EFFECTS OF AGING ON GENE EXPRESSION LEVELS OF INFLAMMATORY, CYTOSKELETAL AND MICROGLIAL MARKERS IN THE BRAIN USING THE ZEBRAFISH (DANIO RERIO) MODEL ORGANISM

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE

OF BILKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF

MASTER OF SCIENCE

IN

NEUROSCIENCE

By

Hande Özge Aydoğan

January 2021

EFFECTS OF AGING ON GENE EXPRESSION LEVELS OF INFLAMMATORY, CYTOSKELETAL AND MICROGLIAL MARKERS IN THE BRAIN USING THE ZEBRAFISH (DANIO RERIO) MODEL ORGANISM

By Hande Özge Aydoğan

January 2021

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

Michelle Marie Adams(Advisor)

Özlen Konu Karakayalı

Çağdaş Devrim Son

Approved for the Graduate School of Engineering and Science:

Ezhan Karaşan
Director of the Graduate School

ABSTRACT

EFFECTS OF AGING ON GENE EXPRESSION LEVELS OF INFLAMMATORY, CYTOSKELETAL AND MICROGLIAL MARKERS IN THE BRAIN USING THE ZEBRAFISH (DANIO RERIO) MODEL ORGANISM

Hande Özge Aydoğan
M.S. in Neuroscience
Advisor: Michelle Marie Adams
January 2021

Age-related cognitive decline burdens the elderly population, limiting their ability to socialize and be independent. To be able to develop proper treatments, healthy aging should be examined. Previous studies focusing on healthy brain aging revealed that abnormal microglial activation was observed. Aging microglia exhibits 0partial loss of motility due to cytoskeletal changes, leading to decreases in their ability to respond to environmental cues. Thus, a more inflammatory phenotype was observed in microglia. These disruptions of the previously established homeostasis in the brain could be the underlying reason for cognitive decline experienced during aging. To understand these changes during aging in the brain, cytoskeletal, microglial, and inflammation-related markers were investigated by using both *in silico* and *in vivo* approaches. *In silico* analyses were performed on mice hippocampus and the whole brain revealed that the genes involved in the actin cytoskeleton reorganization (Arpc1b), neurogenesis (Erbb4), and proinflammatory related pathways (Il1b, P2x7r, Elf2b) showed differential gene expression levels among different age groups, genders, and tissue of origin. On the other hand, no differential expression was observed in microglial (Coro1a and Aif1) and anti-inflammatory markers (Tgfb1 and Il10). To further validate these results *in vivo*, quantitative polymerase chain reaction (qPCR) was performed on young and old zebrafish brains. According to the results, only two genes showed marginally significant differences among young and old brains: *arpc1b* and *p2x7r*. These results collectively could mean 1) the overall microglia population does not change during aging, 2) the brain does not exhibit imbalances in terms of pro- and anti-inflammatory cytokines, and 3) neurogenesis. Furthermore, the significant changes observed in *arpc1b* and *p2x7r* indicated the iii iv importance of the

cytoskeleton and inflammation-related pathways in the correct functioning of the cells. Therefore, this study showed that *in silico* analysis are the reliable indicators of *in vivo* experiments, zebrafish can be used as a gerontological model, and the importance of cytoskeleton in motile cells. However, to understand these described relations, further investigation on the protein level of these genes should be done.

Keywords: aging, glia, neurons, neurogenesis, qPCR, zebrafish, bioinformatic, mice.

ÖZET

ZEBRA BALIĞI (DANİO RERİO) MODEL ORGANİZMASI KULLANILARAK YAŞLANMANIN BEYİNDEKİ İNFLAMATUAR, HÜCRE İSKELETİ, VE MİKROGLİA BELİRTEÇLERİNDEKİ GEN İFADE DÜZEYLERİ ÜZERİNE ETKİLERİ

Hande Özge Aydoğan
Nörobilim, Yüksek Lisans
Tez Danışmanı: Michelle Marie Adams
Ocak 2021

