araştırmak için ek araştırma çabalarına ihtiyaç vardır.

Bu nedenle, bu çalışma inflamasyonun farklı modellerde nasıl tetiklenebileceğini anlamayı, inflamasyonla ilgili potansiyel biyobelirteçleri meta-analiz ile araştırmayı ve inflamasyonun hem zebra balığı primer beyin hücreleri hem de murin mikroglial hücreler için etkisini gözlemlemeyi amaçladı. Bu amaçla her iki modele de kısa süreli bakır sülfat uygulamaları yaptık. Ayrıca, bir mTOR aşağı regülatörü olan aralıklı oruç ve bir inflamasyon indükleyicisi olan yüksek yağlı diyetin zebra balığı beyni üzerindeki etkilerini primer hücre kültürü yöntemiyle moleküler düzeyde inceledik. Son olarak, inflamasyonun ilerlemesini tersine çevirme olasılığını değerlendirmek için primer hücrelere rapamisin tedavisi uyguladık. Sonuçlar, bakır sülfatın zebra balığı için etkili bir oksidatif stres kaynaklı inflamatuar indükleyici olduğunu göstermiştir; bununla birlikte, murin mikroglial hücrelerinde doğrudan bir inflamatuar yanıta neden olmamıştır. Zebra balığı bakır sülfat+DMSO tedavisi grubu, yaşın hayvanlarda oksidatif stresi etkileyerek Nrf2a mRNA seviyesini arttırdığını ortaya koydu. Diyet ve tedavi grubundan bağımsız olarak, yaşlı hayvanlarda inflamasyon belirteçleri daha yüksekti ve bu da yaşlanma ile kronik inflamasyon arasındaki güçlü ilişkiyi vurgulamaktadır. Genç ve yaşlı hayvanlarda yüksek Lc3b seviyesi, yüksek bakır konsantrasyonlarının otofajiyi tetikleyebileceğini ortaya koymaktadır. Nörogenez belirteçleri için sonuçlar, aşırı besleme veya akut inflamasyonun, yaşamın ileri evrelerinde azalan nörogeneze katkıda bulunabileceğini ortaya çıkarmıştır. Aksine, aralıklı orucun ise yaşlanmada nörojenez potansiyelini geliştirdiği ortaya çıktı. Sonuç olarak, bu çalışma, oksidatif stres, nörojenez ve otofajinin yanı sıra nöroinflamatuar yanıtların modülasyonunun yaşa bağlı bir şekilde meydana geldiğini göstermiştir. Ayrıca, diyet veya farmasötik müdahaleler, yaşlanma sırasında beynin nöroinflamatuar profilinin algılanmasında kapsamlı sonuçlar sağlayacaktır.

Anahtar Kelimeler: zebra balığı, nöroinflamasyon, oksidatif stres, nöral kök hücreler, mTOR sinyali

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Contents

List of Figures

List of Tables

CHAPTER 1

1 INTRODUCTION

1.1. Aging

The concept of aging can be portrayed as a relatively new problem in historical integrity. The elderly population is defined as 65 years and older, and the proportion of the elderly population to the total population has increased from 5.30% in the 1970s to 9.63% in 2021 [1]. It is projected that the elderly population will grow exponentially by 2050 and could reach 38% of the total population.[2] Also, an aging population is the most critical medical and social demographic problem globally.[3] For this reason, The United Nations has declared the Decade of Healthy Aging between 2021 and 2030. Meanwhile, the scientific community's contribution to researching the causes of aging is significant. Despite the combined efforts of modern science, the causes of aging and potential interventions to slow it down have yet to be thoroughly examined. To develop current therapeutic approaches, it is crucial to comprehend the signs of aging and how they affect brain function and general health. This strategy would produce practical answers for the aging population by extending people's lives and enhancing their social involvement and integration.

From a biological perspective, aging is a complex and dynamic process that is characterized by a gradual decline over time in the physiological integrity of organisms.[4] Several cellular mechanisms contribute to aging, including telomere shortening, damage accumulation in DNA, mitochondrial dysfunction, and cellular senescence. These processes, consecutively, lead to impaired cellular function, declined tissue repair, and increased predisposition to age-related diseases such as cardiovascular disease, hypertension, Alzheimer’s disease (AD), and Parkinson’s disease (PD).[5]

1.2. Brain Aging

Brain aging occurs gradually in living organisms' brains and central nervous systems. Many physiological, structural, functional, and molecular changes can occur during brain aging. Although these changes usually occur with advancing age, their accumulation can begin at early ages. [6] Brain aging occurs at different severity and rate in people. This difference is because it is a highly dynamic process that is connected and interactive with various factors. [7]

1.2.1 Structural Changes During Brain Aging

Many structural changes can be observed during brain aging. One of them being synapse loss. However, synapse loss can be classified more as a region-specific synapse loss rather than a global loss.[8] For instance, in the dentate gyrus of the hippocampus.[9] Functional changes in synapses are as important as loss of synapses. By disruptions in the synaptic integrity, several functions such as synaptic signaling, synaptic plasticity, and synapse formation could be impaired.[10] Changes in synaptic transmission and decreased number and maturity of spines in aged organisms may reflect aberrant neuronal plasticity, which could be directly related to impaired brain functions. Also, a mark of the aged brain is the increased level of neuroinflammation produced by glial cells, which may contribute to changes in neuronal/synaptic function. [11]

Decreased blood flow in the brain is another structural change associated with aging. Brains depend on blood flow, which delivers oxygen and nutrients necessary for proper neuronal and synaptic functioning. [12] Changes in pressure and flow of the blood, together with inflammatory processes, could contribute to age-related modifications in the jugular veins, which furthermore increases the age-related progression of venous malfunction. [13]

1.2.2 Cellular and Molecular Alterations During Brain Aging

Numerous differentiations could also be observed at the cellular and molecular levels during aging, one of them being neuronal changes. Expanding synaptic connections is the mechanism to compensate for the loss of neurons in the brain. With pathological conditions, the brain loses synaptic contacts; therefore, compensation for the loss of neurons is getting more complex. [14] However, neuronal loss that occurs in such a significant way is not generally expected in healthy brain aging but is rather observed in pathological conditions. [15] Therefore, in the absence of pathological conditions, the separation and identification of features seen in aging are precious.

1.3 Hallmarks of the Aging

Brain aging is a highly complex and complicated process due to its multifactorial nature. Besides the ones discussed above, many common denominators of aging have been identified. During the time concept of hallmarks of aging was introduced, 9 universal hallmarks were nominated. [4][16] Among these were; genomic instability, telomere attrition, epigenetic modifications, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. The common point of these hallmarks is that they comply with the following three criteria; firstly, their demonstration should be age-associated. Secondly, by experimentally accentuating them, one should accelerate aging; and lastly, therapeutic interventions should either decelerate, stop, or reverse aging.[7] However, in the last decade, in order to keep up with the cumulatively increasing data on aging biology and developing new technologies, 3 new hallmarks were added in 2023; disabled macroautophagy, chronic inflammation, and dysbiosis.[7] These 12 strongly related hallmarks were classified into three categories.

1.3.1 Primary Hallmarks of Aging

1.3.1.2 Genomic Instability

Genomic integrity is endangered in 2 different mechanisms [7]. The first is the endogenous mechanisms, including DNA replication errors, telomere erosion, epimutations, chromosome structure and number alterations, and oxidative processes that all contribute to age-related decreases in genomic function.[17] The second mechanism is the exogenous mechanism. Examples include physical, chemical, or biological agents. DNA repair mechanisms developed to fight against these mechanisms have been shown to weaken with age, resulting in genomic mosaicism.[18]

1.3.1.3 Telomere Attrition

Telomeres are repetitive DNA sequences that are found towards the ends of chromosomes, and they support maintaining the stability and integrity of chromosomes. [19] During each cell division, telomeres get shortened, affecting the gene expression in subtelomeric regions, and this has been associated with aging. [20] Telomere attrition could lead to dysfunctional cellular changes, blockage of cell division, as well as interference with tissue renewal. Telomerase is an enzyme that helps the lengthening of the telomeres. Telomerase activation can prolong the cell life cycle and division ability by lengthening telomeres. This could help prevent or delay cell aging and age-related diseases.[21]

1.3.1.4. Epigenetic Alterations

Epigenetic alterations are modifications in the physical structure of the DNA molecule that influence gene expression. These alterations create changes in the expression level of genes. The epigenome is directly related to environmental factors and has also been shown to have an impact during aging.[22] These alterations can lead to changes and deterioration of age-related cellular functions and also increase the risk of several diseases. For example, some diseases like cancer have been associated with epigenetic changes.[23] Examples of critical epigenetic alterations include DNA methylation, histone modifications, and non-coding RNAs (ncRNAs).[24] Epigenetic modifications indicate potential therapeutic targets regarding aging processes.

1.3.1.5. Loss of Proteostasis

Homeostasis of protein metabolism, or proteostasis, is the total of the mechanisms that regulate the correct production, folding, processing, transport, and destruction of proteins in cells.[25] With aging, the capability of cells to fold and process proteins adequately becomes atrophied.[25] This can lead to the accumulation of misfolded or degraded proteins and the formation of toxic protein aggregates that adversely affect cell health, as is also known from Alzheimer's disease tau pathology.[26] The degradation of autophagic processes may be involved in the aging process due to protein accumulation in cells. Nutrition can also affect protein metabolism. For instance, limited calorie intake is shown to increase cell protein breakdown, while overfeeding can provoke toxic protein buildup. [27] Moreover, increased oxidative stress during the aging process can negatively affect the structure and function of proteins.[27]

1.3.1.6. Disabled macroautophagy

As a catabolic process, macroautophagy degrades lipid droplets and glycogen for cellular metabolism while degrading and recycling damaged proteins, organelles, and invading pathogens to maintain cell homeostasis. An age-related decline in macroautophagy represents one of the most critical mechanisms of reduced organelle turnover; therefore, it is a hallmark of aging. [7] Hence, enlightening the mechanisms underlying disabled macroautophagy and exploring possible approaches to restore autophagic activity to promote cellular health and potentially influence lifespan is essential.

Once the autophagy is initiated, the formation of double-membrane vesicles (autophagosomes) takes place. The autophagosomes elongate and sequester the cytoplasmic cargo. It then combines with the lysosome to degrade the cargo. Next, the cargo is degraded by lysosomal enzymes, and degradation products are recycled to the cytosol.[28] The entire process is named autophagy flux.[29] Yeast has been used in the majority of studies to elucidate the macroautophagy pathway. [30] Studies in yeast led to the finding of more than 40 autophagy-related proteins (Atg). [31] Homologs of these proteins have been preserved in higher eukaryotes. The proteins that are essential for autophagosome formation are called core molecular machinery. The core molecular machinery consists of; 1) Atg1/unc-51-like kinase (ULK) complex, 2) Atg12-conjugation system, 3) Atg8/microtubule-associated protein light chain 3 (LC3)-conjugation system, 4) the Vps34 phosphoinositide 3-kinase (PI3 kinase) complex, 5) the Atg9 and Atg2-Atg18 complex, and 6) vacuole-membrane-protein-1 (VMP1). [32] This core molecular machinery's Atg1/ULK complex controls macroautophagic activity by detecting shifts in concentrations of various molecules. Furthermore, this complex is known to be directly regulated by several kinases, such as TOR kinase complex 1 and AMP-activated protein kinase.[33]

1.3.1.6.1 LC3B

LC3B (Microtubule-associated protein 1A/1B-light chain 3B) is an RNA-binding protein that plays a vital role in macroautophagy. [34] The primary function of LC3B is to regulate the expansion of the membrane of autophagosomes. LC3B is triggered by a process called lipidation. [35] During the lipidation, a lipid molecule binds to the LC3B protein, and lipidated LC3B is a fundamental component required for the autophagosome membrane to enlarge and form a closed structure. [36] LC3B is also involved in the regulation of autophagic selective degradation. Intracellular targets such as damaged organelles or protein aggregates can be recognized by LC3B-binding receptor proteins, bound to autophagosomes, and guided for destruction. [37] Thus, LC3B is considered an essential component in the macroautophagy process. Macroautophagy is a critical mechanism for maintaining cell health, clearing the intracellular waste, maintaining balance, and LC3B plays a pivotal role in regulating this mechanism.

1.3.2. Integrative Hallmarks of Aging

1.3.2.1. Stem Cell Exhaustion

Stem cells are unspecialized cells that take part in forming different tissues of the body as well as taking part in their regeneration.[38] The main feature of stem cells is that they can replicate themselves by division and also have the capacity to differentiate into more specific cell types. They can exist during embryonic development and also during adult life. There are multiple identified types of stem cells. Examples of these are Pluripotent stem cells (PSCs), Embryonic Stem Cells (ESCs) (a type of PSCs), and induced pluripotent stem cells (iPSCs).[39] Furthermore, stem cells can be classified according to their differentiation ability, which decreases in spectrum starting from completely pluripotent cells such as ESCs and iPSCs and ending with less potency—multi-, oligo- or unipotent cells. [39]

Neural stem cells (NSCs) are stem cells that can differentiate into neurons and glial cells. NSCs are mainly found in neurogenic regions like the hippocampus and the subventricular zone in the brain. The hippocampus has a great deposit of NSCs. [40] A stem cell goes through some distinct stages on the way to becoming a fully mature astrocyte or neuron. [41] The type-1 stem cells, which resemble radial glia, divide to produce type-2 neural progenitor cells or daughter astrocytes. Based on the expression of glia-like vs. early neuronal markers, the type-2 stage has been split into type-2a and type-2b. Type-2a cells are abundant and are the primary beneficiaries of the running stimulus.[42] Doublecortin expression, a marker connected to the migrating neuroblast stage in neuron maturation, marks the beginning of type-2 b. After the Type-3 stage, the last proliferative stage, the cell forms firstly an immature and later a mature neuron, shown in Figure 1.1 It is possible to maintain adult NSCs in cell culture, considering they keep proliferating in the presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF). [43] Neurospheres can form in cell culture, and NSCs can differentiate into neurons, astrocytes, and oligodendrocytes.

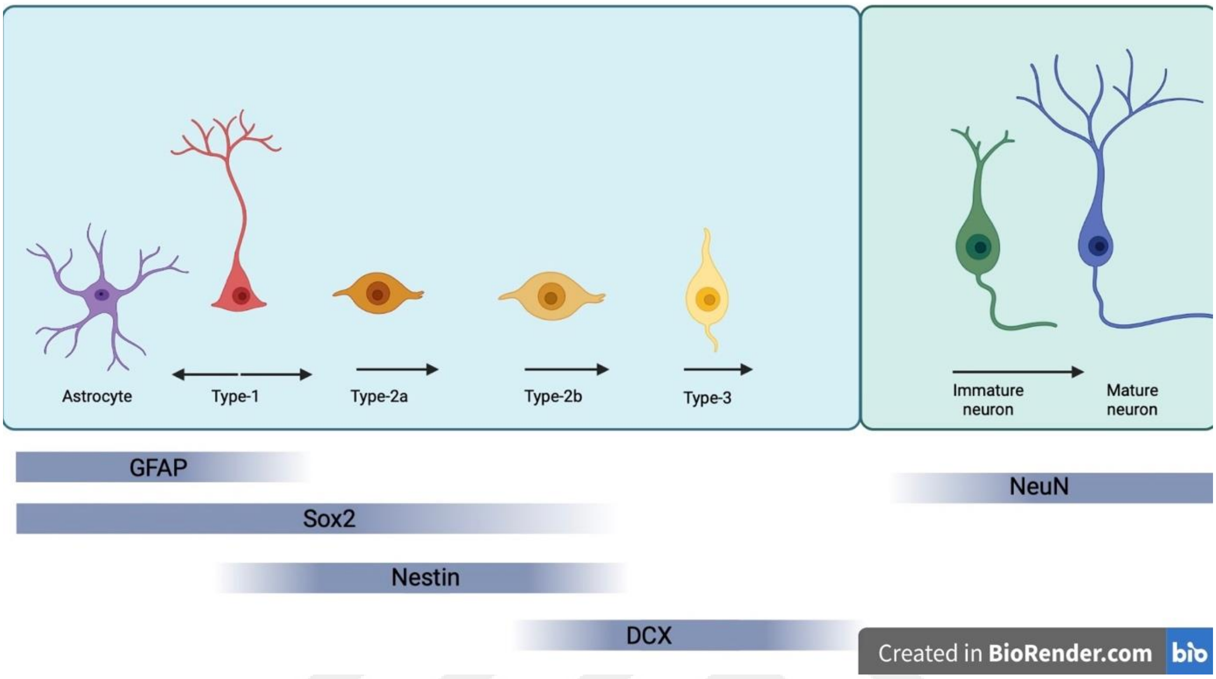


Figure 1.1 Stages of stem cell transformation into mature neuron. The illustration is adapted from Overall (2016) [44] The figure is created with BioRender.

Maintaining neural stem cell function is vital to ensure neurogenesis during adulthood. Throughout aging, a reduction in adult neurogenesis correlates with a decline in cognitive function. Age-related decrease in neurogenesis is primarily due to the reduced reserve of stem cells.[45] Stem cell exhaustion is an essential factor associated with the aging process.[46] [47] As we age, stem cells' number and regeneration capacity are shown to decrease. [48] With stem cell exhaustion, the tissue repair ability of cells may begin to weaken, and the degradation of this process contributes to many diseases, including cancer. [49]

1.3.2.1.1. SOX2

The expression of Sox2 in NSCs provides evidence that the Sox family of transcription factor-encoding genes play a significant role in NSC function. [50] Sox2 also controls the maintenance and proliferation of NSCs, probably by inhibiting the transcription of GFAP. [51] Sox2 expression is observed during Type-1, Type-2a, and Type-2b stages of neurogenesis. [42] Without Sox2, the NSCs differentiate abnormally into neurons, developing fewer and shorter processes and expressing less mature neuronal genes.

1.3.2.1.2. NESTIN

In 1992, for the first time, proliferating cells in the adult rodent brain were shown to express the neural-specific intermediate filament protein nestin.[52] Afterward, these cells developed the morphology of neurons and astroglia. During developmental stages, nestin is expressed by astrocytes and radial glia cells until its expression disappears around postnatal day 11. [53]

However, in the adult brain, nestin is known to be expressed in NSPCs and some astroglia (for example, in the hippocampus). Under pathophysiological conditions, it could also be expressed in reactive astrocytes. Therefore, nestin can be a marker for examining adult neurogenesis. However, there are limitations due to nestin's re-expression during different events, such as traumatic brain injury or neurotoxicity.[54] Thus, looking at Sox2 expression while using nestin as a marker in neurogenesis is beneficial.

1.3.2.1.3. BDNF

Neurotrophic factors are proteins or molecules that promote the health and development of nerve cells and help transmit growth and survival signals. BDNF is a protein also known as the "Brain-Derived Neurotrophic Factor." BDNF is produced in the brain essential for developmental events of the nervous system, such as proliferation, migration, differentiation, survival, apoptosis, and synaptic plasticity.[55] This protein is essential in maintaining and improving nervous system health by promoting neurogenesis and regulating cellular communication.[56]

1.3.2.2. Altered Intercellular Communication

In addition to cell-intrinsic mechanisms, aging can lead to abnormal cell-cell communication patterns. Since senescence signaling could spread throughout the entire organism, the significance of intercellular communication in generating harmful signals released by senescent cells is inevitable. This impact could occur not only locally at the tissue level but also globally.[57] The inflammaging phenomenon, which is characterized by a low-grade chronic type of inflammation seen during aging, illustrates the dysregulation of intercellular communication.[58]

1.3.2.3. Dysbiosis

An imbalance or disruption in the microbiota that live in different areas of the body, as a person ages is referred to as dysbiosis. During dysbiosis, a change from the structure of a healthy or normal microbial population occurs. Changes in the gut microbiota, which are essential for maintaining gut health, nutrient absorption, immune system control, and general wellbeing, can be given as an example of dysbiosis during aging. [4] Interventions that aim to restore a young microbiome may prolong longevity and improve overall health.

1.3.2.4. Chronic Inflammation

Aging, accompanied by low-grade, systemic, chronic inflammation, is identified as "inflammaging". Inflammaging emphasizes the interconnectedness between the aging process and inflammation.[59] As people age, their immune system may undergo changes that result in a state of constant, moderate inflammation without any evidence of infection or injury. This chronic inflammation is thought to contribute to the development of various conditions undermining longevity.