Yaşa bağlı bilişsel gerileme, yaşlı nüfusun sosyalleşme ve bağımsız olma yeteneklerini sınırlar. Doğru tedavi yöntemleri geliştirebilmek için ise sağlıklı yaşlanma incelenmelidir. Sağlıklı beyin yaşlanmasına odaklanan önceki çalışmalar, anormal mikroglial aktivasyonun gözlemlendiğini ortaya koymuştur. Hücre iskeletinde yaşlanmaya bağlı değişikliler yaşan mikroglianın haraket kapasitesinde kısmi bir azalma gerçekleşir ve bu da çevresel sinyallere yanıt verme yeteneğinin azalmasına sebep olur. Böylece mikrogliada inflamasyona daha yatkın bir fenotip gözlenir. Bu şekilde aktive olan mikroglia, beyinde önceden kurulmuş olan homeostazın bozulmasına sebep olur ve bu bozulmaların ise yaşlanma sırasında yaşanan bilişsel gerilemenin altında yatan neden olduğu düşünülmektedir. Beyindeki yaşlanma sırasındaki bu değişiklikleri anlamak için hücre iskeleti, mikroglial ve inflamasyon ile ilgili belirteçler hem *in siliko* hem de *in vivo* yaklaşımlar kullanılarak araştırıldı. Farelerde hipokampus ve tüm beyin üzerinde yapılan in siliko analizler, aktin hücre iskeletinin yeniden düzenlenmesinde (Arpc1b), nörogenezde (Erbb4) ve proinflamatuar ile ilişkili yolaklarda (Il1b, P2x7r, Elf2b) yer alan genlerin farklı yaş, cinsiyet ve dokular arasında, ifade seviyelerinin istatistiksel olarak anlamlı bir fark gösterdiğini ortaya çıkardı. Öte yandan, mikroglial (Coro1a ve Aif1) ve anti-inflamatuar belirteçlerde (Tgfb1 ve Il10) istatistiksel olarak anlamlı bir ifade farkı gözlenmedi. Bu sonuçları *in vivo* yaklaşım ile doğrulamak için, genç ve yaşlı zebra balığı beyinleri üzerinde kantitatif polimeraz zincir reaksiyonu (qPCR) gerçekleştirildi. Sonuçlara göre, genç ve yaşlı beyinler arasında marjinal olarak anlamlı farklılık gösteren sadece iki gen vardı: arpc1b ve p2x7r. Bu sonuçlar toplu olarak 1) genel mikroglia popülasyonunun yaşlanma sırasında değişmediği, 2)

beynin pro- ve anti-inflamatuar sitokinler ve 3) nörogenez açısından dengesizlikler göstermediği anlamına gelebilir. Ayrıca, arpc1b ve p2x7r genlerinde gözlenen anlamlı değişiklikler, hücrelerin doğru işleyişinde, hücre iskeletinin ve inflamasyonla ilişkili yolların önemini göstermiştir. Bu nedenle, bu çalışma, in siliko analizlerinin, *in vivo* deneylerin güvenilir göstergeleri olduğunu, zebra balıklarının gerontolojik bir model olarak kullanılabileceğini ve hareketli hücrelerde hücre iskeletinin önemini gösterdi. Ancak, açıklanan bu ilişkileri daha iyi anlayabilmek için, bu genlerin protein seviyeleri hakkında daha fazla araştırma yapılması gerekmektedir.

Anahtar sözcükler: yaşlanma, glia, nöron, nörogenez, qPCR, zebrabalığı, biyoinformatik, fare.

Acknowledgement

First, I would like to express my sincere and deepest gratitude to my supervisor Professor Michelle Adams for accepting me into her research team, her guidance, support and patience throughout these years. I have learned invaluable academic and life lessons from her.

I want to thank Assoc. Prof. Özlen Konu Karakayalı and Assoc. Prof. Çağdaş Devrim Son for accepting to be in my thesis defence committee. I also want to give a special thank for Assoc. Prof. Özlen Konu Karakayalı for providing the help and guidance when I needed the most.

I also want to thank all the members of Adams research team; Serena Sevdiye Aktürk, Elif Tuğce Karoglu-Eravşar, Begün Erbaba, Naz Mengi, Duygu Mutlu and Melek Umay Tüz-Şaşik. I would like to give special thanks to Duygu Macaroğlu, Beyza Özen and Narin Ilgım Ardıç-Avcı for their endless support and friendship. I would like to especially thank Ergül Dilan Çelebi Birand for her invaluable guidance, support and help. I want to thank my previous intern Ece Tuğlacı for helping me with literature review. From Konu Lab, I want to thank Ayşe Gökçe Keşküş and Melike Tombaz for providing their help while I was performing *in silico* analysis.

I would like to thank my closest friends; Şifa Çelik, Ünzile Çetinceviz and Ali Yasin Vergili. When things are not going well, you were always there for me with your emotional support and friendship.

I want to thank all my family from the bottom of my heart. My parents, Nergiz and Erdal Aydoğan, have supported me in every possible way throughout my life. Kardelen, my favorite person in the whole world, thank you for being the sweetest and most amazing sister. Your unconditional love means everything to me. I also want to thank my fiancée, Burak Balaban. Throughout these years, you have never failed me to surprise, make me laugh and amaze. I could not do this without your endless encouragement, support, patience and love.

I want to acknowledge the Scientific and Technological Research Council of Turkey (TUBITAK) with grant no 119S660 for financially supporting these experiments.

Contents

Abbrevations

ABP	Actin-binding Proteins
AD	Alzheimer's Disease
AKT	Protein Kinase B
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
Aif1	Allograft Inflammatory Factor 1
Arp2/3	Actin Related Protein 2/3
CNS	Central Nervous System
DAMPs	Damage-associated Molecular Patterns
DDR	Dna Damage Response
DEGs	Differentially Expressed Genes
EGFR	Epidermal Growth Factor Receptor
ELF2	E74-like Factor 2
Erbb4/Her4	Erb-b2 Receptor Tyrosine Kinase 4
FMWB	Female Mice Whole Brain
GEO	Gene Expression Omnibus
GO	Gene Ontology
IGF1	Insulin-like Growth Factor 1
IL1β	Interleukin 1 Beta
IL10	Interleukin 10
IL4	Interleukin 4
Iba1	Ionized Calcium-binding Adapter Molecule
Il10rb	Il10 Receptor B Subunit
IHC	Immunohistochemistry
LPS	Lipopolysaccharide
MAVOVA	Multivariate Analysis of Variance
MAPK	Mitogen-activated Protein Kinase
ND	Neurodegenerative Disorders
NFκB	Nuclear Factor Kappa B
NLRP3	Nod-like Receptor Family Pyrin Domain Containing 3

NRG	Neuregulin
OLs	Oligodendrocytes
P2X7R	Ionotropic Purinergic Receptor P2x 7
PAMPs	Pathogen-associated Molecular Patterns
PCA	Principal Component Analysis
PI3K	Phosphatidylinositol 3-kinase
qPCR	Quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SC	Stem Cell
SE	Standard Error
SGF	Sarcoma Growth Factors
SGZ	Subgranular Zone
SMAD	Mothers Against Decapentaplegic Homolog
SVZ	Subventricular Zone
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factor Alpha
VCP	Valosin-containing Protein
limma	Linear Models For Microarray Data
mTOR	Mechanistic Target Of Rapamycin
mtDNA	Mitochondrial Dna

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Aging

The average life expectancy in humans has increased to 72.3 with the development of advanced technologies and medicine. There were 703 million people on earth who are over 65-year-old (elderly population) in 2019. It has been estimated that this number will be around 1.5 billion by 2050 [1]. More specifically, in Turkey, it has been projected that the percentage of people who belong to the elderly population will be 22.6% in 2060. As a result, since aging is one of the risk factors of developing neurodegenerative disorders (ND), the people who died because of Alzheimer's Disease (AD) increased by around 1% in 2018 compared to previous years [2]. Probably, with the addition of other age-related diseases and NDs, this percentage becomes higher, which affects a significant portion of the population. In conclusion, the population in every country is aging rapidly [1]. Modern research on aging has been going on for almost three decades, yet aging has not been fully characterized due to its complex nature [3]. To prevent or even slow down the progression of age-related NDs, healthy aging in the brain should be examined. In other words, to shed light on the complex nature of aging in the brain, studying the effects of aging on the brain without any age-related disease or ND is required.

1.2 Age-Related Biological Changes in the Brain

During the process of aging, the human body, specifically the human brain, experiences changes and disruptions. These changes result in the altered functioning of the organisms, leading organisms to become more vulnerable to diseases and death [4]. Examining the hallmarks of aging first will provide the information required to comprehend the types of changes inside the cells resulting from aging.

1.2.1 Hallmarks of Aging

In 1956, Denham Harman postulated that free radicals in the body are one of the reasons leading to aging. This hypothesis can be explained as the damage caused by the by-products of redox reactions such as reactive oxygen species (ROS), oxygen-free radicals in general, is the main contributor to the processes of aging. Due to disruptions in redox homeostasis, ROS concentration in the tissue could increase and lead to cell and tissue damages [5]. However, the body has a defense system consisting of small molecules and enzymes which are collectively called antioxidants. Their role is to neutralize the harmful effects of ROS if the maximum required concentration is exceeded to protect the cells and tissues. Yet, ROS is used as a signaling molecule in many organisms [6]. Therefore, ROS could be both beneficial and harmful depending on the concentration, and balancing the ROS concentration (ROS homeostasis) has a vital role in the healthy functioning of the organisms [7]. Nevertheless, some organs are more susceptible to damage caused by ROS because they have lower antioxidant concentration compared to others such as the brain. Therefore, evading the deleterious effects of ROS by balancing its concentration is difficult in these organs [8][9].