In the CNS, the cells mainly responsible for neuroinflammation are microglia and astrocytes. [60] Under standard conditions, microglia do not produce nor secrete pro-inflammatory cytokines. Microglia or astrocytes must first be activated/primed to induce an inflammatory reaction. Depending on the incoming stimulus, microglia activation may lead to pro-inflammatory or anti-inflammatory responses.[61] If microglia is polarized to the more pro-inflammatory state (M1 state of microglia), it produces specific cytokines and ROS, followed by detrimental immune responses. However, if microglia become polarized into a more anti-inflammatory state (M2 state of microglia), it releases anti-inflammatory cytokines. During a harmful condition such as injury or bacterial/ viral contamination, it provides debris clearance, enabling repair mechanisms, wound healing, and protection against microorganisms.[62] However, long-term proinflammation interrupts healthy brain functioning. Furthermore, chronic inflammation has been linked to many neurodegenerative diseases, including AD. [63]

1.3.2.4.1. NF-κB

Nuclear factor kappa B (NF-κB), is a protein complex that regulates cellular response mechanisms, acting as a signaling pathway. It is an evolutionarily highly conserved transcription factor. The NF-kB system is the central point connecting the pathogenic attack signals and cellular danger signals and further organizes cellular immunity. [64] It has regulatory effects on angiogenesis, proliferation, inflammatory responses, cell survival, and metastasis. [65]

The modulation of inflammatory responses is a distinguished function of NF-κB. It governs inflammatory T cells' activation and differentiation, and controls the expression of several pro-inflammatory genes in the immune cells. [65] NF-κB is also involved in regulating inflammasome activity. Chronic inflammatory disorders are characterized by dysregulated NF-κB activation. NF-κB is a essential regulator of pro-inflammatory gene expression found in innate and adaptive immune cells. NF-κB is a transcription factor essential for M1 macrophages—required for induction of many inflammatory genes, such as TNF-α, IL-1β, IL-6, and COX2. [66]

1.3.2.4.2. IL1β

IL-1β is a pro-inflammatory cytokine, which is a cellular communication molecule. Cytokines play an essential role in the transmission of cellular responses. IL-1β is released by immune system cells, macrophages and mast cells, and glial cells such as microglia and astrocytes.[67] Conditions, where IL-1β is overactivated, may contribute to chronic inflammation and the development of age-related diseases.[68] Therefore, IL-1β and other inflammation-related molecules are vital for understanding the aging process and addressing age-related diseases.

1.3.2.4.3. IL6

IL-6 is a critical factor in inflammation, autoimmunity, and cancer, and its inhibitors are previously used as therapeutic agents for various diseases. [69] In inflammation, IL-6 is the primary stimulator of STAT3, which plays essential roles in inflammation together with NF-κB. Since IL-6 is the target of NF-κB, the concurrent activation of NF-κB and STAT3 in non-immune cell can trigger a positive feedback loop of NF-κB activation via IL-6–STAT3 axis.[70] This positive feedback loop is named as the IL-6 amplifier (IL-6 Amp). [71] Therefore, NF-κB and STAT3 are the substance of the IL-6 Amp. IL-6 Amp amplifies IL-6 itself, as well as other pro-inflammatory molecules, such as cytokines, chemokines, and growth factors. Infections, injuries, senescence, cell death, and obesity are examples of local progenitors which can activate the IL-6 Amp. This mediates the interaction between non-immune and immune cells, resulting in the expression of inflammatory and autoimmune diseases. Various local facilitators, such as aging, obesity, and infection by IL-6 Amp activation, cause uncontrolled inflammation. [70] These findings suggest an approach for treating disorders that target the IL-6-STAT3 signaling axis.

1.3.2.4.4. TLR2

Toll-like receptor receptors are type I integral transmembrane glycoproteins and their heterodimers generally stimulate an intracellular signaling cascade.[72] This mechanism causes NF-κB to influence gene transcription and, consequently, inflammatory cytokine production. This also activates serine/threonine-specific protein kinases (MAPKs), which can alter the transcription of inflammatory genes. [73] Researchers observed that using bone marrow chimeric Alzheimer's amyloid precursor transgenic mice, TLR2 insufficiency in microglia shifted M1- to M2-inflammatory activation in vivo, which was also related to improved neuronal function. [74] Mechanisms targeting TLR2 deficiency could be promising in longevity research.

1.3.3. Antagonistic Hallmarks of Aging

1.3.3.1. Cellular Senecense

Cellular senescence is a reaction given to short-term or long-term damage.[75] Fibroblasts, endothelial, and immune cells are the primary targets of cellular senescence.[76] However, all cell types can experience senescence during aging. Senescent cells can also be seen in slowly proliferating tissues, for example, the brain or the heart. [77] Oncogene activation, shorter telomeres, mitochondrial damage, differing types of infections, oxidative stress, deregulated nutrient sensing, high-fat diet, and mechanical stress are some of the shapes of damage that can cause primary senescence. Additionally, extracellular mediators of inflammation and fibrosis like TGF-b, IL-6, IL-8, and IL1β can cause secondary senescence. [7] The senescence is a robust tumor-suppressing process in cells. Nevertheless, growing evidence connects cellular senescence to mechanisms of tissue repair in which senescent cells encourage the immune cells to remove damaged cells. In this sense, cellular senescence can be seen as the first step in tissue repair, which is then followed by immune clearance of the senescence. Senescence in this situation is a temporary response that programs a positive consequence. However, when the second stage of immune clearance is not accomplished, the pathogenic implications of senescence only become apparent, and the buildup of senescent cells and their effects on the tissue microenvironment eventually lead to catastrophic events. [78]

1.3.3.2. Mitocondrial Disfunction

Another one of the molecular changes in the brain associated with aging is the reduction of mitochondrial function. Mitochondria are essential organelles that take part in adenosine triphosphate (ATP) production. Because the brain is a high energy-demanding organ, it greatly depends on the proper functioning of the mitochondria.[79] The incompetent operation of the mitochondria is shown to contribute to the aging states as well as the pathology of many neurodegenerative diseases.[80], [81] Damage to the mitochondria is shown to diminish energy production in addition to causing oxidative stress, neuroinflammation, and neuronal damage. Moreover, inadequate functioning of mitochondria in microglia is a feature of the aging brain and AD pathology. [82]

1.3.3.3. Deregulated nutrient-sensing

Nutrients provide energy and building blocks for organisms to survive. Therefore, sensing and responding to fluctuations in the nutrient level is an evolutionarily essential and conserved mechanism. The growth hormone production by hypophysis stimulates the secretion of Insulin-like growth factors (IGFs), especially IGF1. In turn, IGF1 promotes growth and development via its receptor. [83] IFG1 receptor stimulates signals through the regulation of various intracellular signaling pathways, such as PI3K-AKT (Phosphoinositide 3-kinases/ Protein kinase) and the MTORC1 (mammalian target of rapamycin complex 1) networks. [84] Also, transcription factors, including FOXOs (mammalian forkhead members of the class O) and E26, involve and activate the genes in diverse cellular processes.

mTOR fits into the phosphoinositide 3-kinase-related family, which is highly conserved within eukaryotes. Rapamycin, an immunosuppressive drug, can inhibit it. Targeting the downregulation of the mTOR pathway directly via dietary regimen, such as using caloric restriction or intermittent fasting (IF), or using the inhibitor of this pathway could be a beneficial intervention regarding longevity. Evidence from zebrafish shows that even a short-term duration of IF and rapamycin treatment could significantly affect the brain during young and old ages. [85]

1.3.3.4. Oxidative Stress

Even though it is not considered as one of the 12 hallmarks of aging, oxidative stress is an important factor that is associated with many hallmarks and is known to expand with aging. Oxidative stress is the damage caused on biomolecules as a result of the imbalance within free radicals and antioxidant molecules.[86], [87] Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced through oxidative stress. ROS and RNS differ in their reactivity. As an example, ROS including superoxide (O_2^-), hydroxyl ($OH^·$), and peroxyl ($RO_2^·$), characterized as radical ROS, are highly reactive. In contrast, ROS such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are less reactive and thus classified as non-radical ROS. [87] ROS and RNS are eliminated under normal conditions by antioxidant defense mechanisms. Because of the impairment in antioxidant defense mechanisms, free radicals cannot be converted into biologically inactive molecules due to redox impairment. Under physiological conditions, low amounts of ROS can still be involved in the physiological pathway in the brain. [88] However, a balance between ROS production and degradation is essential for standard metabolism.

1.3.3.4.1. Nrf2

Nuclear factor-E2-related factor 2 (Nrf2) is a key regulator of endogenous antioxidant defense mechanism that facilitates the transcription of numerous antioxidant genes. Nrf2 is an internal transcription factor which controls the expression of several genes that code for anti-oxidative enzymes. Under standart conditions, Nrf2 is usually degraded in the cytoplasm by its contact with the Keap1 inhibitor.[89] Under stressful conditions, the redox-stress-sensitive cysteine residues in Keap1 are adjusted, which allows Nrf2 to be released from Keap1. [90] Excessive ROS activates tyrosine kinases, resulting in the dissociation of the Nrf2: Keap1 complex. This results in the nuclear import of Nrf2 and facilitates the expression of various antioxidant genes, involving both enzymatic and nonenzymatic antioxidants.[88] Considering this master regulator role of Nrf2 in oxidative stress, it is a promising therapeutic candidate for age-related impairments.

1.4. Interventions

Following explaining the 12 hallmarks of aging, their complex interrelationships, and more regulators that are not hallmarks but have been associated with aging in previous sections, this intersection discusses potential interventions to mitigate the effects of aging and support brain health by targeting one or more of the hallmarks.

1.4.2. Dietary Interventions

Obesity, due to overeating or a high-fat diet, contributes to something known as "meta inflammation." [91] There is no significant tissue harm, and the inflammatory activation magnitude is minimal. This type of inflammation, which does not exactly fall into the standard definitions of acute or chronic inflammation, can be categorized as low-grade chronic inflammation. [92] Obesity caused by a high-fat diet or overeating has been shown to increase inflammation-related cytokines, expand oxidative stress, trigger the microglia and reduce synaptic proteins. [93], [94] Studying the effects of overnutrition or a high-fat diet on old subjects is particularly important. Aging can result in a state of chronic low-grade inflammation itself, which may interact with diet-induced inflammatory processes, making the individual even more susceptible to inflammation's detrimental effects. This is also a risk of further neurodegenerative disease. While obesity is linked to chronic inflammation, dietary restrictions such as intermittent fasting (IF) can be used as the only non-genetic intervention to promote lifespan. [95] The effects of IF on the brain include the slowdown of the aging process by reducing pro-inflammatory cytokines and ROS and affecting cell proliferation. [96]

1.4.3. Pharmacological interventions

Pharmacological interventions targeting aging are drug or treatment approaches aiming to slow the aging process or prevent age-related decline. Due to the complexity of the aging process, it is essential to develop approaches that target multiple mechanisms at the same time.

1.4.3.1. Inflammation Triggering Interventions

The first intervention to be discussed will purpose to model neuroinflammation *in vitro* and *ex vivo* and aim to understand how the brain is affected by inflammation at the molecular level. In the literature, several strategies are employed to cause inflammation. Because chronic inflammation requires extended research of the neuroinflammatory profile within the brain, acute inflammation may be used and result in an equivalent neuroinflammatory profile. Acute inflammation can be achieved by various strategies that function as inflammatory agents, such as lipopolysaccharide (LPS) and copper sulfate ($CuSO_4$). [97], [98]

1.4.3.1.1. Copper Sulfate ($CuSO_4$)

Despite the fact that oxidative stress and inflammation have interdependent mechanisms, excessive production of ROS can lead to inflammatory responses and the development of diseases, including arteriosclerosis, neurodegenerative disorders such as AD, and cancer. [99], [100] Although copper is a necessary micronutrient, it can harm many different cell types at certain concentrations. Increased copper levels and oxidative stress responses can result from an environment with elevated levels of inorganic copper, which can disrupt the organism's copper homeostasis. The end outcome of this is an inflammatory reaction induced by oxidative stress damage. Neuroinflammatory events and an increase in ROS levels in the brain can result from copper intoxication.[101] Copper can decompose with H_2O_2 to produce free oxidizing agents, resulting in ROS production. These oxidizing agents can produce inflammation and damage macromolecules. In recent literature, copper is applied as a potential inflammation-inducer in zebrafish adults and larvae. [102]–[104]

1.4.3.2. Inflammation Preventing Interventions

1.4.3.2.1. Rapamycin

The second intervention to be discussed will purpose to prevent the inflammatory effects triggered by $CuSO_4$ treatment. In that manner, rapamycin is utilized. The mammalian target of rapamycin (mTOR) signaling pathway is a master regulator of various activities such as cell growth and metabolism. Dysregulation of the mTOR pathway has been involved in several human diseases, such as cancer, diabetes, obesity, neurological diseases as well as genetic disorders. [105] Rapamycin, as a spesific inhibitor of mTOR, is an antibiotic with antifungal and immunosuppressive properties.[106] It was originally discovered as an antifungal metabolite produced by *Streptomyces hygroscopicus* from a soil sample from Rapa Nui island. [105] Afterward, rapamycin was found to hold immunosuppressive and antiproliferative properties in mammalian cells. In the literature, rapamycin treatment has shown to increase lifespan in mice, activate microautophagy in mammals, improve cardiac function, is used to treat acute myeloid leukemia in bone marrow and reverse memory deficits in AD. [107]–[112]

1.5. Microglia

In the CNS, microglia are the primary immune cells, with similar functions to macrophages. Microglia are among the smallest glial cells in CNS. It plays an essential role in the inflammatory reaction as the main effector cells of the central nervous system. Microglia demonstrate a branched phenotype under homeostatic conditions, characterized by multiple processes. These cells can undergo morphological changes in response to inflammation, including shorter, thicker processes and increased production of inflammatory markers. [113] When foreign antigen substances or neuronal cellular debris exist, microglia can quickly be activated and produce cytokines to promote inflammatory reaction progress, in Figure 1.2.

Microglia will return to its resting phenotype once the cell fragments or antigen substances are eliminated.[114] Microglia, as the first immune defense line in CNS, could respond to antigens rapidly and release various inflammatory factors, such as NO, TNF-α, and IL-6.

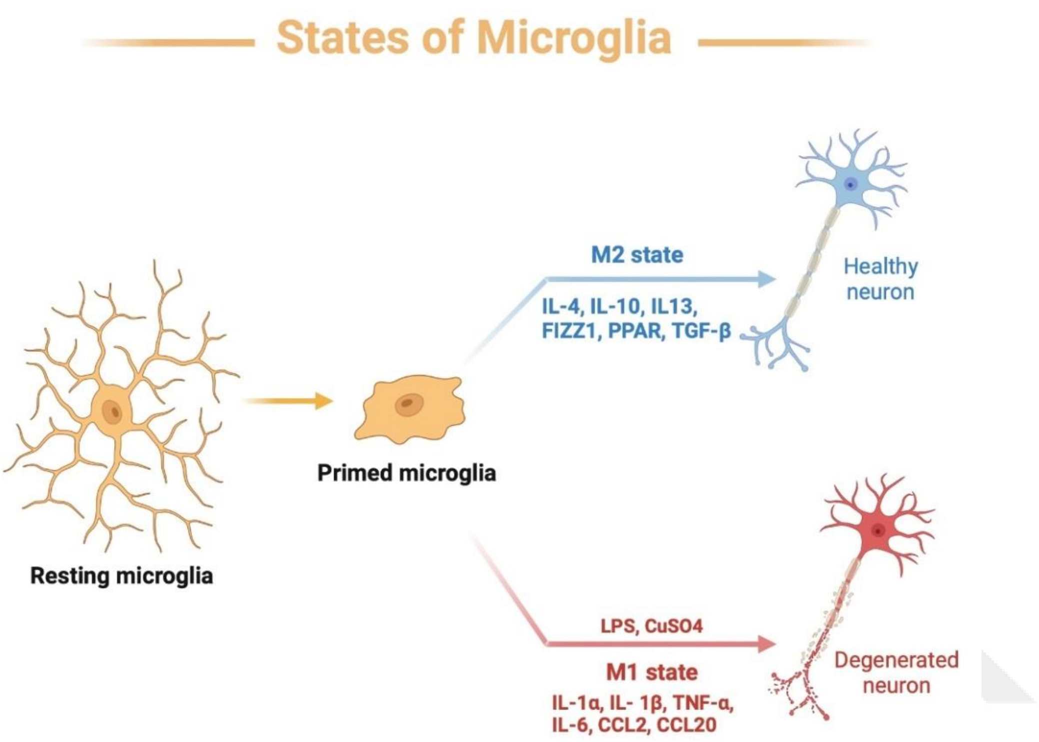


Figure 1.2. States of microglia The figure is created with BioRender.

Cell culture lines are available for the use of microglia in aging research. One of these is the mouse immortalized cell line, BV2 cells. BV2 cells are created by infecting female primary mouse microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus. [115] BV2 cells are well-established mouse microglia cell line used in various research, including inflammation, ischemia, and cancer. [116]–[118]

1.6. Zebrafish as a model organism

The zebrafish (*Danio rerio*) has many advantages as a model organism. Zebrafish embryonic development is rapid. It only takes about 3 months for a fry to turn into an adult fish. [119] Also, a pair of fish will provide up to 300 offspring. [120] Also, zebrafish have a lifespan of between 3 and 5 years. Its genome shows significant similarities to the human genome. The genes in zebrafish are highly conserved with those in humans. Also, zebrafish embryos are transparent and their developing internal organs can be observed until pigmentation formation occurs. [121] This allows for live monitoring of cellular and molecular processes in the embryo. Zebrafish brain development proceeds in a human-like manner and provides a useful model for studying neurological functions.[120] It is advantageous in the study of brain aging because of their similar patterns as mammals with regards to the cellular aging process. For example, zebrafish also have age-related gene declines related to cellular and synaptic structure and growth, neurogenesis, and synaptic alterations. [96] These advantages make zebrafish an effective model organism for many types of research, including brain aging.

1.7. The aim of this thesis

As mentioned in the previous sections, aging is a highly complex process that arises from the interaction of many cellular and intercellular complex pathways. According to the hallmarks of aging, which is defined to explain this complex system, cellular and molecular hallmarks are divided into three groups as primary, integrative, and antagonistic. Hallmarks cannot be completely separated from each other in view of the fact that they interact with each other and can regulate each other.

Aging is associated with increased neuroinflammation and oxidative stress, possibly contributing to age-related cognitive decline. The interaction between inflammation and oxidative stress is thought to play a substantial role in aging. Low-grade chronic inflammation in the brain can lead to oxidative stress, causing proteostasis loss, disabled autophagy, abnormal cell-cell communication, and mitochondrial dysfunction. Oxidative damage can impair cellular function, disrupt signaling pathways such as PI3K/AKT/mTOR, and contribute to ongoing processes. ROS generated during inflammaging can act on signaling molecules to promote the activation of inflammatory pathways. These pathways can further stimulate the release of pro-inflammatory cytokines and chemokines, activate the signaling pathways, and create a positive feedback loop that sustains inflammation. Inflammatory signals can interrupt mitochondrial homeostasis, weakening energy production and increasing ROS production. Mitochondrial dysfunction is a pivotal contributor to oxidative stress in neuroinflammatory conditions. Although mild oxidative stress can trigger beneficial responses that promote neuroprotection and repair mechanisms, chronic or severe oxidative stress can overwhelm the protective mechanisms. Understanding the intricate relationship between inflammation & oxidative stress, and other affected mechanisms in the brain is crucial for developing strategies to maintain longevity.

In light of that information, there were several aims in this thesis. The first objective of the thesis was to select target genes to perform expression analysis by conducting a small bioinformatic analysis using publicly available datasets. The second objective was to use the outcome candidate genes of the bioinformatic analysis to observe the $CuSO_4$'s effect on mRNA levels of genes involved in the oxyinflammaging process. The third aim of this thesis was to employ a BV2 cell line by treating $CuSO_4$ as a brain inflammation model. By using short-term copper treatment, the objective was to assess the optimal period and dosage of the $CuSO_4$ for inducing inflammation.

The 4th objective was to investigate the diet-induced inflammatory effects of different diet regimens in zebrafish by culturing the zebrafish cells. The 5th objective of this thesis was to develop $CuSO_4$ +DMSO for inducing inflammation and rapamycin+DMSO to rescue the pro-inflammatory state that over-feeding induced in long-term dieted zebrafish primary cells. In terms of its impact on the mRNA levels of inflammatory, oxidative stress, neurogenesis, and autophagy markers, the overall goal of the primary cell culture study was to reverse the anti-inflammatory benefit of intermittent fasting and rescue the inflammatory phenotype of the high-fat diet.

CHAPTER 2

The first general objective of this thesis was to utilize a $CuSO_4$-treated BV2 mouse immortalized cell line as an inflammation model. The short-term copper treatment aimed to assess the optimum duration and dosage of the $CuSO_4$ that will be applied to primary cells. In order to select candidate genes, a small bioinformatic analysis was conducted in RStudio using publicly available GEO datasets. The outcome candidate genes of the bioinformatic analysis were implemented into qPCR in order to observe its effect on mRNA levels of various markers. Among these genes determined as a result of bioinformatic analysis, expression analysis was done for those that give the most robust significance, as well as other genes involved in the oxyinflammaging process.

The second general objective of this thesis was to develop $CuSO_4$ as a pro-inflammatory agent and rapamycin as an anti-inflammatory agent in zebrafish primary cell culture. Also, examine the diet-induced inflammatory effects of intermittent fasting and high-fat diets' interaction with inflammatory and anti-inflammatory agents in zebrafish primary neurons. Using the long-term dietary manipulated zebrafish, primary neuronal cell cultures were established and either not treated pharmacologically or exposed to 3 different treatments in 96 well plates: $CuSO_4$, rapamycin, and DMSO. The objective of short-term copper treatment in intermittent fasted zebrafish brains was to induce acute inflammation. In contrast, the short-term rapamycin treatment in high-fat dieted zebrafish brains aimed to emulate successful therapeutic anti-inflammatory outcomes. The overall objective of the primary cell culture study was to reverse the anti-inflammatory advantage of the intermittent fasting group and rescue the inflammatory phenotype of the high-fat group in terms of its effect on mRNA levels of inflammatory, oxidative stress, neurogenesis, and autophagy markers. In the current chapter, the following methods for Chapter 3, Chapter 4, and Chapter 5 are described in detail. While the details are presented here, each individual chapter contains an abbreviated description of the methods.