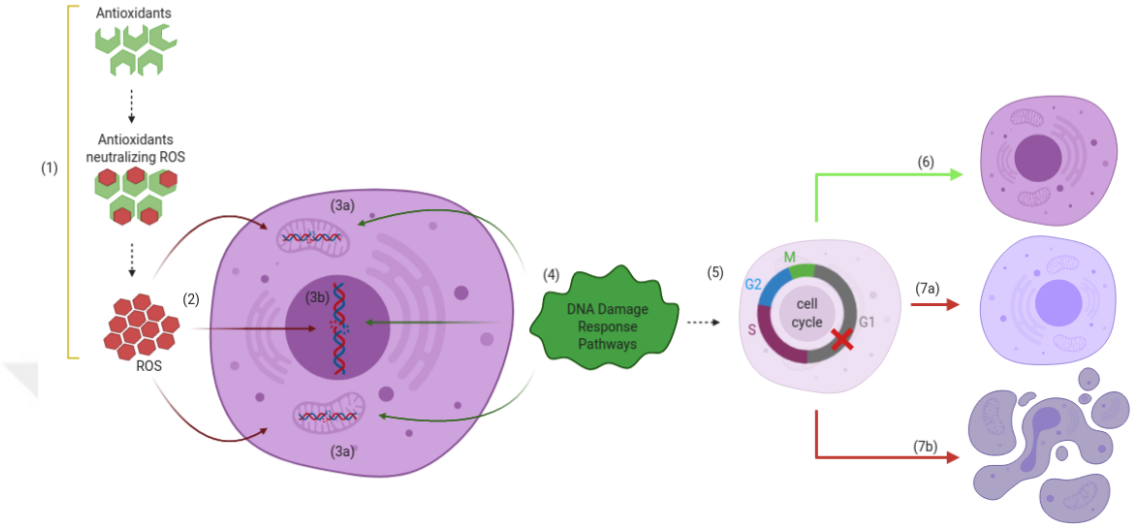


Figure 1.1: Hallmarks of aging. With the increase in ROS concentration, antioxidants initiate the neutralization of ROS. If the antioxidant amount is insufficient, excess ROS freely floats inside the cell (1), reacting with various molecules, proteins and lipids in the cell, especially DNA (2). This reactivity results in DNA damages in both mitochondrial (3a) and nuclear (3b) DNA. Activation of DDR due to DNA damage (4) leads to cell cycle arrest (5). During this period, if the DNA damage is fixed, the cell continues to enter the cell cycle. However, if it cannot be fixed, the cell undergoes either senescence (7a) or apoptosis (7b). This image is created with BioRender.com

In another review by Cadet and Wagner, it was stated that there are many types of mutations found in the DNA as a result of ROS concentration going beyond the antioxidant capacity [10]. Therefore, there is growing evidence that ROS is responsible for DNA damage as well. DNA damage could be in both nuclear DNA and mitochondrial DNA (mtDNA). It is important to note that introducing a mutation into nuclear DNA has far less probability than in mtDNA due to the more error-prone nature of polymerase enzyme in mitochondria and the absence of histones, which normally prevent DNA from damage [11][12]. These mutations could be caused by external DNA-damaging agents and metabolites produced during normal cellular processes. When not repaired, it leads to genomic instability by disrupting the integrity of the genome [13].

The response given to DNA damage is called DNA Damage Response (DDR). DDR includes proteins that are involved in cell cycle arrest, repair mechanisms, senescence and apoptosis. The damaged DNA is recognized by the sensors activating cell cycle regulators by downstream signaling. When the cell is undergoing checkpoints, these regulators signal downstream to stop the cell cycle which is called cell cycle arrest. During this arrest, first, the cell tries to fix the damage by repair mechanisms. If the damage cannot be repaired, then the cell either undergoes senescence or apoptosis [14][15] (**Figure 1.1**).

Imbalance in ROS concentration could also participate in inflammation. Increased ROS level in the cell activates Nod-Like Receptor (NLR) Family Pyrin Domain Containing 3 (NLRP3) which has been known to involve Interleukin 1 beta (IL1β) maturation. IL1β is a proinflammatory cytokine that is released when a cell encounters danger such as pathogens [16][17]. As a result, inflammation is initiated and inflammatory responses given by immune cells could also lead to apoptosis [18]. The cellular deficit caused by the apoptosis of these cells should be compensated with the generating new cells. The type of the cells that provide this compensation are called stem cells (SCs). Like every cell, SCs are also subjected to the hallmarks of aging such as increased concentration of cellular ROS and telomere shortening due to its continuous division. As the organism ages, because of the discussed events, the number of SCs and their functionality decreases. This phenomenon is called stem cell exhaustion, the incidence increases with age [19].

Taken together, mutations in the genome caused by the increased cellular ROS concentration due to aging could lead to cellular apoptosis or senescence. Apoptotic cells can initiate cellular responses including inflammation and it can cause further damage to tissues leading to increased apoptosis. Even though SCs are specialized to close this cellular gap, they are also affected negatively by described cellular events, leading to stem cell exhaustion. Therefore, the hallmarks of aging are linked to each other, triggering one leads others to be induced. These hallmarks are not specific to only one cell type but rather general. To comprehend these hallmarks within the brain, one should be familiar with neuronal and glial changes (major cell types found in the brain) during aging.

1.2.2 Brain Aging

The brain is one of the most vulnerable organs in the body to disruptions in ROS homeostasis. The human brain is responsible for consuming almost one-fifth of the oxygen supplied to the body, on average. However, the number of antioxidant enzymes are insufficient in the brain [8]. Therefore, as humans age, with the combination of high oxygen consumption and an inadequate amount of antioxidant enzymes, the brain becomes more susceptible to oxidative damage, as suggested by Halliwell B [9]. As explained previously, unbalanced ROS concentration could lead to NLRP3 activation, resulting in increased IL1β production and inflammation [17]. It has been thought that inflammation is one of the causes of cognitive decline which occurs during aging, as discussed extensively in Sartori et al. [20]. This is among the most common healths problems among the elderly population, affecting many people including the patients and their families, as well as the whole population [21]. Therefore, examining brain aging has undeniable benefits to both individuals and the society.

To be able to understand brain aging at the cellular level, the cell types found in the brain should be discussed first. There are two major cell types in the brain: neurons and glial cells (neuroglia). These two cell types work in harmony to establish healthy communication among the cells found in the brain [22]. However, during aging, these cell types also go through significant changes disrupting the established harmony.

1.2.2.1 Neuronal Changes

Neurons are the one type of cell that can communicate with each other via electrical and/or chemical signals [23] to perform neurological processes in the brain and central nervous system (CNS). Therefore, these highly specialized cells are essential for the functioning of the CNS. They are one of the most polarized cells in the human body, having subcellular divisions specializing in specific functions [24].

Molecular changes in the cells due to aging could affect the viability and functioning of the cells, as previously discussed. One of the hallmarks of aging is stated as the shrinkage in the volume of the brain and the earliest study focusing on this shrinkage revealed that it is due to decreased neuron numbers [25]. In the studies following the footsteps of Harold Brody, experiments report neuronal number decreases during aging. However, scientists started to question the accuracy of the methods used in these experiments. To count the neurons in the brain more accurately, the development of new methods had started. In 1991, Mark West published an article reporting a new stereological method for counting neurons more accurately without bias [26]. That development led to a burst in the number of published articles using this method which is still being used.

However, the results of these articles indicated that neuron count does not vary during aging with only insignificant changes (**Figure 1.2**). One of the pioneering examples was provided by Rapp and Gallagher in 1996. The authors stated that the number of neurons found in the hippocampus remains stable during aging [27]. The method they used for counting neurons accurately has been still in use. Following this development, the method was used in rats (hippocampal and subicular neurons) [28] and primates (cerebral cortex) [29]. On the other hand, there is a decrease detected in neuron numbers even though it is not statistically significant. For example, this insignificant decrease can be detected in the auditory system of rats during aging [30]. This probably means that, even though neuronal death occurs during the course of aging, there is a mechanism compensating the neuronal deficit in the brain, producing newborn neurons when required. This mechanism is called neurogenesis; birth, maturation and integration into the existing network of new neurons [31].

Previously, it was thought that neurogenesis is restricted to embryonic development, and during adulthood new neurons cannot be generated. However, recently, it is established that neurogenesis can occur in adults as well [32][33]. Unlike neurogenesis, mammalian adult neurogenesis is restricted to several regions, specifically neurogenic niches: the subventricular zone (SVZ) in the lateral ventricle and subgranular zone (SGZ) in the hippocampus. However, due to aging, there is a decline in the rate of neurogenesis [34]. This decline can be

explained with increased inflammation in the tissues during aging.