METHODS

2.1. Bioinformatic Analysis Using GEO Datasets with R

To narrow down and select novel candidate genes associated with neuroinflammation, publicly available glial microarray datasets, which are either anti-inflammatory treated or pro-inflammatory treated, were selected from the Gene Expression Omnibus (GEO) Database were used. Specific requirements were designated for datasets to be comparable with each other. Cross-platform analysis requires different normalizations, therefor only the datasets that used Affymetrix Mouse Gene Array microarray platforms were included. As the second criterion, only the microarray datasets of the *Mus musculus* were used. Considering that the Probe ID names of different organisms may differ, the study is limited to a single organism. The cell types of the datasets used were restricted to glial cell types. The raw CEL files of the datasets were downloaded from GEO Datasets. Since non-raw files are processed by different laboratories using various normalization and background correction methods, all normalization and pre-processing stages were performed using R Studio (Version 1.3.1093) with a handwritten script.

2.1.1. Microarray Data Analysis

2.1.1.1. Pre-processing and Normalization

Using the GEOquery package, raw files of all the datasets were uploaded in R Studio. CEL files are read into raw intensity files using the Bioconductor package named 'oligo.' Normalization of the datasets is executed by The Robust Multi-array Average (RMA) method. The overall variability of each sample's reflection to their groups was examined by Principal Component Analysis (PCA), and PCA plots for each dataset were compared (Not shown).

2.1.1.2.Differentially Expressed Gene Analysis

DEGs were analyzed following the pre-processing and normalization steps. The output of the RMA normalization results was provided as input to the limma package. A moderated t-test on each gene was performed using the eBayes method in limma. Calculation of the t-statistic and a corresponding p-value for each gene were obtained. By combining data from the entire dataset through empirical Bayes moderation, limma improves the reliability of raw p-values from t-tests. As a result, moderated p-values are produced, which are generally more reliable and consistent than raw p-values, particularly with small sample numbers. For visualization purposes, The Volcano Plot, which is a scatterplot that shows the P-value versus log2 fold changes, was prepared separately for each dataset. Volcano plots revealed genes with large-fold changes that can be interpreted as statistically significant. The criteria for defining a table for the DEGs, moderated p-value <0.05, and fold change> 1.5 or <-1.5 requirements were set. A list was established from each dataset's probe set IDs of the DEGs by filtering the DEGs that comply with the inclusion criteria. The annotations of the acquired DEGs were made using the "mogene10sttranscriptcluster" and "mogene21sttranscriptcluster" packages in RStudio.

Probe IDs of the genes were converted into ENTREZ IDs. A text file was saved for each individual dataset. A meta-analysis approach was used to compile independent yet relevant results. (7) The common genes for proinflammation-triggered states were analyzed by comparing the datasets GSE103156 and GSE73022. Likewise, common genes for the anti-inflammation state were explored by comparing the datasets GSE55627 and GSE115652.

DAVID Annotation Analysis

The list of genes, including the DEGs for pro-inflammation and anti-inflammation states, was transferred into DAVID Bioinformatics Resources 6.8. The species section was just restricted to *Mus musculus*. A functional annotation clustering report was established. (8) The annotations of the DEGs obtained in RStudio were completed in DAVID, and the data were combined.

2.2. Gene Expression Experiments with BV2 Cells

2.2.1. Cell Culture

Murine microglial cells (BV2 cell line) were previously provided by Dr. Güneş Özhan Baykan (Izmir Biomedicine and Genome Center). The medium for BV2 cells was Dulbecco's modified Eagle's medium (DMEM). A complete medium for cell culture was prepared by the addition of heat-inactivated 10% Fetal Bovine Serum (BIOWEST), 1% Penicillin-Streptomycin (BIOWEST), and 1% L-Glutamine. The cells were placed in an incubator fixed at 37^{o}C, 5% CO_2. Cells were checked every 2-3 days. When cells reached %80 confluency, they were subcultured by detachment with Trypsin-EDTA (BIOWEST), centrifugation of the falcon at 1000 rpm for 5 minutes, and resuspension with fresh complete medium. For the experiments, the cells were seeded at $5x10^6$ density on 6 well plates.

2.2.2. qPCR primers used for BV2 Cells

Primer pairs (Nfkbie, Tlr2) were designed using the Primer Blast (NCBI) tool. The most suitable primer pair for each gene was selected according to the difference in the melting temperature (Tm) of the primer pairs, the length of the primers, the percentage of GC, self-complementarity level, and whether there were undesirable targets or not. After these analysis, each gene's most suitable pair was selected. Additionally, IL6 and IL1β primers which our group previously designed, were added into experiments. (Table 2.1) As the housekeeping gene, ribosomal protein L13a (RPL13-a) was used, and the relative gene expressions were calculated based on the RPL13-a gene.

Table 2.1 Primers List Used in the BV2 qPCR Experiments

Gene Name	Forward Primer	Reverse Primer	Concentration in reaction	Sample Dilution
Rpl13-a	AGCCTACCAGAAAGTTTGCTTAC	GCTTCTTCTTCCGATAGTGCATC	0.5 µM	1:4
Nfkbie	TCATCCACTCTGTGTGAGGC	TTTCAGGTCATCGAAGGGCA	0.5 µM	1:8
Tlr2	TGTGCCACCATTTCCACG	AAAGGGCGGGTCAGAGTT	0.5 µM	1:8
IL6	AGCCCACCAAGAACGATAGTC	GCAACTGGATGGAAGTCTCTTGC	0.5 µM	1:8
IL1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	0.5 µM	1:4

2.3. Gene Expression Experiments with Primary Cells

2.3.1. Animal Husbandry

ZebTech Housing System (Tecniplast, Italy) in İhsan Doğramacı Bilkent University Molecular Biology and Genetics Department Zebrafish Facility, Ankara, Turkey, is used to maintain the zebrafish under standard conditions. The temperature and pH level of the circulating water was controlled through carbon and mechanical filters regularly. The temperature of the system was 28 C°, and pH levels were kept between 7.0-7.5. The fish were fed with standard dry flakes (Sera, Germany) twice and artemia (Sera, Germany) once a day. They were kept in either 3.5 liters or 8 liters of tanks. Fish were housed with 14 hours of light and 10 hours of the dark cycle. There were 50 old and 29 young adult fish that belonged to wild type background (AB strain) used in this study.

2.3.2. Habituation Period

For the dietary restriction experiments, the wild-type fish were separated based on their age (young and old group). Fish were selected randomly for each age group and distributed into 3 different treatment conditions; intermittent fasting (IF), *ad libitum* (AL), and over-feeding (OF). Fish were habituated in 8L glass tanks containing 4L of system water up to 2 weeks. During the habituation period, all animals were fed *ad libitum* and water temperatures were recorded each day.

2.3.3. Feeding Procedures

The feeding experiments continued for 6 weeks and were completed in an isolated room in Zebrafish Facility to prevent contamination of the main system. During the feeding experiments, fish were maintained under standart temperature (27 C° – 29 C°) and light cycles (14L: 10D). Fish were kept in 8L glass tanks filled with 4L of system water. Since the aquaria was not connected to the ZebTech Housing System with an automatic recirculation system, there was a need to supply all treatment groups with fresh water. In order to clean the aquarium water, 2L of the tanks water is replaced with fresh system water every other day. The tanks temperatures are regulated by 100 W aquarium heaters (Ada; Catalog #: HL-100) and regularly measured by a glass thermometer. Tank's oxygenation and circulation were regulated by air filters (Venusaqua, China; Catalog #: VS-410F) and cleaned every other day during tank cleanings performed. Each treatment group was housed in separate tanks during the feeding experiments. The feeding schedule was adapted from previous work by our research group. [122]

Old treatment group's food amounts and type is written in Table 2.2. In summary, intermittent fasting (IF) group animals were fed every other day with 90 mg fish flakes, 180 mg egg yolk, and 3 mL artemia. Animals in *ad libitum* group were fed same amount of food as the IF group but each day. The over-feeding (OF) animals were fed 1.25 times more food than the AL group. For the young treatment group, same kind of logic was applied as in the old group, but the amounts were adapted according to the animal numbers in each group. Young treatment group's food amounts and type is written in Table 2.3.

Table 2.2 Feeding schedule and daily feeding amounts for the old treatment groups. IF: intermittent fasting group, AL: *ad libitum*-fed control group, OF: over-feeding group

	Monday	Tuesday	Wednesday	Thurday	Friday	Saturday	Sunday	Monday
IF (n:20)	90 mg dry flakes, 180 mg egg yolk and 3 mL artemia		90 mg dry flakes, 180 mg egg yolk and 3 mL artemia		90 mg dry flakes, 180 mg egg yolk and 3 mL artemia		90 mg dry flakes, 180 mg egg yolk and 3 mL artemia	

AL (n: 10)	60 mg dry flakes, 120 mg egg yolk and 2 mL artemia	60 mg dry flakes, 120 mg egg yolk and 2 mL artemia	60 mg dry flakes, 120 mg egg yolk and 2 mL artemia	60 mg dry flakes, 120 mg egg yolk and 2 mL artemia	60 mg dry flakes, 120 mg egg yolk and 2 mL artemia	60 mg dry flakes, 120 mg egg yolk and 2 mL artemia	60 mg dry flakes, 120 mg egg yolk and 2 mL artemia	60 mg dry flakes, 120 mg egg yolk and 2 mL artemia
OF (n:20)	112,5 mg dry flakes, 225 mg egg yolk and 3,75 mL artemia	112,5 mg dry flakes, 225 mg egg yolk and 3,75 mL artemia	112,5 mg dry flakes, 225 mg egg yolk and 3,75 mL artemia	112,5 mg dry flakes, 225 mg egg yolk and 3,75 mL artemia	112,5 mg dry flakes, 225 mg egg yolk and 3,75 mL artemia	112,5 mg dry flakes, 225 mg egg yolk and 3,75 mL artemia	112,5 mg dry flakes, 225 mg egg yolk and 3,75 mL artemia	112,5 mg dry flakes, 225 mg egg yolk and 3,75 mL artemia

Table 2.3. Feeding schedule and daily feeding amounts for the young treatment groups.

IF: intermittent fasting group, AL: *ad libitum*-fed control group, OF: over-feeding group

	Monday	Tuesday	Wednesday	Thurday	Friday	Saturday	Sunday	Monday

IF (n:10)	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia		45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia		45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia		45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia	
AL (n: 9)	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia	45 mg dry flakes, 90 mg egg yolk and 1,5 mL artemia
OF (n:10)	55 mg dry flakes, 110 mg egg yolk and 1,8	55 mg dry flakes, 110 mg egg yolk and 1,8	55 mg dry flakes, 110 mg egg yolk and 1,8 mL artemia	55 mg dry flakes, 110 mg egg yolk and 1,8	55 mg dry flakes, 110 mg egg yolk and 1,8	55 mg dry flakes, 110 mg egg yolk and 1,8	55 mg dry flakes, 110 mg egg yolk and 1,8	55 mg dry flakes, 110 mg egg yolk and 1,8

	mL artemia	mL artemia		mL artemia	mL artemia	mL artemia	mL artemia	mL artemia

Artemia was prepared by mixing 3X salt with 3X artemia eggs in 0.5 L distilled H2O. The eggs were sustained with a constant air supply to let them hatch. After 48 hours, hatched shells were discarded, live artemia was rinsed with distilled H_2O to remove salt, and fed to the animals. Dry fish flakes were weighed weekly, aliquoted in 1.5 mL Eppendorf tubes, and stored at room temperature. The egg yolk was also weighed, aliquoted in 1.5 mL Eppendorf tubes, and stored at +4 C^o.

2.3.4. NO_3 level measurements

The levels of NO_3 in water samples were measured for each tank by using a commercially available kit (Sera, Germany). According to the manufacturer's protocol, the measurement vial was filled with water samples up to 10 mL. Six drops of reagent 1 were added, and the vial was shaken after adding the reagent. Six drops of reagent 2 were added, and the vial was shaken again. Then, one measurement spoon of reagent 3 was added, and the vial was shaken vigorously for 15 seconds. Lastly, six drops of reagent 4 were added and mixed. After 5 minutes of incubation at room temperature, the color of the vial was compared to the color chart, and the NO_3 concentration was recorded.

2.3.5. Euthanasia and Dissections

Following the 1-week habituation and 6-week feeding period, animals were dissected. Before dissections, the fish was euthanized by adding iced system water to small plastic tanks filled with system water. Prior to dissection, fish were monitored until their gills stopped moving, and the tail-pinch reflex test was used to confirm that fish had lost their reflex ability. Bodies of the fish were dried with tissue paper, and body weights and lengths were measured. After weighting, each fish body was sterilized by dipping it into a 70% EtOH-filled Petri dish. Fish were then decapitated by a sterile surgical blade. Gender confirmation was achieved by visualizing the trunks under a dissection microscope. After gender confirmation, trunks were collected into Eppendorf tubes and weighted. Gill tissue and eyes were removed from the head, collected into Eppendorf tubes, weighted, and immediately snap-frozen in liquid nitrogen. The brains were dissected and collected in Hank's Buffered Saline Solution (BIOWEST) filled (supplied with 1%Antimytotic-Antibiotic and 1% Penicilin-Streptomycin) 1.5 mL Eppendorf tubes on ice (5 brains pooled into one Eppendorf). Gills, eyes, and trunk tissues were stored at -80°C until further experiments. Body Mass Index (BMI) calculations were used to observe body parameter changes. BMI was obtained by dividing body weight (gr) by height (cm) squared for each animal and then compared between dietary groups. Pooled-brain tissues proceeded to enzymatic dissociation to establish primary zebrafish cell cultures. Primary cell culture was established using the enzymatic dissociation method with isolated brain tissues collected in HBSS-filled Eppendorf tubes after the dissections were performed. In the days before primary cell culture was established, 96-well plates coated with PDL and laminin were prepared for the primary cells.

2.3.6. Poly-D-Lysine (PDL) and Laminin Coating

2.3.6.1. PDL Coating

Poly D Lysine (Sigma,USA, P7280) stock solution (1000 µg/ml) was diluted to 10 µg/ml working concentration with nuclease-free water prior to coating. Diluted PDL added 100 µl per well for 96-well plate. The plate was left at the cell culture hood for overnight (O/N).

2.3.6.2. Laminin Coating

Laminin (Sigma, USA) (1000 µg/ml) was diluted to 10 µg/ml working concentration with Hank's Buffered Saline Solution (HBSS). PDL was removed from each well by micropipette. Laminin working solution added 100 µl per well for a 96-well plate. The plate was left in a 37 ºC + no CO_2 incubator for O/N. On the day primary cell culture was established, laminin was removed from each well, and wells were washed 3x with Dulbecco's phosphate-buffered saline (DPBS) solution. Plates were placed into 28.5 ºC + no CO_2 incubator until used.

2.3.7. Primary Brain Cell Culture Establishment by Enzymatic Dissociation

The DPBS solution, washing medium (Table 2.4), and first-day medium (Table 2.5) were pre-heated at a 37 ºC water bath. The enzyme mix 1 (EM1) (Miltenyi Biotec Neural Tissue Dissociation Kit P, Germany) was prepared by adding 50 µl Enzyme P to 1900 µl Buffer X. EM1 was pre-heated for 15 minutes in the 37 ºC rotating chamber. The isolated brains were centrifuged at 1300 rpm for 2 minutes at room temperature (RT). HBSS solutions were sucked with a micropipette, 1950 µl of EM1 was added to each Eppendorf and placed in the rotator at 37 ºC chamber for 15 minutes. Enzyme mix 2 (EM2) (Miltenyi Biotec Neural Tissue Dissociation Kit P, Germany) was prepared by mixing 10 µl Enzyme A and 20 µl Buffer Y. Samples containing EM1 were placed in a hood, and 30 µl EM2 was added into each reaction. Samples were homogenized by pipetting up and down 60x using a glass pipette with a small opening. After homogenization, the samples were incubated for 20 minutes in the 37 ºC chamber while rotating. The cell suspensions were applied to the 70 µm cell strainers placed on 50 mL falcon tubes. The falcons were rinsed with 10 mL washing (LDF) medium and centrifuged at room temperature for 10 minutes at 1300 rpm. Supernatants were discarded carefully, and pellets were washed with a 10 mL washing medium again. Samples were centrifuged at room temperature for 8 minutes at 1200 rpm. Plates were taken from the 28.5 ºC + no CO2 incubator, and laminin was removed. Wells were washed 3x with warm DPBS.

Samples were resuspended in 150 µl of first-day medium per falcon. Plates were placed in 28.5 ºC + no CO2 incubator.

Table 2.4 LDF (Washing) Medium (1X)

Substance	Amount
DMEM: Hank's F12 medium (1:1)	15 mL
DMEM (1X) Medium	10 mL
Leibovitz's L-15 Medium	25 mL
Gentamicin (10 mg/mL)	500 µl

Table 2.5 Firsy-day Medium

Substance	Amount
LDF Medium (1X)	1000 µl
N-2 supplement (100X)	10 µl
Human EGF (20 mg/mL)	1 µl
Human FGF-2 (2 mg/mL)	10 µl

2.3.8. Maintenance of Zebrafish Brain Primary Cell Culture

2.3.8.1. Day 1

After 1x washing with warm DPBS, the first-day medium was changed to 150 µl complete Leibovitz's L-15 (with N-2 supplement) + Growth factors (EGF and FGF) (Table 2.6) if no signs of bacterial or fungal contamination were observed under the microscope. The plate was placed in 28.5 ºC + no CO_2 incubator.

Table 2.6 Complete Leibovitz's L-15 Medium

Substance	Amount
Leibovitz’s (L15) Medium (1X)	1000 µl
N-2 supplement (100X)	10 µl
Human EGF (20 mg/mL)	1 µl
Human FGF-2 (2 mg/mL)	10 µl

2.3.8.2. Day 2

After 1x washing with warm DPBS, culture media was changed with 150 µl complete L15 medium. Cells were left over the weekend in 28.5 ºC + no CO2 incubator.

2.3.8.3. Day 5

Culture media was changed with 150 µl complete L15 medium if there was no contamination. The plate was placed in 28.5 ºC + no CO2 incubator.

2.3.8.4. Day 6 and further

Cell confluency and neurosphere distribution were analyzed under the microscope (Figure 2.1.). If cells were reached enough confluency (70% confluent or more), the pro-inflammatory and anti-inflammatory treatments were applied. If not confluent, cells were washed 1x warm DPBS, and the medium was changed to Complete L15 medium without N2 supplement. The plate was placed in 28.5 ºC + no CO2 incubator, and the confluency were examined each day until enough confluency was achieved.

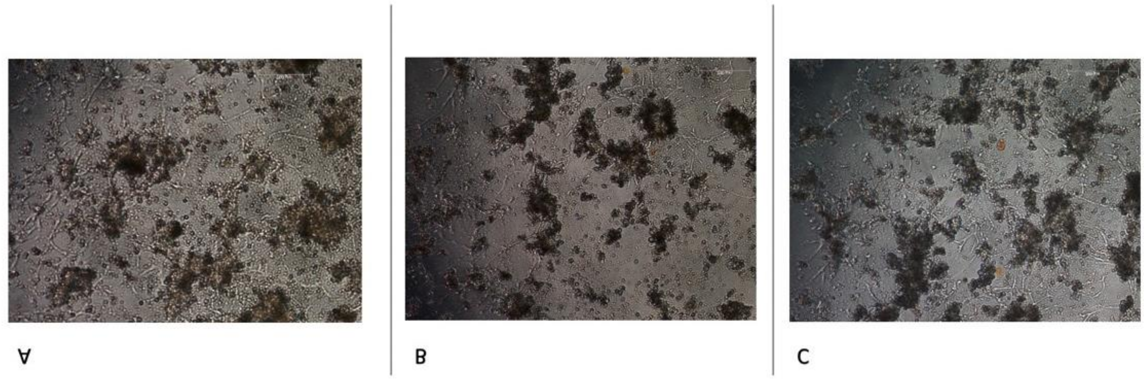


Figure 2.1. Light microscope image of diet-manipulated primary cells during Day 5. A: IF (intermittent fasting), B: AL (*Ad libitum*), C: OF (overfeeding) group. (10x magnitude)

2.3.9. RNA Isolation from Primary Cells

For RNA isolation, established cell cultures formed from 5 pooled zebrafish brains were used to form each technical replica for each treatment condition and time point. Firstly, conditioned growth mediums were removed from each well, and 300 µl of TRIzol reagent (Trizol ® Reagent, Ambion, 19367501) was added per 1 × 10^5 – 10^6 cells directly to the culture dish to lyse the cells. Cells were washed several times with TRIzol by pipetting the lysate up and down in order to homogenize. Then, samples were incubated at room temperature for 5 minutes to allow complete dissociation of the nucleoproteins complex. Then, 60 µl of chloroform (Chloroform, Merck, 1.02245.2500) was added into each tube, and Eppendorf tubes were thoroughly mixed by shaking 20 times. Samples were incubated for 3 minutes at room temperature, followed by centrifugation for 15 minutes at 12,000 × g at 4°C. Afterward, the mixture was separated into a lower red phenol-chloroform phase, interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred into a new tube by angling the tube at 45° and pipetting the solution out. 150 µl isopropanol was added for lysis, and samples were incubated for 10 minutes at 4°C. Again, samples were centrifuged for 10 minutes at 12,000 × g at 4°C. Total RNA precipitated and formed a white gel-like pellet at the bottom of the tube. The supernatant was discarded with a micropipette, and the pellet was resuspended in 300 µl 75% EtOH. Samples were vortexed briefly, then centrifuged for 5 minutes at 7500 × g at 4°C. The supernatant was removed with a micropipette. The RNA pellets were air-dried for 5 minutes and resuspended in 10 µl of RNase-free water (Thermo Fisher Scientific, USA, AM9937) by pipetting up and down. Final RNA concentrations and ratios were determined on NanoDropTM 2000, and samples were stored at -80 °C.

2.3.10. DNase Treatment and Complementary DNA (cDNA) Synthesis

For DNase treatment, the TURBO DNA-freeTM Kit (AM1907, Ambion, USA) was used to exclude the DNA residues within the RNA samples. After a quick spin of RNA samples, 7 µl nuclease-free water (Thermo Fisher Scientific, USA, AM9937), 2 µl 10X buffer from the kit, and 1 µl DNase enzyme were added to the sample, followed by incubation at 37 °C for 30 minutes. After the incubation, 2 µl DNase inactivation buffer was added immediately to stop the reaction. Samples were incubated at room temperature for 5 minutes. Then, samples were centrifuged for 2 minutes at 11.000 x g. The inactivation buffer precipitated at the bottom of the tube, and the supernatant containing the DNase-free RNA was transferred into the new tube. Again, the RNA concentration was measured on NanoDropTM 2000.

After the DNase treatment, cDNA synthesis was performed. cDNA synthesis kit (iScript cDNA Synthesis Kit, BioRad, USA, 1708891) was used to synthesize cDNA. The final RNA concentrations measured in the previous steps were used to calculate the required amount to synthesize 500 ng cDNA. To obtain 20 µl cDNA from each reaction, the samples were diluted with 15 µl nuclease-free water. Then, 4 µl 5X buffer and 1 µl reverse- transcriptase enzyme were added. Samples were centrifuged briefly and incubated at 25°C for 5 minutes, at 46°C for

20 minutes and at 95°C for 1 minute at the thermal cycler (Thermal Cycler C1000, BioRad) machine. Later, samples were kept at -80°C for continuing experiments.

2.3.11. Quantitative Polymerase Chain Reaction (qPCR)

2.3.11.1. Primer Design

The gene expression levels were measured by qPCR. The primers Lc3b and Nrf2a were designed using Primer-Blast (National Center for Biotechnology Information, (NCBI) [123]. Other primers, such as RPL13-a, SOX-2, NF-κB, BDNF, and Nestin, were previously validated by our group. As the housekeeping gene, ribosomal protein L13a (RPL13-a) was used, and the relative gene expressions were calculated based on the RPL13-a gene. The primers were optimized using trial Primary Cell Culture cDNA which were treated for RAPA, Cuso4, or non-treated. Forward and reverse sequences, the final concentrations of primers, and the cDNA concentration used in the reaction are stated in Table 2.7

Table 2.7 Primers List Used in the Zebrafish qPCR Experiments

Gene Name	Forward Primer	Reverse Primer	Concentration in reaction	Sample Dilution
Rpl13-a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	0.5 µM	1:4
Lc3-b	CCTCCAACTCAACTCCAACC	GCCGTCTTCGTCTCTTTCC	1 µM	1:4
Nf-κB	GGTCGGACAGAGATCACGGATT	TGCTGTTCTTCACGTCCTCT	1 µM	Undiluted
Sox2	CCTCCGGGGTCTGTATTTGT	GACCATTCATCGACGAAGCC	1 µM	1:2
Nestin	GAGAAAGACTGTCTGGGGCA	TGTTTGGTGTTGGTCTGTCG	1 µM	1:2

Bdnf	AGAGCGGACGAAT ATCGCAG	GTTGGAACTTTACTGTC CAGTCG	1 µM	1:2
Nrf2a	TCGGGTTTGTCCCT AGATG	AGGTTTGGAGTGTCCGC TA	1 µM	1:4

2.3.11.2. Quantitative Polymerase Chain Reaction (qPCR) Protocol

The qPCR protocol for both optimization and actual experiments was identical, with a total reaction volume of 20 µl for each sample. The reaction mix included 10 µl LightCycler® 480 SYBR Green 1 Master (Roche, Switzerland, 04707516001), 6 µl nuclease-free water, 2 µl premix which consists of diluted primers and 2 µl of template cDNA. We then combined SYBR Green 1 Master and nuclease-free water to make MasterMix and mixed it with premix for each specific target mRNA. Next, we filled each well of the Roche 96 well plate (Roche, Switzerland, 04707516001) with 18 µl of master mix and added cDNA to each well according to the agreed sample dilutions. The plate was then sealed and centrifuged at 1500 rpm for 2 minutes. After the centrifugation step, we placed the plate into the LightCycler® 480 Instrument (Roche) and initiated the reaction.

2.4. Protein Isolation Optimization with Primary Cells

The protein isolation experiments aimed to optimize the use of the remaining organic phase during RNA isolation with TRIzol, so that the data on both mRNA and protein levels could be obtained from the same biological sample.

2.4.1. Subjects

All of the primary cell culture animals were taken from the Bilkent University Zebrafish Facility, whose conditions were mentioned previously. This study was obtained from zebrafish with AB background, which was used during optimization experiments for primary cell culture isolation.

2.4.2. Protein Isolation from Primary Cells

The fresh TRIzol-chloroform extracts remaining from the RNA extraction protocol were collected into 1.5 mL Eppendorf tubes for protein extraction. Samples were centrifuged for 15 minutes at 12,000 × g at 4°C. After the centrifuge, pellets were discarded, and the proteins were precipitated by adding 90 µl of %100 EtOH. Samples were mixed by inverting several times. Then, samples were incubated for 2 minutes at room temperature, followed by a 5-minute centrifuge at 2000 × g at 4°C. After the centrifugation, supernatants were transferred into new Eppendorf tubes and 450 µl of %100 isopropanol. Later, samples were incubated for 10 minutes at room temperature and centrifuged for 15 minutes at 12,000 × g at 4°C. The supernatant was removed with a micropipette. The protein pellets were washed three times with 600 µl 0.3 M guanidine hydrochloride in %95 EtOH. Samples were incubated for 20 minutes at room temperature, followed by a 5-minute centrifuge at 7500 × g at 4°C. Supernatants were discarded. A 1:1 solution of 8M urea (in 1M Tris-HCl, pH: 8,0) and 1% SDS was added to the protein pellets. Samples that were kept on the ice were sonicated for 3 x 10 seconds. Lastly, samples were centrifuged for 10 minutes at 3200 × g at 4°C. Clear supernatant containing the solubilized protein was stored at + 4°C until the protein concentration were measured on DC Assay.

2.4.3. DC Assay

DC Assay was used to measure the protein concentrations which were isolated from primary cultured cells. As the standard control, 1 mg/ml bovine serum albumin (BSA) protein was used in this measurement. In hard-shell 96-well plates, BSA (Sigma, USA, A2153) standards were prepared by diluting with ddH_2O. BSA standards can be seen in Table 2.8

Table 2.8 BSA Standars (µl) for DC Assay

	0	0.1	0.2	0.4	0.6	0.8	1
BSA amount	0	0.5	1	2	3	4	5
ddH_2O amount	5	4.5	4	3	2	1	0

All protein samples were prepared as 2.5 µl protein and 2.5 µl ddH_2O. Standards and proteins were prepared as duplicates. Then, 25 µl of reagent A' (reagent A + reagent S) was added into each well. Later, 200 µl reagent B was added to each well. The plate was gently agitated and incubated for 15 minutes. Using the Microplate Reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA), the absorbance values at 750 nm were recorded. A standard curve was obtained from microplate reader recordings based on the BSA values. The concentration of the proteins was calculated according to the standard curve. (Figure 2.2.)

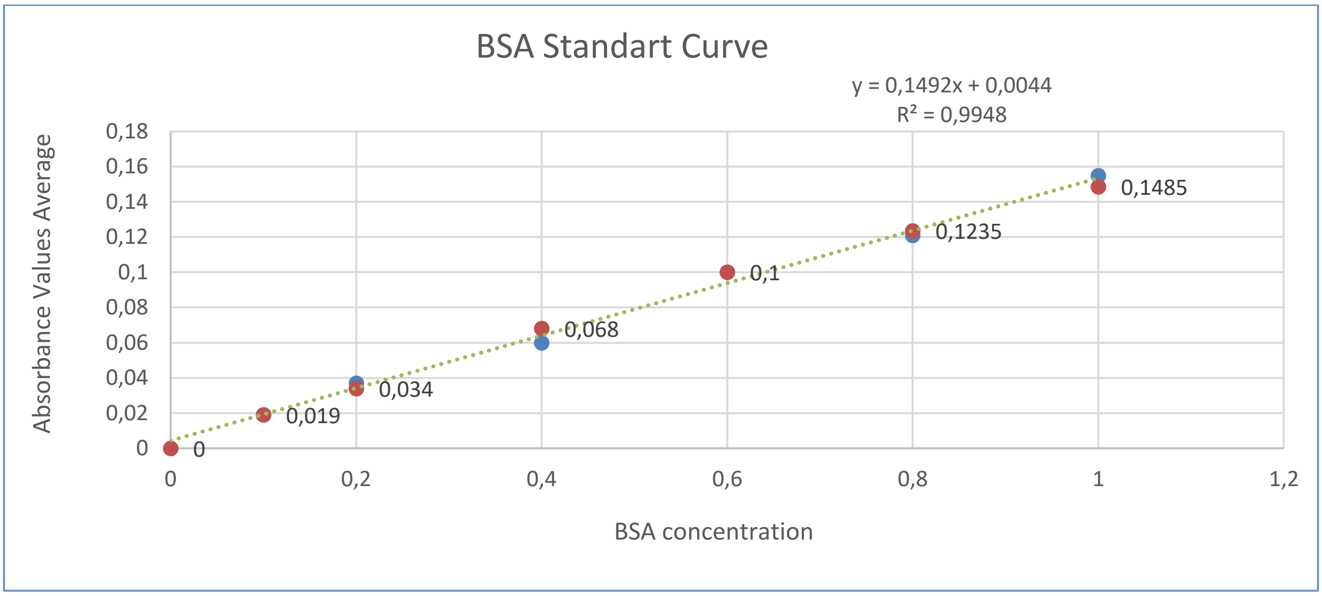


Figure 2.2. BSA Standard Curve

2.4.4. Western Blot Experiments

2.4.4.1. Sample Preparation

The optimization experiments resulted in a determination to load a total of 40 µg of proteins for each sample in order to prevent protein saturation. To achieve this, protein samples were appropriately diluted using ddH_2O and mixed with 2X loading dye to attain a final volume of 40 µl. Prior to the preparation of samples, 10% dithiothreitol (DTT) was added to the loading dye mixture. The samples were subsequently heated to 95 C°, boiled for 10 minutes, cooled down, and then loaded into an SDS-PAGE gel.

2.4.4.2. SDS-PAGE Gel Preparation

As part of Western Blot experiments, two types of gels were formulated. Resolving gels with a 10% concentration and stacking gels with a 5% concentration. (Table 2.9) The purpose of the stacking gel was to facilitate the creation of wells, protein loading, and focusing the protein sample into a tightly confined band, whereas the resolving gels were utilized for protein separation during the electrophoresis. The selection of gel concentration was dependent on the size of the target protein, with higher concentrations of gels being more efficacious for the separation of smaller proteins.

Table 2.9 Stacking and Resolving Gels

Reagent Name	**Amount (For 1 gel)**	
	Stacking Gel (5%)	**Resolving Gel (10%)**
ddH_2O	2.38 ml	4.65 ml
Tris-HCl	1 ml (0.5 M, pH=6.8)	2.5 ml (1.5 M, pH=8.8)
10% SDS	40 µl	100 µl
30% Acrylamide/bis-acrylamide mix (Sigma, A6050)	530 µl	2,65 ml
10% Ammonium persulfate (APS)	40 µl	100 µl
N,N,N'N'-tetramethylethane-1,2-	4 µl	15 µl

diamine (TEMED) (Sigma, T9281)		

2.4.4.3. Loading and Gel Electrophoresis

The PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, USA; Catalog #: 26616) was used as a molecular weight marker for the electrophoretic separation of the proteins. The Mini-PROTEAN Tetra Cell Electrophoresis System (BioRad, USA) was used for gel attachment, and the tank was filled with ice-cold 1X running buffer. Protein stacking was carried out at 90 V for 30 minutes until the point the loading dye reached the resolving gel. The voltage was then raised to 120 V, and the gels were allowed to run for a duration of 90-120 minutes, depending on the protein under examination.

2.4.4.4. Transfer of Proteins

Prior to the gel transfer, gel holder cassettes, Whatman papers, and foam pads were soaked in 1X Transfer buffer. In parallel, Polyvinylidene difluoride (PVDF) membranes were activated by soaking in 100% methanol for 1 minute before being immersed in the 1X Transfer buffer as other materials. A sandwich system was then assembled to transfer the gel. The running was stopped, and the cassette sandwiches were opened using the gel releasers to expose the resolving gel, which was placed in the gel holder cassette on top of the Whatman paper. The stacking gel was removed, and the PVDF membrane was placed on top of the resolving gel. Two layers of Whatman paper and a sponge were then added on top of the PVDF membrane. The gel holder cassettes were then closed and placed into the Mini Trans-Blot Cell Module (BioRad, CA, USA). The transfer process was carried out at 4°C for 90 minutes using a voltage of 100 V. Regular monitoring was conducted on the mA values to prevent any increase. In the event of an increase, the quantity of fresh transfer buffer added to the transfer tank was increased in the next runs.

2.4.4.5. Blocking and Primary Antibody Incubation

In order to prepare the blocking buffers and wash the membranes, a 10X concentrated Tris-buffered saline (TBS) solution was appropriately diluted to achieve 1X concentration using double-distilled water (ddH2O), followed by the addition of Tween-20 (Sigma; 8221841000) to attain a final concentration of 0.003% TBS-Tween-20 (TBS-T). The blocking buffers were formulated by dissolving either 5% non-fat milk powder or bovine serum albumin (BSA) in TBS-T, depending on the specific needs of the respective antibodies. To ensure maximum efficacy, the blocking buffers were freshly prepared each time and subsequently stored at 4°C until the blocking procedure. To minimize non-specific binding, blocking of the membranes was necessary, and it was performed on a shaker for 1 hour at room temperature. After blocking, the membranes were incubated with primary antibodies on a shaker overnight at 4°C. (Table 2.10)

2.4.4.6. Secondary Antibody Incubation

After the overnight incubation with primary antibodies, the membranes were thoroughly washed with 1X TBS-T for 5, 5, 10, 5, and 5 minutes, respectively. The membranes were exposed to the secondary antibody and incubated for 1 hour at room temperature. Subsequently, the membranes were subjected to additional washing with 1X TBS-T using the identical durations specified previously. As a result, the membranes were primed for detection through chemiluminescence.

Table 2.10 List of the antibodies utilized in the optimization study

Antigen	Supplier	Catalog #	Use	Dilution
β-Tubulin	CST	2146	Primary	1:5000
Rabbit-HRP	CST	7074	Secondary	1:5000

2.4.4.7. Chemiluminescent Detection

The detection of bands was performed using Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095). A combination of peroxide and enhancer detection solutions was used in a 1:1 ratio to cover the membranes, which were then incubated in the dark for 5 minutes to develop a signal. The membranes were visualized using the BioRad ChemiDocTM XRS+ Imaging System (BioRad, CA, USA) in automatic exposure and signal accumulation modes since the suitable exposure time was contingent on the protein of interest.

2.4.4.8. Preliminary Western Blot Experiments from Primary Cells

In order to assess the success of this developed method, a preliminary western blot experiment was conducted to compare with the RIPA isolation method, which is currently used in our laboratory. Four different methods were compared with each other for the housekeeping gene Tubulin. The protein levels were compared for the band intensity. The results for these comparisons revealed that protein expression level and concentration were most prominent in the newly developed protocols (from the remaining organic phase of the TRIzol reagent with the sonication method). (Figure 2.3.)

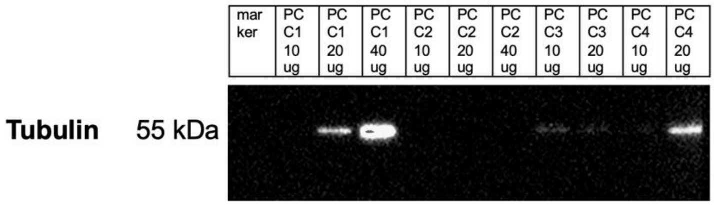


Figure 2.3. Western blot band image of the preliminary protein isolation experiments. PCC1: Primary cell protein sample isolated with the remaining extract from Trizol reagent + lysis with sonicator, PCC2: Primary cell protein sample isolated with the remaining extract from Trizol reagent, PCC3: Primary cell protein sample by isolation with RIPA + lysis with sonicator, PCC4: Primary cell protein sample by isolation with RIPA.

2.5. Statistical Analysis

Assumptions of normality were checked with Shapiro- Wilk and homogeneity of variance was checked with Levene's test in the analyses in Chapters 4 and 5. In cases where normality and homogeneity of variance assumptions were violated, non-parametric tests such as the Mann-Whitney U and Kruskal-Wallis tests were applied. When the assumptions of normality were fulfilled, a one-way analysis of variance (ANOVA) was applied to Chapter 4 experiments. A two-way ANOVA was carried out for Chapter 5 experiments with only diet-treated cells with the factors of age with two levels (young and old) and the feeding regimen with three levels (intermittent fasting, *Ad libitum,* and overfeeding) on the mRNA expression levels of the stated genes. The significance level was set to $p<.05$. Tukey HSD was used as a post-hoc comparison. Kruskal-Wallis was carried out for Chapter 5 experiments with pharmacologically treated cells for age and treatment effects. Mann Whitney U test was carried out for pairwise comparisons in the cases of the significant Kruskal- Wallis results. Adjusted p-values were used for Mann

Whitney U test. All of the analyses mentioned were carried out with SPSS 26. software (IBM, Istanbul, Turkey).

CHAPTER 3

Meta-analysis on Publicly Available GEO Datasets in RStudio

3.1. INTRODUCTION

This chapter focuses on the meta-analysis of publicly available microarray datasets to identify differentially expressed genes associated with neuroinflammation-related gene expression profiles. In the previous chapters, evidence suggests that inflammation is an increased immune response to defend the body against possible endogenous and exogenous harm. Several cell types, such as monocytes, lymphocytes, mast cells, and tissue-specific macrophages, communicate mutually in order to form an immune reaction. Neuroinflammation is a sort of inflammation seen in the CNS. The primary macrophage-like cells in the CNS are microglia. Microglia's primary duty in the CNS is maintaining a healthy neural environment and homeostasis. Microglia play the leading role in starting the inflammation response in return for injury/infection. The principal purpose of inflammation is to protect the nervous system's homeostasis. However, rather than protecting the nervous system, prolonged inflammation damages and leads to disrupted homeostasis. Chronic inflammation is common in neurodegenerative disorders such as AD and PD. [124] Moreover, normal aging is also affiliated with enhanced expression levels of inflammatory agents such as pro-inflammatory cytokines. [125] Therefore, it is crucial to discover biomarkers to be used in investigating the role of inflammation in brain aging.

A key objective in molecular science is the identification of reliable biomarkers from microarray data that have several therapeutic applications. Early disease detection in healthy people, disease classification, and prognosis can be some of the potential uses for biomarkers. Given the massive amount of existing gene expression studies, publicly accessible microarray data has enormous potential to be a source of biomarker findings. For example, Gene Expression Omnibus (GEO), which has a repository of array-based and sequence-based expression data, currently has 6,630,194 samples from 25,269 platforms. [126]

Microarray technology is used to observe the expression profiles of many genes concurrently. Through this technology, thousands of gene sequences can be placed on sites called gene chips. [127] The expressed genes in the sample can be demonstrated by reading the gene chips. Even though microarrays monitor the expression of thousands of genes at the same time, it is predicted that only a tiny portion of these genes would be linked to the required biological outcome, and this group of genes has the potential to serve as biomarkers. [128] The statistical power to get a more accurate estimate and to reduce the impact of study-specific biases grows by including multiple datasets with higher sample sizes. [128] Meta-analysis aims to find differentially expressed genes (DEG), which could be potential candidates for biomarkers. The objective of this chapter was to select target genes (candidate biomarkers) to perform expression analysis by conducting a small bioinformatic analysis using publicly available datasets.

3.2. METHODS

3.2.1. Subjects

The subject of this chapter is selected GEO datasets according to elimination criteria as indicated in Chapter 2.1. Three microglial and one astroglial publicly available microarray datasets were selected for the analysis. The detailed information about datasets included in the study is explained below.

3.2.2. Datasets Used in the Bioinformatic Analysis

GSE103156

In this dataset, BV2 murine microglial cells were incubated with an endotoxin of gram-negative bacteria called lipopolysaccharide (LPS). LPS is often used as an agent to trigger pro-inflammation. 24 hours of incubation with LPS (1ug / ml) was applied to BV2 cells. the results of expression profiling by array and non-coding RNA profiling by array were obtained.

GSE115652

Microglial gene expressions following TGFb1 treatment were assessed in this dataset. Recombined microglia were utilized for evaluating the pathways involved in inflammatory responses. Supernatants were collected to measure the release of chemokines and cytokines. Tgfbr2-deficient microglia cells showed anti-inflammatory characteristics. Expression profiling was carried out by high throughput sequencing.