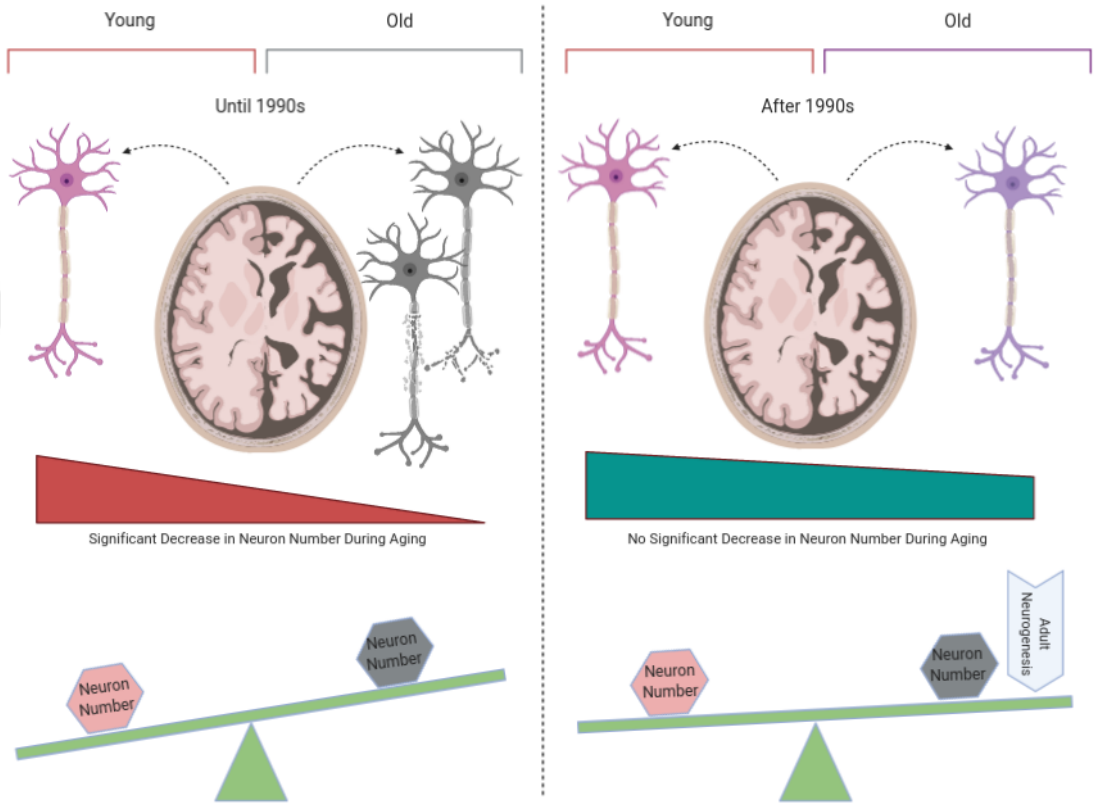


Figure 1.2: The timeline of studies using different strategies for counting neurons and corresponding results. Until the 1990s, it was thought that the neuron number decreases during aging in the brain. However, with the development of new stereological methods, it was proved that the neuron number does not decrease significantly during aging. This balance could be accomplished by adult neurogenesis. This image is created with BioRender.com

The link between neurogenesis and neuroinflammation is complex due to several reasons. As explained in Russo et. al, neurogenesis can be affected negatively or positively depending on the duration of inflammation and the type of activation of glial cells (neurotoxic or neuroprotective), including microglia and astrocytes. Mainly, proinflammatory cytokines released by microglia can reduce neurogenesis; however, exercise, environmental enrichment, or release of

an anti-inflammatory cytokine such as interleukin 4 (IL4) can increase neurogenesis [35]. As mentioned previously, proinflammatory cytokine IL1β is generated due to the age-related increase in cellular ROS level through NLRP3 [17]. The release of proinflammatory cytokines shifts the inflammation to a more neurotoxic state [36]. Therefore, these have been thought to be the possible causes of decreased neurogenesis. However, neurogenesis continues throughout the life of organisms. This could be the reason why even though the number of neurons decreases, the change is not statistically significant.

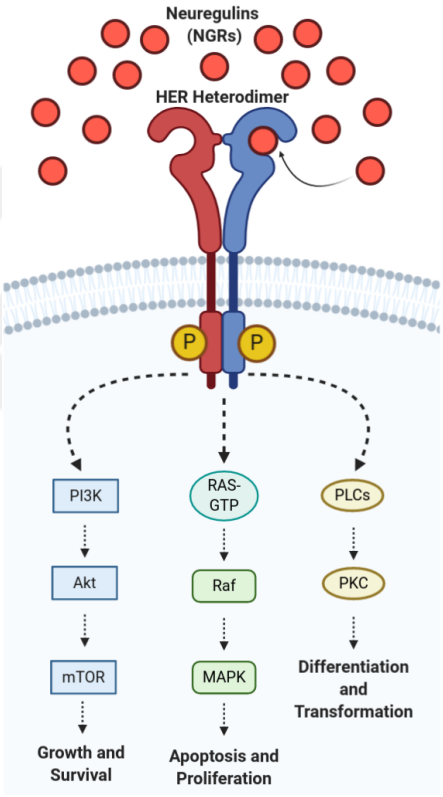


Figure 1.3: The pathways initiated by Erbb receptors. Upon receptor activation through NRGs initiates signaling cascades leading to various cellular responses. This image is created with BioRender.com