GSE73022

This study triggers the pro-inflammatory stimulus by treating mouse astrocyte cells for 24 h with TNF (50 ng/ml). Expression profiling by the array is obtained in the study using two conditions, including three control and 3 TNF-treated replicates.

GSE55627

The investigators treated microglia in this dataset with Aβ42 (10uM) and the EP4 agonist AE1-329 (100nM). Four treatment conditions, Aβ + AE1, Aβ only, AE1 only, and vehicle only, were determined, and three replicates were used for each group. Aβ42 treatment aimed to induce pro-inflammation, whereas EP4 stimulation was applied to decrease the inflammatory effects of Aβ42 treatment.

3.2.3. Microarray Data Analysis

The microarray data analysis was conducted in RStudio by downloading each dataset using the "GEOquery" package. Pre-processing and data normalization steps were conducted by using a

Bioconductor package called “oligo”. Pre-processing steps were conducted as indicated in Chapter 2.1.1.1 Using the “limma” package, a moderated t-test was performed, and t-statistic and a corresponding moderated p-value for each gene were obtained, as indicated in Chapter 2.1.1.2. Limma package uses an empirical Bayes approach to compute moderated t-statistics. In order to filter the most biologically significant genes, p-value <0.05 and fold change> 1.5 or <−1.5 were set as criteria, and only genes that comply with this rule were utilized in further experiments. A list was created for the differentially expressed genes, and biological annotations were conducted in DAVID Bioinformatics Resources 6.8, as indicated in Chapter 2.1.1.3.

3.3. RESULTS

3.3.1. Normalization of Raw Data

In microarray studies, it is essential to normalize the data before further analysis. Normalization ensures that biological differences are adequately measured by eliminating non-biological variations. In Figure 3.1, the effect of normalization is shown. Boxplots of before-normalization and boxplots of normalized data can be observed. In Figure 3.1 for (A), (C), (E), and (G), the median gene expression value represented by the horizontal black lines was varied for each sample. On the contrary, for (B), (D), (F), and (H) had comparable median expression levels and comparable scales of the boxes, suggesting that the data following normalization were suitable for further analysis.

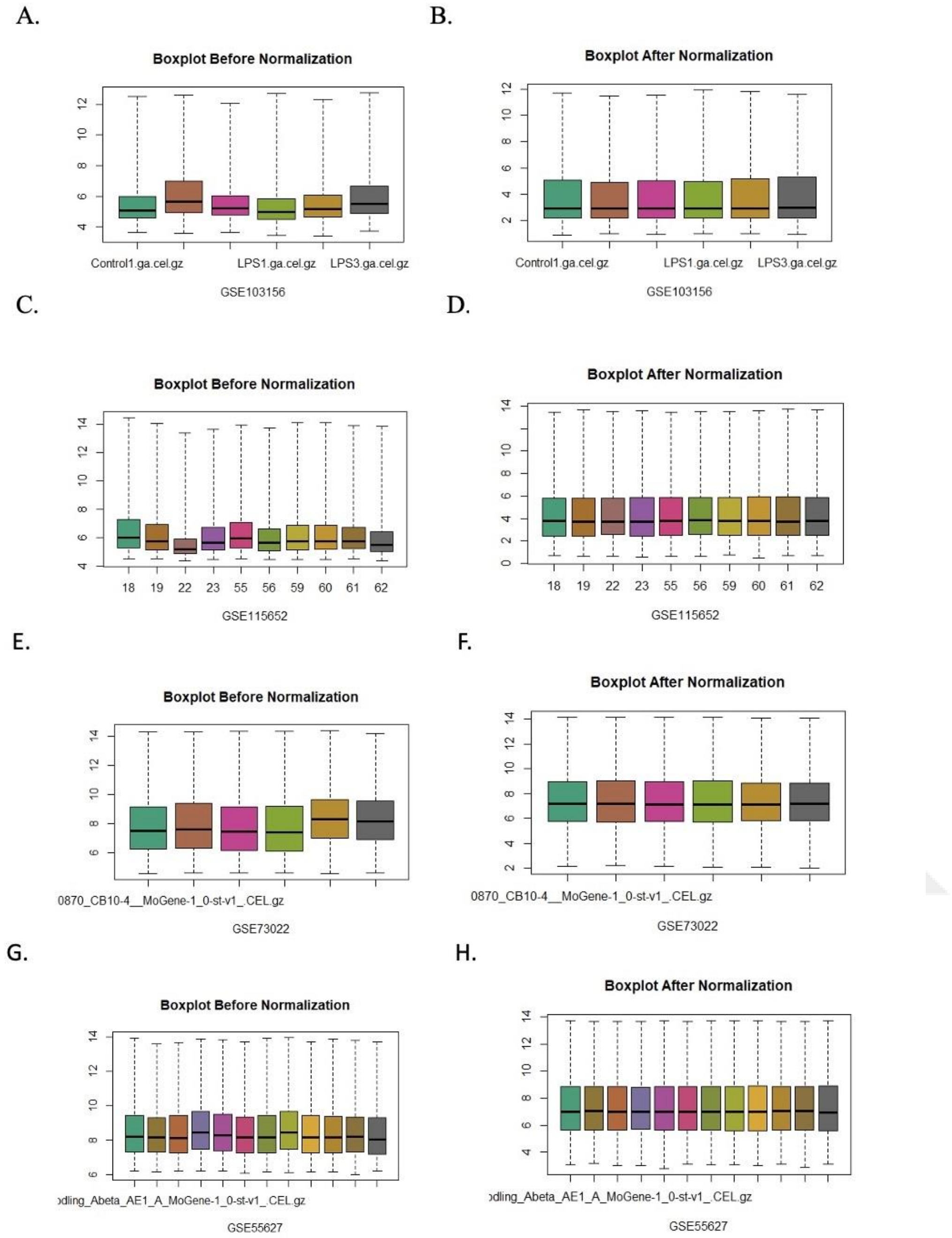


Figure 3.1. Boxplots of before-normalization and after normalization. (A),(C),(E),(G) demonstrate boxplots for the gene expression data prior to normalization. (B),(D),(F),(H) demonstrate boxplots for the gene expression data after the RMA normalization. Expression values were determined using oligo package in RStudio software. The black bar indicates the median value. NCBI's GEO2R is used for graphical illustration.

3.3.2. Analysis of Differentially Expressed Genes

As a result of a meta-analysis of DEG's obtained from GSE103156 and GSE73022 datasets, 12 common genes in pro-inflammation triggered cells were found. Their gene description, gene name, Entrez gene ID, regulation type, and log fold change values are shown in Table 3.1. Only 1 common gene was found in anti-inflammation-triggered cells due to a meta-analysis of DEG'S obtained from GSE55627 and GSE115652 datasets, as shown in Table 3.2.

Table 3.1. Common genes in pro-inflammation triggered cells

Gene Description	Gene Name	Gene IDs	Regulation	Fold Change
Tumor necrosis factor, alpha-induced prote	Tnfaip3	9068	Upregulation	3.42
Tumor necrosis factor, alpha-induced prote	Tnfaip2	12165	Upregulation	3,95
Toll-like receptor 2	Tlr2	23023	Upregulation	4.01
TNFAIP3 interacting protein 1	Tnip1	10886	Upregulation	2.22
Syntaxin 11	Stx11	9036	Upregulation	2.62
Receptor (TNFRSF)-interacting serine-thre kinase 2	Ripk2	24405	Upregulation	3.25
Prostaglandin E synthase	Ptges	21157	Upregulation	2.59
Nuclear factor of kappa light polypeptide g enhancer in B cells inhibitor, epsilon	Nfkbie	17347	Upregulation	4.30
Nuclear factor of kappa light polypeptide g enhancer in B cells inhibitor, alpha	Nfkbia	12360	Upregulation	3.35

Baculoviral IAP repeat-containing 3	Birc3	33236	Upregulation	3.04
Adenosine monophosphate deaminase 3	Ampd3	29304	Upregulation	1.90

Table 3.2. Common genes in anti- inflammation treated cells

Gene Description	Gene Name	Gene IDs	Regulation	Fold Change
PTPRF interacting protein, binding protein (liprin beta 2)	Ppfibp2	29261	Downregulation	-1,81

3.3.3. DAVID Bioinformatics Analysis

DEGs uploaded to DAVID Bioinformatics Resources 6.8 for each dataset were submitted by selecting *Mus musculus* as the organism background. Continuation of annotation of genes performed at DAVID. Functional Annotation, Cluster reports of DEGs, were created. It has been observed that genes are involved in various pathways and activities. Genes associated with TNF signaling pathway, Chemokine signaling pathway, Toll-like receptor signaling pathway, and NF-kappa B signaling pathway among the KEGG pathways were observed for DEGs. Figure 3.2 shows the annotation report for the DEGs found in GSE73022 dataset as a representative Functional Annotation Cluster report from DAVID.

Annotation Cluster 1	Enrichment Score: 17.66		Count	P_Value	Benjamini
UP_KEYWORDS	Immunity	RT	22	5.8E-22	6.8E-20
GOTERM_BP_DIRECT	immune system process	RT	22	6.4E-21	3.2E-18
UP_KEYWORDS	Innate immunity	RT	16	7.1E-17	4.2E-15
GOTERM_BP_DIRECT	innate immune response	RT	17	8.4E-14	1.4E-11
Annotation Cluster 2	**Enrichment Score: 4.51**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	cellular response to interferon-beta	RT	10	6.0E-15	1.5E-12
GOTERM_CC_DIRECT	symbiont-containing vacuole membrane	RT	4	1.4E-6	1.2E-4
GOTERM_MF_DIRECT	GTPase activity	RT	8	5.7E-6	9.4E-4
INTERPRO	P-loop containing nucleoside triphosphate hydrolase	RT	13	6.9E-6	4.5E-4
INTERPRO	Guanylate-binding protein, C-terminal	RT	4	7.5E-6	4.5E-4
INTERPRO	Guanylate-binding protein, N-terminal	RT	4	9.4E-6	4.5E-4
INTERPRO	Interferon-inducible GTPase	RT	4	1.7E-5	5.3E-4
GOTERM_BP_DIRECT	cellular response to interferon-gamma	RT	5	6.7E-5	3.7E-3
GOTERM_BP_DIRECT	defense response to protozoan	RT	4	1.2E-4	5.5E-3
GOTERM_BP_DIRECT	defense response to Gram-positive bacterium	RT	5	2.2E-4	9.5E-3
GOTERM_MF_DIRECT	GTP binding	RT	8	2.7E-4	1.4E-2
GOTERM_BP_DIRECT	adhesion of symbiont to host	RT	3	3.6E-4	1.3E-2
UP_KEYWORDS	GTP-binding	RT	5	1.2E-2	1.2E-1
GOTERM_CC_DIRECT	cytoplasmic vesicle	RT	5	1.3E-1	6.2E-1
UP_SEQ_FEATURE	nucleotide phosphate-binding region:GTP	RT	3	2.2E-1	1.0E0
Annotation Cluster 3	**Enrichment Score: 4**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	negative regulation of viral genome replication	RT	6	5.4E-8	6.8E-6
GOTERM_BP_DIRECT	defense response to virus	RT	8	1.0E-6	1.0E-4
GOTERM_BP_DIRECT	response to virus	RT	6	7.2E-6	6.1E-4
UP_KEYWORDS	Antiviral defense	RT	6	7.4E-6	2.2E-4
UP_KEYWORDS	RNA-binding	RT	3	4.8E-1	1.0E0
GOTERM_MF_DIRECT	RNA binding	RT	3	7.4E-1	1.0E0
Annotation Cluster 4	**Enrichment Score: 3.59**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	positive regulation of inflammatory response	RT	5	4.9E-5	3.1E-3
UP_KEYWORDS	Inflammatory response	RT	6	7.3E-5	1.7E-3
GOTERM_BP_DIRECT	inflammatory response	RT	6	4.9E-3	1.2E-1

Figure 3.2. Functional Annotation Cluster report for the GSE73022 dataset. In the annotation cluster with the highest enrichment score, terms such as immunity, immune system process, and innate immune response are together. In the second cluster, terms related to the defense response to different organisms are together. Annotation cluster 4 is related to inflammation response and positive regulation of inflammatory response. The figure is from DAVID.

3.4. DISCUSSION

In bioinformatics research, it is important to understand the biological significance of DEGs. In the functional annotation results, DAVID Bioinformatic Resources were used. Among the KEGG pathways, DEGs were shown to be involved in the TNF signaling pathway, Chemokine signaling pathway, Toll-like receptor signaling pathway, and NF-kB signaling pathway. Observing TNF signaling among pathways was to be expected since the TNF-α treatment was used to induce pro-inflammation.

Among the most promising pro-inflammatory candidate genes, TLR2 is a gene that is frequently found in pro-inflammation-triggered cells. This receptor, which is a membrane protein, is known to be involved in the immune system and is in charge of producing signals by identifying foreign substances. Together with TLR1 and TLR6, TLR2 produces heterodimers, which is the first step in a series of processes that result in innate immune responses, the establishment of adaptive immunity to infections, and defense against immunological residuals brought on by infection with these pathogens. [129] TLR2 heterodimers usually start an intracellular signaling cascade after ligand stimulation. This pathway triggers nuclear factor-B (NF-kB) translocation to control gene transcription and the subsequent generation of inflammatory cytokines. Furthermore, the cascade activates MAPKs, which could impact inflammatory gene transcription and the stability of those transcripts' mRNA. [129] Literature has shown that, in vivo, TLR2 deficiency in microglia was shifting M1- to M2-inflammatory activation, that was associated with improved neuronal function.[74] Therefore, the strong outcome for TLR2 of this meta-analysis should be assesed further with experimentation, whether a similar pattern is observed for aging.

Another promising candidate gene, which is increased after NF-kB activation is nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor epsilon (Nfkbie). Nfkbie is a protein that inhibits NF-kB activity. by encoding IκBε, a negative feedback regulator of NF-kB. [130] It was not anticipated that this cellular protein would be one of the frequently occurring genes of cells induced by pro-inflammation. This protein may be accompanied by the polarization of microglia in the M1 to M2 to a more anti-inflammatory state. In the literature, Microarray studies following stimulation with IFN-α, using Affymetrix mouse genome array, revealed that the Nfkbie gene was found to be involved in transcriptional regulators encoded by microglia-enriched genes rather than encoded by astrocyte-enriched genes.[131] These results warrant further experimentation for Nfkbie in inflammation-induced microglia cells.

Even though the DAVID Bioinformatic Resources were used in this chapter, additionally, Enrichr and Reactome can be utilized in the future as two effective online resources frequently used in the literature for functional annotation. [132][133] Reactome focuses on route analysis, while Enrichr simplifies gene set enrichment analysis. These tools could help with a deeper understanding of DEGs data as essential resources since they contain the key to uncovering the functional context of the current DEGs.

CHAPTER 4

Expression Analysis of Nfkbie, Tlr2, IL-6 and IL1β in $CuSO_4$ Treated BV2 Cell Culture

4.1. INTRODUCTION

At standard conditions, microglia are stagnant due to neuron and astrocyte-derived factors. Once it encounters an injury or infection, microglia becomes active and take part in the defense of the immune system. It has been revealed that two different activated states of microglia exist. Those two states of microglia are highly dynamic and can alter their functions while generating an immune response. When encountered with cellular or bacterial debris, the stagnant microglia is polarized into a pro-inflammatory state known as the M1 state of microglia. Once it encounters a harmful stimulus, it responds by generating inflammatory cytokines such as TNFα and IFNγ and various chemokines. Microglia with the M1 phenotype can cause the generation of ROS in addition to the generation of pro-inflammatory cytokines such as IL-1α, IL- 1β, TNF-α, and IL-6, besides the chemokines such as CCL2 and CCL20. [134] M2 state microglia is polarized into anti-inflammation with results like suppresed inflammation, wound healing, and matrix deposition. The main duty of M2 microglia is to inactivate pro-inflammatory consequences, suppress inflammation, and restore homeostasis. These are accompanied by cytokines such as IL-4 and IL-10. M2 microglia shifted to the anti-inflammatory phenotype, can release anti-inflammatory factors such as IL-4, IL-10, IL-13, FIZZ1, TGF-β, and PPAR. [135]

Nfkbie and Tlr2 genes, among the prominent genes from bioinformatics analysis, were further selected for assesing mRNA levels. Toll-like receptor 2 (TLR2) is a common gene found in pro-inflammation-triggered cells [136]. This gene is a receptor membrane protein known to play a role in the immune system[136]. It generates signals suitable for the immune system by recognizing foreign substances. Nfkbie is a protein that is upregulated after the NF-kB activation. NF-kB gene expression is associated with pro-inflammation [137]. These two genes have provided potential biomarkers in differing inflammation states to study the oxyinflammaging process in cell culture.

This chapter focuses on the expression analysis of the most promising candidate genes of the previous chapter, as well as two inflammation-related genes, to observe the $CuSO_4$'s effect on mRNA levels of BV2 mouse microglia cells. Also, by treating $CuSO_4$, utilize a BV2 cell line as a microglial inflammation model. Using short-term copper treatment, we focused on assessing the optimal period and dosage of $CuSO_4$ for inflammation induction.

4.2. METHODS

4.2.1. Cell Culture

Dulbecco's modified Eagle's medium (DMEM) was used as the culture medium for BV2 cells. Complete medium recipes for BV2 cells were indicated in Chapter 2.2.1. The cell culture conditions and subculturing protocol is also indicated in Chapter 2.2.1 On six-well plates, the cells were seeded at a density of $5x10^6$ for the treatments and qPCR experiments.

4.2.2. $CuSO_4$ Treatments

BV2 cells received differing concentrations of $CuSO_4$ dissolved in the final concentration of 1 μM DMSO in complete DMEM for 3 hours. (Table 4.1) Later, after observing significant cell death in 3-hour treatments, the duration was decreased to 1 hour. After treatment, cells were washed with room temperature PBS and then collected by applying TRIzol (Trizol ® Reagent, Ambion, 19367501) for RNA isolation.

Table 4.1 Copper sulfate concentrations applied to the BV2 cell line

1. Control	2. Dose	3.Dose	4.Dose	5.Dose
0 µM	2.5 µM	5 µM	7.5 µM	10 µM

4.2.3. Quantitative Polymerase Chain Reaction (qPCR) Experiments

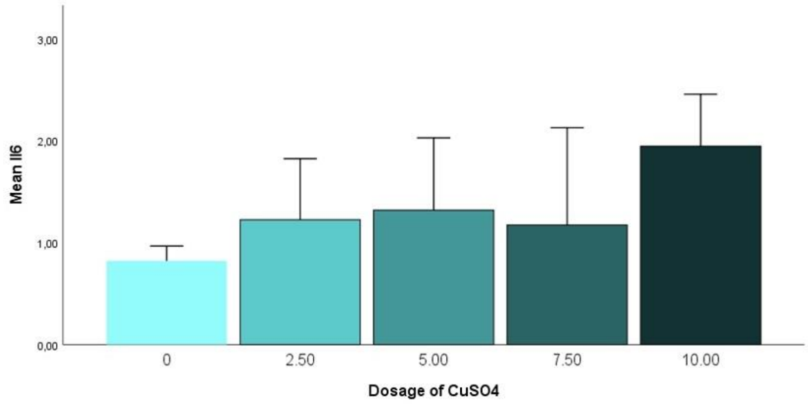
RNA isolation experiments were performed similarly, as indicated in Chapter 2.3.9. The TURBO DNA-freeTM Kit (AM1907, Ambion, USA) was used to remove the DNA remnants from the RNA samples. The synthesis of cDNA was carried out following the DNase treatment. Chapter 2.3.10 provides further explanations for DNase treatment and cDNA synthesis. The Primer Blast (NCBI) program was used to create the primer pairs (Nfkbie, Tlr2). Additionally, experiments included IL6 and IL1β primers that our team had previously designed. Further explanation on qPCR primers was indicated in Chapter 2.3.11.1. The RPL13-a gene served as the basis for calculating relative gene expressions. The qPCR procedure followed as described in Chapter 2.3.11.2

4.3. RESULTS

The main objective of this study was to asses the capability of CuSO4 to induce an inflammatory response in BV2 cells and further experimentation on the 2 genes, which found significant from the meta-analysis. Therefore, we checked the gene expression levels of two pro-inflammatory cytokines, IL-6 and IL1β, and Nfkbie and TLR2 in the short-term CuSO4 exposed microglia cells. There was no significant effect of CuSO4 dosage ($H(4) = 6.218$, p =

0.183) on the log2 fold change values of IL-6 (Figure 4.1 (A)). Also, there was no significant effect of CuSO4 treatment (H (4) = 7.216, p = 0.125) on log2 fold change values of IL1β (Figure 4.1 (B)).

A.



B.