To be able to have a better understanding of the relation between neurogenesis and neuroinflammation, Erb-b2 receptor tyrosine kinase 4 (Erbb4) can be used. Errb4 is a cell surface kinase receptor belonging to the epidermal growth factor receptor (EGFR) subfamily. Binding to its ligands such as neuregulins (NRGs) activates Erbb receptors and initiates various cellular responses including growth and survival through PI3K/Akt/mTOR pathway, apoptosis and proliferation through Ras/Raf/MAPK pathway, and finally differentiation and transformation through PLCγ1/PKC Pathway (**Figure 1.3**). As can be interpreted, the EGFR family can trigger many different pathways and result in various cellular responses [37]. In terms of CNS, the reports on the functions of NRG1 and Erbb4 (Her4) indicated that they are involved in both embryonic [38][39] and adult neurogenesis [40][41][42]. Deletion of Erbb4 in parvalbumin-positive

interneurons results in inhibition of adult neurogenesis in the hippocampus by decreasing the survival along with the maturation of neurons that are newly generated, indicating the importance of Erbb4 [43]. Furthermore, disruption in Erbb signaling is associated with several NDs that are related to neuroinflammation, such as schizophrenia [44], AD [45] and amyotrophic lateral sclerosis [46]. Additionally, other than ND, Erbb4 also is involved in many cancer types including breast cancer as explained thoroughly in Sundvall et al. [47]. Moreover, inflammation induced by lipopolysaccharide (LPS) results in differential expression in NGR1/Erbb4 in the hippocampus, being one of the regions showing active neurogenesis during adulthood. Furthermore, it has been shown that neuroinflammation, apoptosis of neurons, and cognitive impairment could be caused by the disruption in the signaling of NGR1/Erbb4 [48].

Even though there are abundant articles and research on this issue, this area is still under investigation. As reviewed by Alsegiani and Shah, synapses found in neurons show altered, specifically decreased plasticity due to aging, and this results in impaired neuronal communication and could be the reason for cognitive decline. Furthermore, this decreased plasticity could be initiated by the altered inflammatory response produced by microglia [49]. Moreover, a human study examining transcriptional differences during aging indicated that most of the differentially expressed genes are specific to the glial cells, but not neurons [50]. In light of these findings, scientists have started to investigate glial changes, especially microglial changes due to the altered inflammatory response during aging.

1.2.2.2 Glial Changes

Glia function as supporting cells to establish an environment to make neurotransmission possible and faster, as well as protecting neurons from damages. Glial cells can be divided into three categories depending on their function in the brain and CNS in general: oligodendrocytes (OLs), astrocytes, and microglia. These three cell types can only be found in CNS [51].

Even though these cells are all specialized to perform specific functions and have specific transcriptome signatures, they are affected by aging similarly. The number of glial cells could be changing like neurons as discussed in the neuronal changes section (1.2.2.1), due to apoptosis and decreased rate of proliferation of progenitor cells and producing mature OLs, astrocytes, and microglia. These could be again related to stem cell exhaustion. Other common changes can be categorized as morphological changes, senescence, and becoming dysfunctional [52] (**Figure 1.4**).

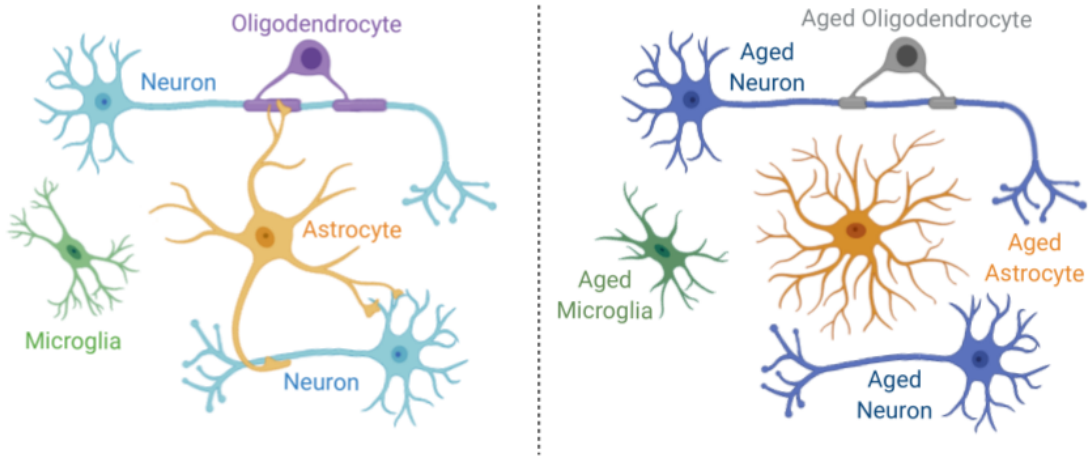


Figure 1.4: During aging, both neurons and glial cells experience many changes, ranging from morphological to functional. Oligodendrocytes could lose their sufficient myelin sheath production and become more prone to senescence, while astrocytes adopt reactive morphology and could lose their connection with both neurons and other glial cells. At the same time, like astrocytes, microglia adopt activated morphology and become cytotoxic. This image is created with BioRender.com