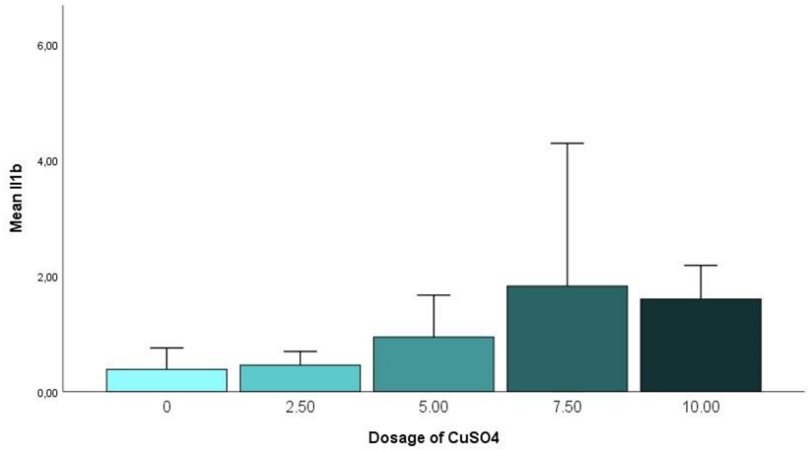
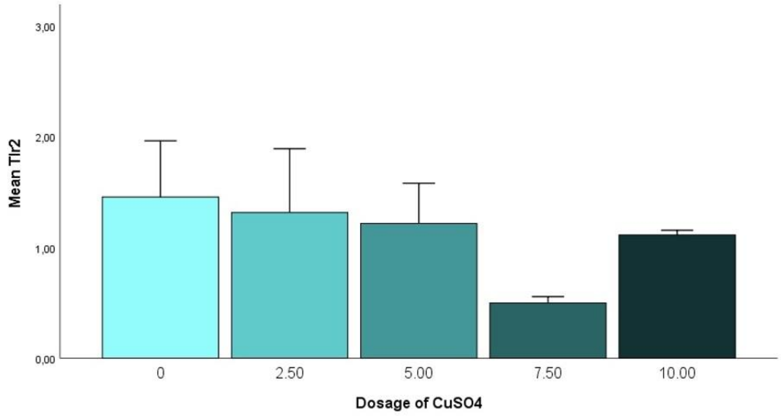


Figure 4.1. **Log2 fold change values in the expression levels of two pro-inflammatory cytokines, IL-6 and IL1β**. There were no significant effects of CuSO4 dose on IL6 (A) and IL1β (B). Error bars represent +SE

In terms of the gene expression levels of Tlr2, there was no significant effect of CuSO4 dosage (H (4) = 5.564, p = 0.234) on the log2 fold change values of Tlr2 (Figure 4.2 (A)). Also, there was no significant effect of CuSO4 treatment (H (4) = 6.000, p = 0.199) on log2 fold change values of Nfkbie (Figure 4.2 (B)).

A.



B.

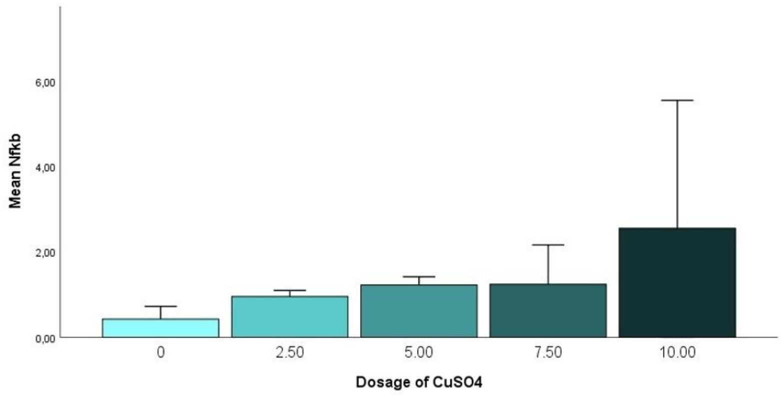


Figure 4.2. Log2 fold change values in the expression levels of Tlr2 and Nfkbie. There were no significant effects of CuSO4 dose on Tlr2 (A) and Nfkbie (B). Error bars represent +SE ,

4.4. DISCUSSION

The effect of $CuSO_4$ treatment to increase inflammation in mouse immortal cell line BV2 cells was investigated. As a result, mRNA expression analyses for the pro-inflammatory cytokines IL-6 and IL1β, as well as Tlr2 and Nfkbie, were not found to be significant. In the literature, LPS is frequently used for the induction of BV2 cells by inflammation. Lipopolysaccharide (LPS) is a complex molecule that is a component of the bacterial cell wall. [138] It is usually found in gram-negative bacteria and can affect the immune system. It can be a trigger in cases of infection or inflammation in the body. Literature on BV2 cells has shown altered levels of pro-inflammatory cytokines (IL-6 and TNF-α) in response to LPS-induced inflammation in mouse microglia cells. [139]

There are also models induced by inflammation using $CuSO_4$. Extreme levels of inorganic copper in the environment can interrupt the natural copper balance in zebrafish, resulting in an inflammatory response. [140] This response is caused by oxidative stress-induced damage because of elevated copper levels. The literature documented that exposing zebrafish embryos to $CuSO_4$ inhibits their survival and development.[141] In the literature, $CuSO_4$ administration to zebrafish embryos induced inflammation and resulted in embryo mortality. The direct assessment of inflammation severity was assessed by monitoring the mortality rate in embryos.[140]

The fact that mRNA levels of pro-inflammatory cytokines were not significantly increased with the $CuSO_4$ treatment on mouse microglia cell-line can be explained in many ways. First, there may be differences between experimental conditions and protocols. Fish embryos develop in aquatic environments, while mouse microglia cells are found in the complex nervous systems of mammals. Microglia cells play a role in immune functions as part of the central nervous system in mammals.[134] Cells in fish embryos perform immune functions during embryonic development. The effect of these cells on the nervous system may differ from that of mammalian microglia cells. These differences may have led to different responses. Secondly, the effect of the stimulant used can be taken into account. The capacity of the stimulus ($CuSO_4$) to induce inflammation in mouse cells may differ from fish embryos. LPS may trigger a more effective inflammatory response in mouse cells, while $CuSO_4$ can affect different mechanisms or induce a lower inflammatory response.

Also, there could be differences between protein levels and mRNA expression. [142] Therefore, looking at mRNA expression alone may not fully reflect the results. Proteins can be affected by post-transcriptional regulation, environmental factors, and cellular mechanisms. From these perspectives, the fact that no significant changes were observed in mRNA levels does not change the fact that there may be differences in protein levels. Therefore, examining both mRNA and protein levels simultaneously in future studies by using more successful inflammation models specific to the organism may be helpful in obtaining a complete picture of cellular responses during inflammation. Therefore, these results led us to further experiments with zebrafish primary cells to observe the effect of $CuSO_4$ on the inflammation process.

CHAPTER 5

Expression of Various Oxidative Stress, Inflammation, Autophagy, Neural Stem Cell, and Neurogenesis Related Markers in Dietary and Pharmacologically Manipulated Zebrafish Primary Cell Culture

5.1. INTRODUCTION

Zebrafish's numerous advantages, including its capacity to regenerate injured brain tissue, led to its emergence as a reliable and widely used model organism over the course of time. Brain tissue regeneration can be induced by activating progenitor stem cells, which eventually migrate to the lesion/degeneration site and mature into more mature neurons. Throughout the lifespan of zebrafish, radial glial cell progenitors, which are dorsally located along the ventricle at the telencephalon, exhibit persistent neurogenic activity. Understanding the capacity of zebrafish neurons may help better understand the reduction of adult neurogenesis of mammalians. Age-related decrease in neurogenesis capacity is mainly due to a reduced reserve of stem cells (stem cell exhaustion), an essential factor associated with the aging process. [47] Therefore, understanding and developing treatment strategies for stem cell exhaustion in aging by identifying the proper regeneration cues is essential. As ideal models of neurodegeneration, zebrafish primary brain cell cultures may provide insights into the ability of zebrafish neuronal regeneretaion and enabling to study neurogenic plasticity. The ratio of undifferentiated cells (neural stem cells in neurospheres) to fully developed cells (neurons) must be maintained in order to maintain the primary cell culture. In order to maintain the undifferentiated state of the cells in the culture, growth factors and N-2 supplement utilized. In the zebrafish primary cell culture, there are diverse populations of cells in neurospheres with differing differentiation levels. Neurospheres may influence radial glial cells toward de-differentiation and redifferentiation when sufficient cues (EGF and bFGF) are provided or in the event of acute injury. This can result in the formation of mature neurons and astrocytes. Neurospheres are clusters of neural stem cells (NSCs) that possess fundamental abilities like proliferation and self-renewal. They develop by adding supplemental fibroblast growth factor (FGF) and epidermal growth factor (EGF). [143] FGF is involved in differentiation, proliferation, wound healing, and angiogenesis, and EGF stimulates cell growth, proliferation, and differentiation. Together, these two factors support the proliferation and development of NSCs. Additionally, N-2 supplementation can help with preserving the stem-cell character.

Nestin, which is abundantly expressed in the subgranular zone of the brain with neuronal precursor cells, is an intermediate filament (IF). Nestin serves as a marker of multipotent NSCs and influences radial axon formation. In cells that are undergoing mitosis, nestin is present. For efficient self-renewal and survival of NSCs, nestin is essential.

Sox2 (SRY (sex determining region Y)-box 2) is a critical transcription factor for self-renewal, differentiation, and development in NSCs during neurogenesis. This transcription factor significantly affects the formation and functional differentiation of neurons. Sox2 leads the maintenance and proliferation of NSCs, probably by inhibiting the transcription of GFAP. [51] Sox2 expression is observed during various stages of neurogenesis. [42] Without Sox2, NSCs develop improperly into neurons, expressing less mature neuronal genes and growing fewer and shorter processes.

BDNF (Brain-Derived Neurotrophic Factor) is a neurotrophic factor that also plays an essential role in the process of neurogenesis. As a neurotrophin, BDNF supports adult neurogenesis, brain plasticity, and neuronal differentiation. It also supports neuronal survival throughout early development. BDNF is a growth factor that belongs to the neurotrophin family. Initially produced as 32–35 kDa pro-isoforms, neurotrophins are later cleaved to mature forms that function as receptor ligands. [144] The biologically active form of BDNF is mature BDNF, which has a strong affinity for the tropomyosin-related kinase B (TrkB) receptor. Like other neurotrophic factors, BDNF promotes long-term potentiation (LTP), neuron survival, and the growth and differentiation of new neurons. In addition to its main effects, BDNF also has other effects on energy expenditure and glucose metabolism. In the literature, it has been shown that energy balance influences BDNF and neurogenesis in the hippocampus. [145] For example, dietary restriction causes an increase in neurogenesis in the adult mouse hippocampus, which is linked to increased BDNF expression. On the other hand, a high-fat diet is shown to reduce BDNF and impair cognitive functions.

Lc3b, (Microtubule-associated protein 1A/1B-light chain 3B), plays a critical role in autophagy as an RNA-binding protein. [34] Previous sections discussed lipidated LC3B as a fundamental component required for the autophagosome membrane to enlarge and form a closed structure. [36] Furthermore, LC3B involves in the regulation of autophagic selective degradation. As a result, LC3B is regarded as a crucial part of the macroautophagy process. In order to keep cells healthy, eliminate intracellular waste, and maintain homeostasis, macroautophagy is a crucial mechanism, and LC3B is a key player in its regulation. Disabled macroautophagy, as a hallmark of aging, is crucial to research since an age-related decline in macroautophagy represents one of the most critical mechanisms of aging.

Nuclear factor-E2-related factor 2 (Nrf2) is discussed in previous chapters as a central regulator of the antioxidant defense mechanism. Genes that code for detoxification enzymes and antioxidant proteins are coordinated and controlled by the Nrf2, which also regulates their expression. The survival and protection of cells depend critically on this system. Excessive ROS trigger the nuclear import of Nrf2 and shall provide for a variety of antioxidant genes, including enzymatic and nonenzymatic antioxidants, to be expressed.[87] Considering this master regulator role of Nrf2 in oxidative stress, and since the $CuSO_4$-induced inflammation is an inflammatory reaction induced by oxidative stress damage, it is an essential marker for inflammation-induced age-related impairments. In previous sections, a protein complex known as nuclear factor kappa B (NF-kB) was discussed as it controls cellular response processes by functioning as a signaling pathway. Angiogenesis, proliferation, inflammatory reactions, cell survival, and metastasis are all regulated by it. In the aging brain, neuroinflammation is most likely caused by the buildup of oxidative damage in tissues, the excessive activation of the innate and adaptive immune systems, the increased production of pro-inflammatory cytokines by the pool of senescent cells, and the buildup of defective proteins as a result of dysregulated autophagy in response to excessive mTOR activation. Due to these early changes, inflammasome activation of various pro-inflammatory signaling pathways, including the PI3K/Akt/mTOR pathway, NF-kB pathway, and MAPK signaling pathways, occur. Therefore an objective of this chapter was to develop $CuSO_4$ as a pro-inflammatory agent and rapamycin as an anti-inflammatory agent in zebrafish primary cell culture. Using the long-term dietary manipulated zebrafish brains, primary neuronal cell cultures were established. Cells were either not treated or exposed to 3 different treatments: $CuSO_4$, rapamycin, and DMSO (control)—copper treatment in intermittent fasted zebrafish cells aimed to mimic acute inflammation. In contrast, the aim of the rapamycin treatment in high-fat dieted zebrafish cells was to emulate rehabilitative outcomes. The main aim of this chapter was to assess the anti-inflammatory dietary advantage of the intermittent fasting group and assess the rapamycins effect on the inflammatory phenotype of the high-fat group in terms of its effect on mRNA levels of inflammatory, oxidative stress, neurogenesis, and autophagy markers.

5.2 METHODS

5.2.1 Subjects and BMI Calculations

The subject of primary cell culture experiments was dietary manipulated young and old zebrafish belonging to AB background. Animal distribution across feeding groups is summarized in Table 5.1. There were two age (old and young adult) groups, and both males and females were included in the experiments.

The detailed information about animal husbandry, feeding procedures, and dissections were mentioned in 2.3.2- 2.3.5. The body mass indexes of young and old fish, which were exposed to 3 different diet regimens for 6 weeks and then taken into cell culture, were measured. Body Mass Index (BMI) calculations were used to observe body parameter changes. BMI was obtained by dividing body weight (gr) by height (cm) squared. The amount of food used in the 6-week diet periods was explained in Table 2.2 and Table 2.3.

Table 5.1 Number of animals exposed to different diet regimens

	Young	**Old**
IF	10	20
AL	9	10
OF	10	20

5.2.2 Primary Cell Culture Establishment and Maintenance

Brain tissues obtained from long-term diet-manipulated animals were taken into tubes as five brains/tube. Three primary neuronal cell culture groups were obtained for each cohort: IF, AL, and OF groups. Each neuronal culture group was seeded in 96-well plates and grown for 6 days, as mentioned in detail in Chapter 2.3.8, and then subjected to different drug treatments.

5.2.3. Treatment Conditions

Using the long-term dietary manipulated zebrafish, three different primary neuronal cell culture groups were established; IF treated, AL treated, and OF treated. Each neuronal culture group was exposed to 3 different treatments in 96 well plates: $CuSO_4$, rapamycin, and DMSO. Short-term copper treatment in intermittent fasted zebrafish primary neuronal cultures aimed to mimic acute inflammation. In contrast, the aim of the rapamycin treatment in high-fat dieted zebrafish cells was to assess rehabilitative outcomes. DMSO was used at 15 µM final concentration to prepare stock solutions of rapamycin and $CuSO_{4;\ \text{therefore}}$, as a third (control) group, DMSO treatment was applied in *ad libitum* dieted zebrafish primary neuronal cultures.

At day 6 of cell culture, the complete L15 medium was removed, and each group of primary cells was washed with 150 µl warm DPBS. Later, the IF group was subjected to a 150 µl complete L15 medium containing 5 uM dissolved $CuSO_4$. These well plates were again kept in 28.5 ºC + no CO2 incubator for 1 hour. OF group was subjected to 150 µl complete L15 medium, which contained 1 uM dissolved rapamycin, and these well plates were kept in 28.5 ºC + no CO2 incubator for 3 hours. AL group was subjected to 150 µl complete L15 medium, which contained 1,5 µl dissolved DMSO, and as control, and these well plates were kept in 28.5 ºC + no CO2 incubator for 1 hour and 3-hour durations (summarised in Table 5.2). After the short-term treatments, conditioned mediums were removed, and each well proceeded to downstream applications.

Table 5.2 Time, dosage and group information of treatments applied in Primary Cell Culture

Chemical	Copper Sulfate ($CuSO_4$)	DMSO	Rapamycin (RAPA)
Duration	1 hour	3 hours	3 hours
Dosage	5 uM	5 uM	1 uM
Applied Group	IF	AL	OF

5.2.4. Quantitative Polymerase Chain Reaction (qPCR) Experiments

RNA isolation experiments were performed in same manner, as indicated in Chapter 2.3.9 The synthesis of cDNA was carried out following the DNase treatment as indicated in Chapter 2.3.10 The Primer Blast (NCBI) program was used to create the primer pairs (Lc3b and Nrf2a). Additionally, experiments included Nestin, BDNF, NfkB and Sox2 primers that our team had previously designed. All of the primers were optimized for cell culture usage. Dilutions of each primer pair, except for BDNF and Nrf2a, were prepared in separate tubes with a final concentration of 0.5 µM. For BDNF and Nrf2a, the primer pair concentration was determined as 1 µM. It was tested for 5 different dilutions (undiluted, 1:2, 1:4, 1:8 and 1:16 diluted) of the same sample for all primer pairs. The ct values obtained in the qPCR reactions of all primer pairs were analyzed by fitting the standard curve plot, and gene expression analyzes were continued only with primers with the appropriate PCR efficiency range. Further explanation on qPCR primers was indicated in Table 2.7. The RPL13-a gene served as the basis for calculating relative gene expressions. The qPCR procedure followed in same manner as described in Chapter 2.3.11.2.

5.3. RESULTS

5.3.1 Body Mass Index Results

This study aimed to culture the zebrafish cells to examine whether there are molecular alterations in the brains of IF, *ad libitum,* and OF diet-treated zebrafish. Furthermore, another aim was to assess the molecular alterations after short-term pharmacological manipulations. Firstly, a two-way ANOVA analysis was performed to evaluate the effect of diet and age factors on body mass index (BMI) between young and old animals after the diet treatment. There was a significant diet effect ($F_{(2,71)}$ = 7,710, p <0.001)) and a significant age effect on BMI ($F_{(1,71)}$ = 4,836, p = 0.031) but no significant diet and age interaction ($F_{(2,71)}$ = 0.092, p = 0.912) (Figure 5.1) Pairwise comparisons by the Tukey HSD test in the young group revealed a significant difference between IF and AL groups (p<.001).

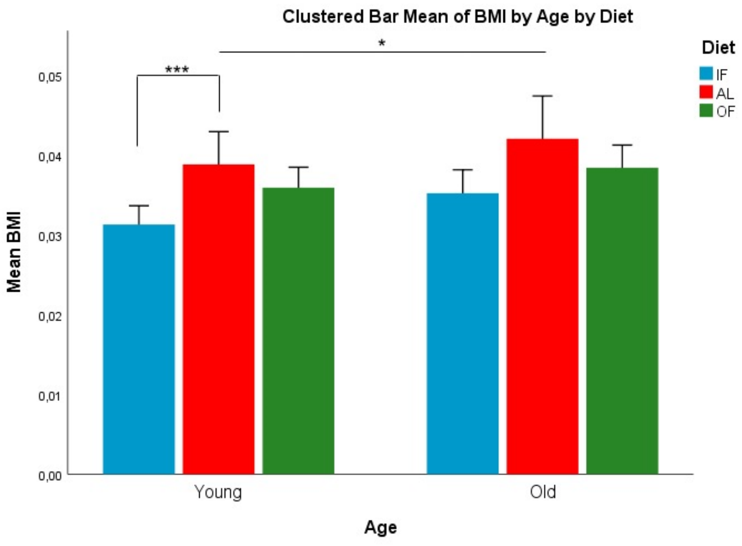


Figure 5.1 Body mass index differences depending on age and diet (BMI: Body mass index, IF: intermittent fasting, AL: ad libitum, OF: overnutrition, Error bars represent +SE (***: diet effect (p≤ 0.001), *: age effect (p≤ 0.05), ns: p> 0.05)

5.3.2 Diet-Treated Gene Expression Levels

The objective of the primary cell culture study was to assess the different diet regimens via their impact on the mRNA levels of inflammatory, oxidative stress, neurogenesis, and autophagy markers. Therefore, we checked the gene expression level of oxidative stress marker, Nrf2a in the diet-treated young and old primary cells. (Figure 5.2) There was no significant effect of diet (H(2)=1.309, *p*=.320) or age (H(1)=1.176, *p*=.278).

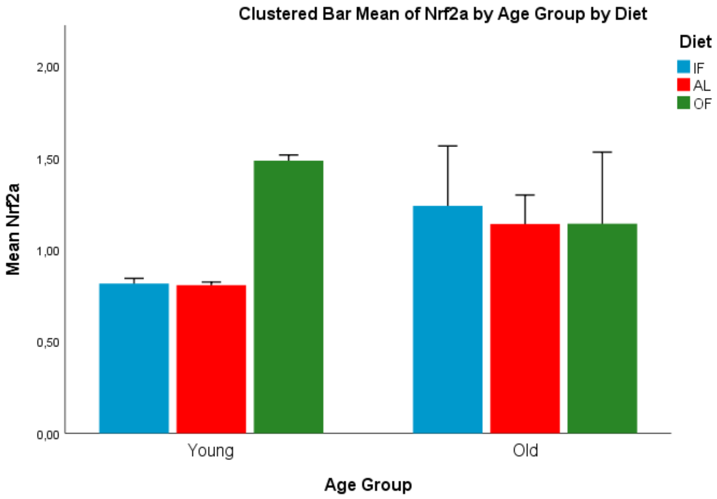


Figure 5.2. Distribution of non-treated Nrf2a gene expression levels by age. Error bars represent +SE

In terms of gene expression levels of an inflammation-related marker, NfkB, there was a significant effect of age (Figure 5.3). Analyzed by the Kruskal Wallis H test, mRNA levels of NfkB were significant (H(1)=9.894, p=.002). The Mann-Whitney U test revealed a significant difference between the young and old groups U(N_{young}=6, N_{old}=10)=1.00, z=-3,145 p=.002) However, there was not a signigicant effect of diet (H(2)=.548, p=0.760)

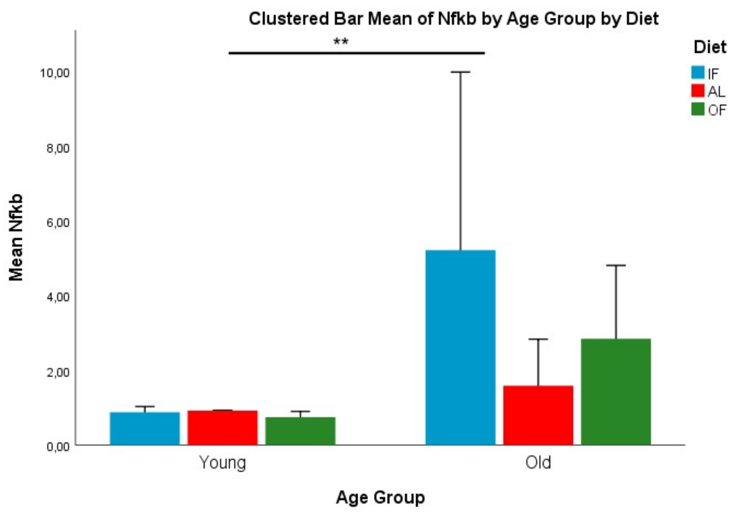


Figure 5.3. Distribution of non-treated NfkB gene expression levels by age. Error bars represent +SE (**: p≤ 0.01, *: p≤ 0.05, ns: p> 0.05)

In terms of gene expression levels of the neurogenesis marker, Sox2, there was a significant difference in the diet (H(2)=8.838, *p*=.012). (Figure 5.4 The Mann-Whitney U test revealed a significant difference between young AL and OF ($U(N_{AL}=1, N_{OF}=2)$=,00 , z=-2.558, *p*=.01)) and between young OF and old OF ($U(N_{OFY}=2, N_{OFO}=4)$=,00 z=-2,152, *p*=.043)) groups. However, there was no significant effect of age (H(1)=.047, p=.828)

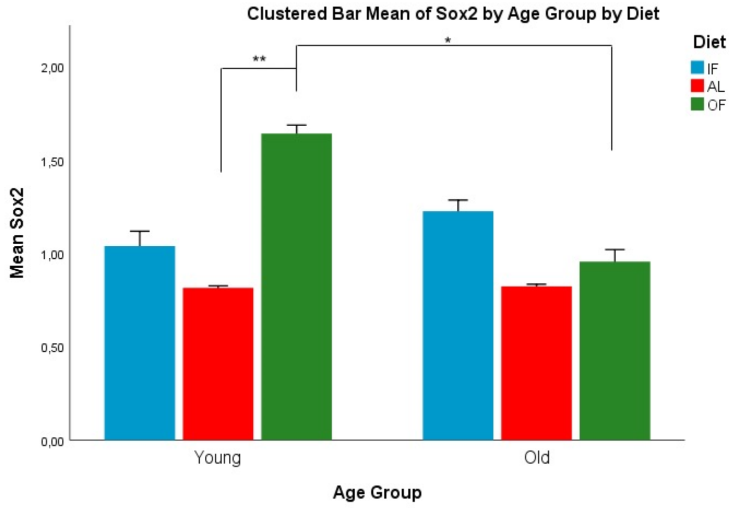


Figure 5.4. Distribution of non-treated Sox2 gene expression levels by age. Error bars represent +SE (**: p≤ 0.01, *: p≤ 0.05, ns: p> 0.05)

For the neurogenesis marker, BDNF, Two-way ANOVA revealed a significant diet effect ($F_{(2,16)}$=13.845, p=.001), a significant age effect ($F_{(1,16)}$=8.350, *p*=.016), and a significant diet and age interaction ($F_{(2,16)}$=6.865, *p*=.013) (Figure 5.5) Multiple comparisons by the Tukey HSD test revealed a significant difference between following groups; the young AL and OF groups (p=.014), the old IF and AL groups (p=.015), the old IF and OF groups (p=.013), and in between young and old OF groups (p=.007).

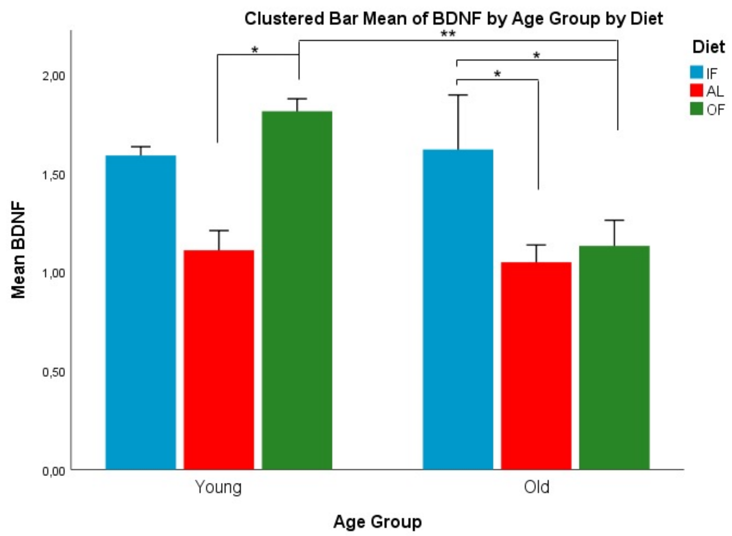


Figure 5.5. Distribution of non-treated BDNF gene expression levels by age. Error bars represent +SE (**: p≤ 0.01, *: p≤ 0.05, ns: p> 0.05)

In terms of gene expression levels of autophagy marker, Lc3b, Two-way ANOVA revealed no significant diet effect ($F_{(2,36)}$=2.258, p=.155) and no significant age ($F_{(1,16)}$=.633, p=.445) effect or age and diet interaction ($F_{2,16)}$=.102, p=.904) (Figure 5.6)

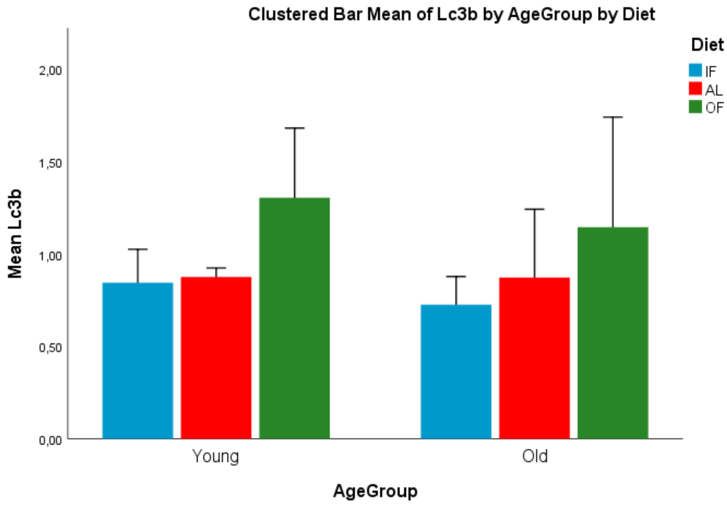


Figure 5.6. Distribution of non-treated Lc3b gene expression levels by age. Error bars represent +SE (ns: p> 0.05)

In terms of gene expression levels of neural stem cell markers, Nestin, analyzed by the Kruskal Wallis H test, revealed a significant diet effect (H (2) =11.222, *p*=.006). The Mann-Whitney U test revealed a significant difference between the young IF and OF groups (U (N_{IF}=2, N_{OF} =2) =.00, z=-2,846 p=0.011)) and in between young and old OF groups (U(N_{OFY}=2, N_{OFO} =4)=3,00 z=-2,818, *p*=.012)). Nevertheless, there was no significant age effect (H (1) =.295, p=.587). (Figure 5.7)

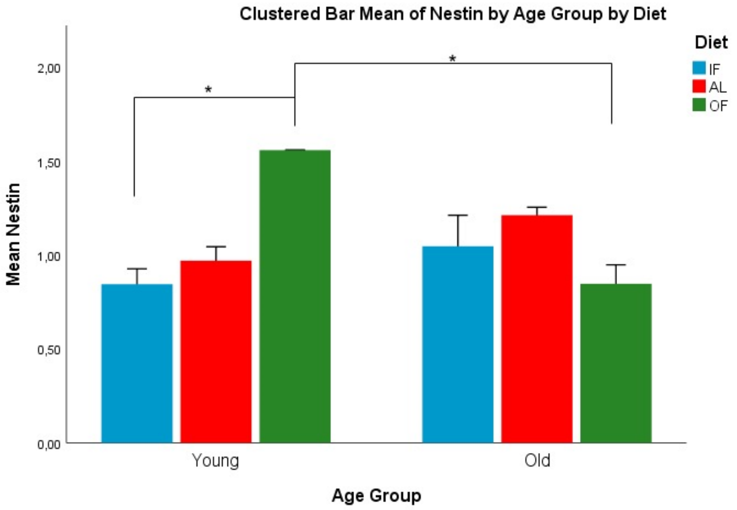


Figure 5.7 Distribution of Nestin gene expression levels by age. Error bars represent +SE (*: ($p \leq 0.05$, ns: $p > 0.05$)

5.3.3 Diet and Pharmacologically Treated Gene Expression Levels

The main objective of second part of the primary cell culture study was to assess the anti-inflammatory advantage of intermittent fasting under inflammation and rescue the inflammatory phenotype of the high-fat diet by rapamycin via their impact on the mRNA levels of inflammatory, oxidative stress, neurogenesis, and autophagy markers. Hence, we checked the gene expression level of Nrf2a, an antioxidant marker. There was a significant effect of age for intermittently fasted cells ($H(1)=7.385$, $p=.007$). However, treatment had no significant effect ($H(3)=8.654$, $p=.054$). (Figure 5.8, A) For the *Ad libitum* fed cells, Nrf2a did not have a significant effect on age ($H(1)=0.029$, $p=.865$) or treatment ($H(3)=7.212$, $p=.065$). (Figure 5.8, B) The same was observed for the overfeeding group on age ($H(1)=0.083$, $p=.773$) and treatment ($H(3)=6.167$, $p=.104$).(Figure 5.8, C)

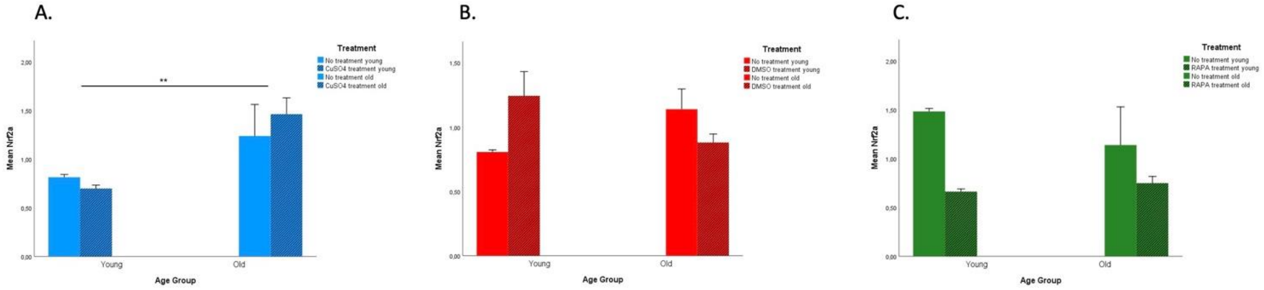


Figure 5.8. Distribution of pharmacologically treated and non-treated Nrf2a gene expression levels by age. A: Intermittent fasting, B: *Ad libitum*, C: Overfeeding, Dashed lines indicate treatment groups, Error bars represent +SE (**: (p<0.01), ns: p> 0.05)

Relating to gene expression levels of inflammation marker, NfkB, age (H(1)=3.490, p=.062) and treatment (H(3)=3.750, p=.290), did not have a significant effect on mRNA levels of for IF group. (Figure 5.9, A) In *Ad libitum* group, similarly age (H(1)=.333, p=.564) and treatment (H(3)=2.000, p=.572) did not had an significant effect on NfkB mRNA levels. (Figure 5.9, B) However, in overfeeding group, age was significantly effected (H(1)=6.108, p=.013), but treatments effect was not significant (H(3)=6.754, p=0.080). (Figure 5.9, C)

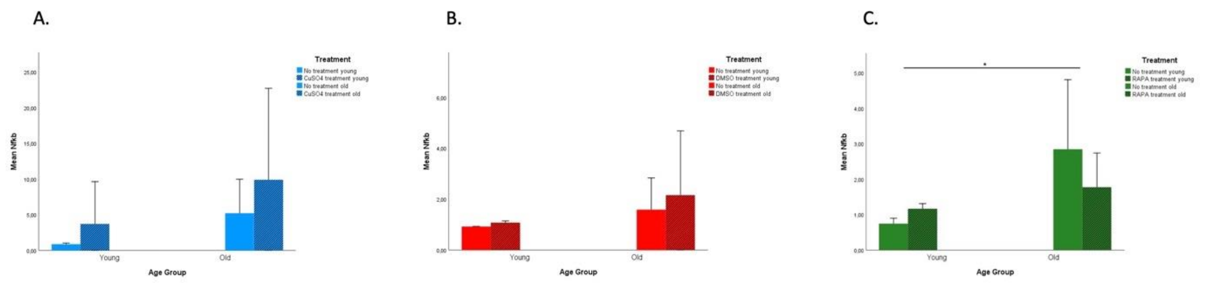


Figure 5.9. Distribution of pharmacologically treated and non-treated NfkB gene expression levels by age. A: Intermittent fasting, B: *Ad libitum*, C: Overfeeding, Dashed lines indicate treatment groups, Error bars represent +SE (*: ($p<0.05$), ns: $p> 0.05$)

For the gene expression levels of the neurogenesis marker, Sox2, age had a significant effect on the intermittent fasting group (H(1)=7.385, p=.007). But no significant effect of treatments (H(3)=9.077, p=.058 was observed. (Figure 5.10, A) Similar significance for OF group was observed on age (H(1)=5.674, p=0.017), and the treatment effect was insignificant (H(3)=7.999, p=0.056). (Figure 5.10, C) However, in the AL group, neither age (H(1)=.00, p=1.00, nor treatment (H(3)=5.667, p=.129) was significant. (Figure 5.10, B)

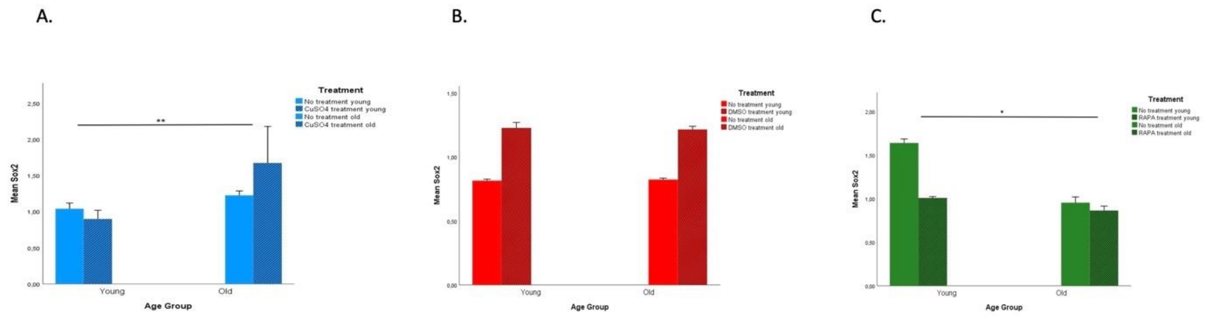


Figure 5.10. Distribution of pharmacologically treated and non-treated Sox2 gene expression levels by age. A: Intermittent fasting, B: *Ad libitum*, C: Overfeeding, Dashed lines indicate treatment groups, Error bars represent +SE (**: ($p<0.01$), *: ($p<0.05$), ns: $p> 0.05$)

For a different neurogenesis marker, BDNF, there was no significant effect of treatment (H(3) =4.519, p=.211) or age (H(1) =0.029, p=.865) for the IF group. (Figure 5.11, A) Similarly, for the AL group, no significant effect of treatment (H(3)=3.922, p=.270) or age (H(1)=.021, p=.885) was observed.(Figure 5.11, B) For the OF group, there was no significant effect of age (H(1)=.029, p=865) however, there was a significant treatment effect for Rapamycin+ DMSO (H(3)=9.135, p=0.028). (Figure 5.11, C) Mann Whitney U test revealed that BDNF mRNA levels of no treatment young group were altered compared to Rapamycin+DMSO treated young group (U($N_{notreatment}$=4, $N_{RAPA+DMSO=}$4)=1.00, z=-2.021, p=0,043).

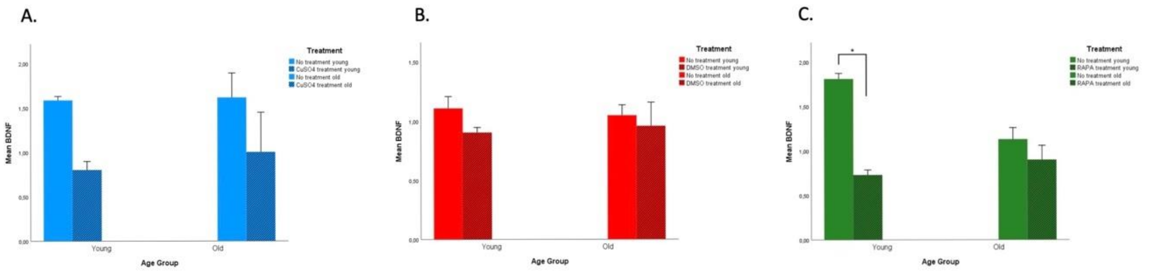


Figure 5.11. Distribution of pharmacologically treated and non-treated BDNF gene expression levels by age. A: Intermittent fasting, B: *Ad libitum*, C: Overfeeding, Dashed lines indicate treatment groups, Error bars represent +SE (*: (p<0.05), ns: p> 0.05)

For the gene expression levels of the autophagy marker, Lc3b, in the IF group, there was a significant effect of treatment (H(3)=8.538, p=.036). Mann Whitney U test revealed the $CuSO_4$+DMSO treatment significantly altered the Lc3b levels in old cells U($N_{notreatment}$=4, $N_{CuSO4+DMSO}$=4) =.00, z=-2.309, p=0.021). (Figure 5.12, A) However, there was no significant age effect (H(1)=.115, p=.734) Furthermore, there was no significant effect of treatment (H(3) =4.167, p=.244) or age (H(1) =.00, p=1.00) for AL group. (Figure 5.12, B) Also, no significant significant effect of treatment (H(3) =1.596, p=.660) or age (H(1) =.029, p=.865) for OF group was observed. (Figure 5.12, C)

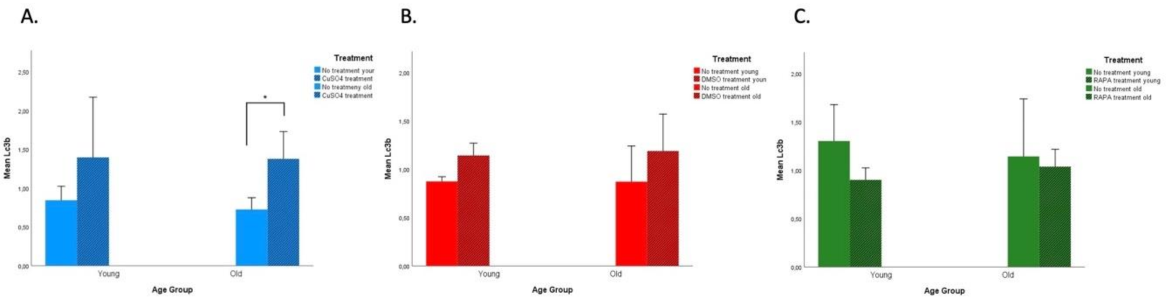


Figure 5.12. Distribution of pharmacologically treated and non-treated Lc3b gene expression levels by age. A: Intermittent fasting, B: *Ad libitum*, C: Overfeeding, Dashed lines indicate treatment groups, Error bars represent +SE (*: (p<0.05), ns: p> 0.05)

The gene expression levels of Nestin, a neural stem cell marker, revealed a significant treatment effect (H(3)=10.154, p=.017) and age effect (H(1)=7.385, p=.007) for the IF group. The Mann-Whitney U test revealed a significant difference between no CuSO4-treated old cells and CuSO4+DMSO treated old cells ($U(N_{notreatment}=4, N_{CuSO4+DMSO}=4)=.00$, z=-2.409 p=.023). (Figure 5.13, A) However, no significant effect of treatment (H(3)=6.167, p=.104) or age (H(1)= 115, p=.734) was observed for AL group. (Figure 5.13, B) For OF group, a significant treatment effect (H(3)=9.495, p=.023) was observed as elevated Nestin mRNA levels in no treated young cells compared to rapamycin+DMSO treated young cells ($U(N_{notreatment}=4, N_{RAPA+DMSO}=4)=.00$, z=-2.309 p=.021) But, no age effect was observed in OF group (H(1)=1.042, p=.307) (Figure 5.13, C)

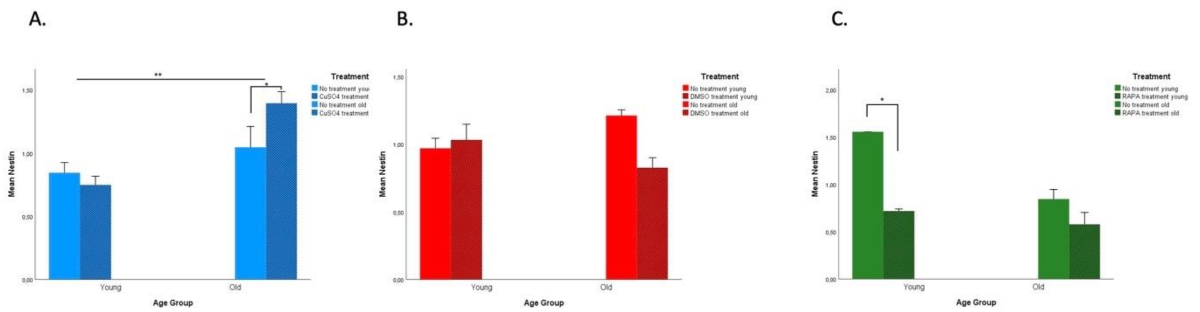


Figure 5.13 Distribution of pharmacologically treated and non-treated Nestin gene expression levels by age. A: Intermittent fasting, B: *Ad libitum*, C: Overfeeding, Dashed lines indicate treatment groups, Error bars represent +SE (**: (p<0.01), *: (p<0.05), ns: p> 0.05)

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5.4. DISCUSSION

The present study was designated to investigate whether there are molecular alterations in the IF, AL (*Ad libitum)*, and OF diet-treated young and old zebrafish with or without short-term pharmacological manipulations. While the main effects of the untreated group can be inferred in the analysis, this is not possible for the treatment groups. Due to the lack of DMSO-only control in the treatment groups, the significant effect in the conclusions to be drawn is the effect of drug+DMSO. The effect of DMSO cannot be distinguished in the current design.

Current data in Nrf2a expression revealed a significant difference between young and old groups in pharmacologically treated cells. A significant increase in the old group was observed. However, a significant treatment effect was not observed. Furthermore, there was no diet effect observed in the non-treated groups. Previous studies reported a significant Nrf2 reduction of mRNA levels in HFD, concluding that HFD feeding impairs the Nrf2 pathway to weaken the antioxidant defense system in the liver of tilapia. [47] Another study observed that the DNA-binding activity of Nrf was reduced in $Tlr4^{-/-}$ mice hippocampus. IF did not revert this effect, suggesting intermittent fasting effects on oxidative stress could be TLR4 dependent. [146] Consistent with the current study, intermittent fasting may not be regulating the Nrf2a expression. Furthermore, studies with mice in the literature have shown that $CuSO_4$-induced nephrotoxicity can be treated with quercetin. Quercetin treatment increased Nrf2 levels and inhibited the NfkB pathway. [147] However, in order to compare the studies in the literature with the current study, the results of $CuSO_4$ treatment after an anti-inflammatory treatment like intermittent fasting with proper DMSO control are necessary. Nrf2a's regulation pathway and its potential interaction with the mTOR pathway require further investigation. Subsequent research efforts can shed light on the underlying mechanisms driving Nrf2a activation and whether mTOR plays a role in influencing this process.

In terms of NfkB expression, a significant effect of age was revealed for dietary manipulated primary cells and rapamycin+DMSO treated cells. Significant increases in the old groups were observed in both cases. Coherent with the present data, previous studies have shown in Drosophila an age-related pattern of increased NfkB-controlled immune activity, and silencing NfkB in the brain of predisposed flies restored their lifespan. [148] [149]

In the Sox2 expression profile, a significant effect of diet was revealed. In young animals, the OF group's mRNA levels were significantly elevated compared to the AL groups. Furthermore, young OF group's mRNA levels were significantly altered compared to old OF groups. A significant effect of age was observed in treatment groups. In the IF cells, Sox2 levels were increased during aging, but in OF group, Sox2 levels were decreased during aging. Previous studies proposed that intermittent fasting enhanced hippocampal proliferation of NSCs in mice, and Sox2-positive cells were increased in the subgranular zone after traumatic brain injury. [150] The current study revealed the neurogenesis potential under OF diet was decreased during older ages.

In terms of BDNF, a significant effect of diet was observed for both young and old animals. In old animals, BDNF expression in the intermittent fasting group was significantly higher than in the AL and OF groups. However, the OF group showed a significant increase in young animals compared to the AL group. Besides, overfeeded young cell's mRNA levels were significantly higher than overfeeded old cells. Furthermore, rapamycin+DMSO treatment significantly downregulated BDNF mRNA levels in the young treatment group. Consistent with current study, previous research suggested that BNDF-induced long-term potentiation in rat hippocampus can be inhibited by rapamycin.[151] Current data shows in the aging brain, overfeeding may downregulate BDNF levels. Consistent with our findings, current literature on the zebrafish model of diet-induced obesity suggests decreased levels of BDNF mRNA in the gastrointestinal tract, implying the involvement of the BDNF/TrkB system in regulating food intake and energy balance in zebrafish, as well as mammals. [152]

Current data in Lc3b expression revealed no significant effect on the no-treatment group. However, a significant increase in the old $CuSO_4$+DMSO treated group was observed. Previous literature has shown a significant increase of LC3B-II at high $CuSO_4$ concentrations (600 μM and 800 μM) in the HepG2 WT cells, suggesting high copper concentrations can trigger autophagy in the cell. Although 5 μM $CuSO_4$ was used in the intermittently fasted cells, and the DMSO's effect can not be distinguished in the current study, a similar effect was shown for old animals, and the same pattern was observed for young animals. [153]

In terms of Nestin expression, a significant effect of treatment was observed for pharmacologically induced cells. A significant increase in the $CuSO_4$+DMSO treated group was revealed in old animals. Also, a significant decrease was observed in young animals for the rapamycin+DMSO-treated group. Furthermore, a significant diet effect was observed for dietary-manipulated cells. The OF group's expression was significantly higher in young cells than in the IF group. Furthermore, the OF old group's Nestin mRNA levels were significantly lower compared to its young counterpart. Suggesting overfeeding during older ages may lead to stem cell exhaustion. Previous research suggested rapamycin stimulating gene transcription related to neuronal differentiation while inhibiting nestin in U87MG cells, and a progressive dose-dependent decrease of nestin mRNA was also observed in U251MG and A172 cells at 24 h after rapamycin administration, which was coherent with current data. [154] However, contradictory data in the literature revealed that rapamycin prevented Ang II-induced podocyte damage via upregulating nestin. [155] Furthermore, previous studies demonstrate that exposure to a high-fat/high-fructose diet was associated with increased expression of TGF-β protein and decreased podocin and nestin expression, consistent with our results. [156]

CHAPTER 6

6. DISCUSSION

Inflammation is a cellular response mechanism that occurs due to the interaction of many different factors, as discussed in previous chapters. This thesis aimed to understand how inflammation can be triggered on different models, investigate potential inflammation-related biomarkers with the power of meta-analysis and observe the effect of inflammation in immortalized microglia cells. Moreover, to examine the effects of intermittent fasting, which is an mTOR downregulator, and high-fat diet, which induces inflammation, on the brain of zebrafish at the molecular level by primary cell culture method. Furthermore, the objective of short-term copper treatment in intermittent fasted zebrafish brains was to induce acute inflammation. In contrast, the short-term rapamycin treatment in high-fat treated zebrafish brains aimed to emulate successful therapeutic anti-inflammatory outcomes. The overall purpose of the primary cell culture study was to reverse the anti-inflammatory advantage of the intermittent fasting group and rescue the inflammatory phenotype of the high-fat group in terms of its effect on mRNA levels of various markers. The previous chapters outlined discussions on the outcomes of bioinformatic analysis, inflammation studies on the microglial immortalized mouse cell line, and zebrafish primary cell culture studies. (See Chapters 3.4, 4.4, and 5.4 for discussion within each study) Hence this chapter aims to give a comprehensive discussion and bind the outcomes of all studies. The comparison of the results to the human system also aimed to be covered. Although an inflammatory response is anticipated in both cell types, this chapter will address the variations in these responses and how they might affect the human system. Highlights of each chapter and their comparison to the human systems are as follows.

6.1. The Meta-analysis Results

The meta-analysis results found 11 common differentially expressed genes (DEGs) in pro-inflammation-induced cells. One common differentially expressed gene was found as a result of anti-inflammation treatment. The magnitude of fold changes observed in DEGs as a consequence of pro-inflammatory stimulation exhibited a notably heightened level compared to those arising from the anti-inflammatory stimulus, yielding a more striking outcome. In the KEGG database results, DEGs were shown to be involved in various pathways, including the TNF signaling pathway, the Toll-like receptor signaling pathway, and the NF-kB signaling pathway. From the pool of acquired DEGs, particular attention was directed toward identifying and selecting genes characterized by the most substantial fold changes, concluded as Tlr2 and Nfkbie. Human literature has shown that, in cell culture of differentiated human SHSY5Y cells, activation of neuronal TLR2 induced an inflammatory response, including the secretion of TNFα mRNA and the production of reactive oxygen species, supporting the current meta-analysis. [157] Human literature for Nfkbie, which used Metabolic Gene Rapid Visualizer data from more than 4000 microarrays, revealed Nfkbie was the only NFκB inhibitor in its family with high expression and improved prognosis in hepatocellular carcinoma. GSCA database analysis suggested that Nfkbie could inhibit the PI3K/AKT, RAS/MAPK, RTK, and TSC/mTOR pathways. [158] Hence, these highly modulated genes could be selected as promising candidates and subjected to assessment in subsequent studies. Other bioinformatics tools such as Enrichr and Reactome can be employed in the future as two efficient online resources often used in the literature for functional annotation since, in the current study, the DAVID Bioinformatic Resources were used for KEGG pathway analysis. [132], [133]

6.2. The Response of Mouse Microglia Cells to Inflammation

mRNA expression assessments for the pro-inflammatory cytokines IL-6 and IL1b, as well as Tlr2 and Nfkbie, were not found to be significant on $CuSO_4$ studies with the BV2 mouse microglia cell line. However, an increasing trend was observed for IL1b and IL-6 mRNA levels depending on the $CuSO_4$ dose. This trend changes when it reaches the 7.5 μM level. It was also observed that Nfkbie was in a dose-dependent increasing trend. Since Nfkbie is an inhibitor of NfkB, it may be inferred that in 1-hour treatment, the anti-inflammatory response may already dose-dependently increase in response to $CuSO_4$ treatment in mouse immortalized cells. In future studies, more precise results can be obtained by measuring the gene expression of anti-inflammatory cytokines. According to the literature, inflammation is regularly induced in mouse cells using LPS (lipopolysaccharide), and models using $CuSO_4$ result in inflammation in an oxidative stress-related manner in zebrafish. Fish embryos develop in aquatic environments, but mouse microglia cells are situated in intricate mammalian neurological systems. It is also important to consider how the stimulant was utilized. $CuSO_4$ may have a different capacity to cause inflammation in mouse cells than it does in fish embryos. $CuSO_4$ targets oxidative-stress-related inflammation that can activate different pathways or create a milder inflammatory response for mouse cells when compared to LPS, which may cause a more potent inflammatory response. As a result, variations in experimental conditioning and biological variations among models could demonstrate that mouse microglia cells may undergo a similar inflammatory process quickly. When comparing inflammatory stimulants such as LPS and $CuSO_4$, it should be considered that $CuSO_4$ triggers inflammation as a result of oxidative stress. In this context, for aquatic organisms such as zebrafish, oxidative stress may be effective as a more pronounced stress factor in their biological systems. Aquatic organisms may be more sensitive to environmental factors related to oxidative stress that can be caused by various chemical and physical changes in their habitats. On the other hand, using a bacterial stimulant such as LPS for mice might mean that it can activate their defense systems more rapidly and show a more acute inflammatory response. LPS can activate defense mechanisms against bacterial pathogens, initiating a robust immune response. As a result, oxidative stress may be a more effective stress factor for zebrafish, an aquatic organism. At the same time, the robust immune response against bacterial infections may be more pronounced for mice. The biological characteristics and defense mechanisms of these two different organisms may influence the nature of inflammatory responses. In addition, studies in the literature show that viral-derived agents are commonly used to initiate a robust inflammatory cellular response for humans.[159],[160] In conclusion, it is essential to develop an inflammation model while accounting for the variances intrinsic to the biological systems of the chosen model organism. These findings led us to the third study of the thesis.

6.3. The Response of Diet-Treated and Pharmacologically Induced Primary Cells to Inflammation

With or without short-term pharmacological manipulations, molecular alterations in the diet-treated young and old zebrafish brains were investigated. Comparing the results obtained in the current study for Nrf2a, a marker of antioxidant capacity, with the literature, it can be inferred that OF may reduce antioxidative capacity, especially in old age. Other pathways could regulate intermittent fasting effects on oxidative stress, perhaps TLR4. It is necessary to investigate the regulation mechanism of Nrf2a and its potential interactions with the mTOR pathway. Nrf2/Keap1 signaling expression was evaluated in literature in human iPSC-derived NSCs differentiating into neurons and astrocyte-like cells. Consistent with current results on pharmacologically treated cells, the literature showed when NCSs were differentiated following exposure to rotenone (oxidative stress inducer), researchers noticed a progressive activation of the Nrf2 pathway. [161] It can be concluded that another avenue to explore is the relationship between Nrf2 signaling and neuronal differentiation. The fundamental mechanisms causing

Nrf2a activation and whether mTOR has an impact on this process can be revealed by future studies, with the inclusion of proper DMSO control groups.

Age significantly impacted NfkB expression for dietary- and pharmacologically induced primary cells. There were significant increases in the older animals. Moreover, human literature has demonstrated age-related NfkB activity raises. For example, human fibroblasts from older adults show excessive NfkB activation. [162] Also, older mice have increased DNA binding to NfkB in their skin, liver, kidney, cerebellum, and heart muscle. [163] Thus, the utilization of NfkB as a marker in examining age-related inflammation proves to be efficient.

A significant effect of diet on the Sox2 expression profile was revealed. Overfeeding significantly altered mRNA levels in young animals compared to the *Ad libitum* in terms of Sox2 expression; furthermore, during overfeeding, Sox2 mRNA expression was reduced in older animals, revealing diminished neurogenesis potential. Consistent with our old group results, it is widely recognized that intermittent fasting after acute inflammation increases neurogenesis by affecting the expression of Sox2 in other mammals. [164] Additionally, pharmacologically manipulated cells revealed age was affecting the Sox2 expression levels differently according to treatment type. However, the existence of this mechanism in humans NSCs should be clarified with future studies.

BDNF expressions in young and old cells revealed different significant responses to diet. In young animals, OF upregulated the BDNF mRNA compared to AL, but in old animals, IF upregulated the BDNF levels compared to both AL and OF. Furthermore, OF was significantly downregulating BDNF levels during aging. Earlier studies have hinted at rapamycin's potential to hinder BDNF-induced long-term potentiation for the rat hippocampus. Human literature considers direct and indirect pathways by which decreased activity of BDNF may confer vulnerability to overeating and weight gain in an obesogenic environment.[165] However, further exploration in human studies is needed to clarify the interplay between BDNF and mTOR pathway in the context of aging. [151]

There was no significant evidence that dietary stimulated cells are significantly affected by Lc3b expression in the current study. High $CuSO_4$ concentrations have been found in the literature to significantly enhance LC3B-II in HepG2 WT cells, indicating that high copper concentrations can cause autophagy in the cell. Although 5 µM $CuSO_4$ was utilized in the intermittently fasted animals in the current work, and the DMSO's effect can not be separated, both young and old animals had the same pattern as literature, the old being significant. This result can be interpreted as a higher defense mechanism against inflammation, and increased autophagy at older ages may be needed. Human literature for Alzheimer's disease showed that Aβ25-35 induced inflammatory responses and induced Lc3b, and β-asarone protected cells from Aβ25-35 induced inflammation and attenuated autophagy. [166] However, the significance of Lc3b and dysregulated macroautophagy in healthy brain aging requires sustained investigation.

Our current work highlighted a significant downregulation of Nestin in the overfed old group compared to their younger counterparts. Furthermore, a significant effect of age and treatment was observed for pharmacologically induced cells. In old animals, a significant increase in the $CuSO_4$+DMSO-treated group was revealed. Also, in young animals, rapamycin+DMSO treatment significantly decreased Nestin mRNA levels. However, the DMSO's effect should be separated in future studies by the addition of DMSO control in each diet group. There are contradicting data in the literature about Nestin's expression under rapamycin treatment.[154], [155] While some studies have shown that rapamycin inhibits Nestin, others have shown that rapamycin inhibits Ang II-induced podocyte damage by upregulating Nestin. Considering potential variations in Nestin expression profiles between kidney and brain tissues, it is essential that findings about brain aging in the literature should be confirmed through human studies and elucidated at a molecular level.

The comprehensive dataset showed that, with or without pharmacological interventions, in the cerebral context of both young and old, as well as intermittently fasted and high-fat diet-exposed subjects, inflammation exerted diverse effects on various cellular mechanisms, including oxidative stress, macroautophagy, and neurogenesis in distinctive patterns.

CHAPTER 7

7. Conclusions and Future Perspectives

This study revealed that copper sulfate+DMSO is an efficient inflammatory reagent for zebrafish. Copper sulfate+DMSO treatment was successful in increasing mRNA levels of Nrf2a, suggesting altered oxidative stress in old animals. Furthermore, regardless of diet and treatment group, NfkB expression levels were higher in whole old animals. This noteworthy finding underscores the association between aging and chronic inflammation. Lc3b levels were in an upregulation pattern in the copper+DMSO-treated groups in young and old animals, and it was significant for old animals. These results capture the higher defense mechanism against inflammation, and increased autophagy at older ages is essential. Nestin's mRNA levels were decreased during aging in the OF group. Also, BDNF's mRNA levels were downregulated in the old OF group. This observation may suggest that overfeeding could contribute to compromised neurogenesis in advanced stages of life. On the contrary, the enhanced neurogenesis potential of intermittent fasting in old animals was revealed by increased mRNA levels in Sox2, even under acute stress, and age was affecting the Sox2 expression levels differently according to treatment type.

Copper sulfate treatment did not cause a direct inflammatory response in BV2 mouse microglia cells. However, depending on the $CuSO_4$ dose, an upward trend for IL1b and IL-6 mRNA levels was observed. Nfkbie was also noticed to be developing in a dose-dependent manner. After just one hour of treatment, the anti-inflammatory response to $CuSO_4$ treatment in mouse immortalized cells may already be rising in a dose-dependent manner. Oxidative stress may function as a more potent stress factor in the biological systems of aquatic species like zebrafish. Targeting defensive mechanisms against bacterial infections (such as LPS stimulation) may cause mice to develop a rapid and potent immune response. In conclusion, these results reveal that it is critical to establish an inflammation model considering the variations inherent to the model organism's biological systems.

This study has some limitations, and future research could eliminate them. First of all, for the meta-analysis, Enrichr and Reactome can also be employed in the future since only the DAVID Bioinformatic Resources were used for functional annotation. The sample size for each treatment group in the BV2 and primary cell culture studies can be increased. There were some data points that exhibited considerable heterogeneity, indicating some individual variability within the dataset. These results may have been significant by adding more cohorts. Also, copper's effectiveness was not enough to capture a significant inflammatory response in Chapter 4, possibly due to the biological characteristics of different models. Anti-inflammation-related cytokines may added in future research on BV2 cells. The usage of only young and old animals for Chapter 5 is another limitation. Adding a middle-aged group in between can help better capture the effects of inflammation and preventive strategies over the course of aging. In the context of the experiments conducted in Chapter 5, including DMSO-treated feeding groups is deemed essential to facilitate a meaningful comparison between pharmacologically treated and untreated groups. In the present experimental design, any conclusions drawn would not be attributable solely to the impact of the drug itself; rather, they would reflect the combined effect of the drug and DMSO. Although male and female animals were used in the Chapter 5 study, sexually dimorphic characteristics were not examined. Males and females may experience neuroinflammation development differently. [167] Hence more animals should be included in the study to undertake this type of analysis. Moreover, certain effects observed within the marker profiles might exhibit distinct variations across diverse brain regions. For example, the hippocampus in mice or the telencephalon in zebrafish brains are mainly responsible for age-related neurobiological changes. [168] Region-specific alterations may be monitored by dissection of the brain regions or immunohistochemical methods. Additionally, changes in mRNA levels may not always correspond to changes in protein expression. Following mRNA synthesis, post-translational modifications could alter the protein expression levels. [169] As a result, the outcome of qPCR may not correspond to those of protein expression. Further research can examine related post-translational modifications and how they affect protein expression in inflammaging. Ultimately, all of the studies have been done in mouse immortal cell lines or zebrafish primary cell cultures. In this regard, primary human cell cultures or cerebral organoids may provide a closer resemblance to in vivo conditions. However, it should be noted that no *in vitro* model can fully reflect *in vivo* conditions. Therefore, in vivo experiments in zebrafish or mice can be performed to strengthen the study's findings.

Consequently, this study has demonstrated that the modulation of neuroinflammatory responses, as well as oxidative stress, neurogenesis, and autophagy, occurs in an age-related manner. Moreover, it suggests that dietary or pharmaceutical interventions could yield comprehensive outcomes in perceiving the inflammatory profile of the aging brain.

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