1.2.2.2.1 Oligodendrocytes

OLs are the main factory for CNS myelin sheath production in vertebrates. Wrapping around axons with myelin sheath successfully decreases the time spent during neurotransmission from one cell to another, thus providing efficient conduction of nerve impulses [51]. Other than the myelination of axons, OLs have a pivotal role in neuronal survival. To accomplish neuronal survival, while OLs

synthesize trophic factors such as insulin-like growth factor (IGF1) [53][54], brain-derived neurotrophic factor (BDNF) [55], and glial cell line-derived neurotrophic factor (GDNF) [56][54]), they also provide energy to neurons since neurons require a significant amount of energy to initiate neurotransmission, yet they cannot store the sufficient amounts [57].

Myelin sheath production is an energy-dependent process and thus the oxygen requirement of OLs is higher [58]. Producing adenosine triphosphate (ATP) from oxygen generates byproducts such as hydrogen peroxide. If generated hydrogen peroxide cannot be eliminated or neutralized, it was shown to induce DNA damage and apoptosis [59]. Neutralization of hydrogen peroxide is established by glutathione, an antioxidative enzyme. However, it has been shown that OLs have lower glutathione levels [60]. This lethal combination causes apoptosis or dysfunctional OLs. Since increased oxidative damage is one of the hallmarks of aging, OLs are one of the cells that are vulnerable to aging. OLs have been shown to undergo changes in terms of morphology, myelin dynamics, rate of oligodendrogenesis (birth of new OLs), and therefore changes in cell number as discussed in Salas et al. [52].

1.2.2.2.2 Astrocyte

Star-shaped glia named astrocytes is the most abundant cell type in the CNS, comprising one-fifth of the total brain cells. In some regions, astrocytes outnumber the neurons [61]. Even though it was believed that astrocytes are only important for holding brain cells together by providing structural support, previously known as the brain glue, now it is known for regulating essential functions such as maintaining the signaling among neurons and the healthy functioning of the cells [62]. More specifically, they are required for (i) neurogenesis by guiding axons and aiding synapse formation, (ii) protecting homeostasis of synaptic transmission, and (iii) synapse functioning, (iv) providing the required energy to neurons when needed by breaking down glycogen from its storage, and (v) maintaining the blood brain barrier [63]. Therefore, it is not surprising that during the course of aging, astrocytes can function abnormally.

During aging, processes of astrocytes become shorter and thicker in rodents, primates and humans [64][65][66]. There is still a debate on whether the number of astrocytes is increased, decreased, or exhibiting no change during aging [67]. These changes could depend on the brain region, counting method, and marker that has been used to identify astrocytes. Additionally, they adopt a reactive (i.e. activated) phenotype, shifting towards a proinflammatory state and affecting expression levels of many genes involved in various pathways [68]. There is also evidence for an interplay among glial cells, especially between astrocytes and microglia. It has been shown that proinflammatory microglia could have an impact on astrocytes, shifting them to a reactive state by releasing cytokines during inflammation [69]. Therefore it is important to understand how microglia are affected by aging to be able to understand aging as a whole.

1.2.2.2.3 Microglia

Microglia are derived from yolk sack during embryogenesis and have a role in protecting brain-resident cells from any damage whose function is similar to macrophages, their immune system counterpart. Since their gene expressions are unique, they are identified as a specific cell population [70]. They constantly surveil the environment to obtain information about the resident cells and the environment. They can phagocyte to clear pathogens, viruses, and debris to create a safe environment for other cells. However, the most significant function of the microglia is to secrete many cytokines to initiate inflammation, which results in the protection of healthy cells while destroying the infected or abnormal ones. Therefore, it is the first and only line of immune defense in the CNS [22]. The next section will be based on microglial changes during aging.

1.3 Microglia and Aging

During aging, one of the most affected cell types found in the brain is microglia. Since aging is one of the risk factors of developing neurodegenerative disorders

(ND) [20], one of the proposed models for the mechanisms underlying ND is impaired functioning of microglia. This impairment results in altered inflammatory responses given by microglia to the pathogens, viruses and injuries [71]. In this section, how cytoskeleton, morphology and function are related with each other and affected by aging will be covered.

1.3.1 Motility Requirements and Its Relation with Cytoskeleton

As previously mentioned, microglia are mobile cells performing two types of motility: surveillance and directed motility [72]. On the contrary to former thought that microglia is active only in the presence of an infection or pathogen, microglia continue to be active under normal physiological conditions which is called baseline activity, resting state, or surveillance mode. When a microglia is in surveillance mode, it inspects the environment with its processes extending and retracting for a substance that could damage the other cells and be a sign of inflammation. Via this inspection, microglia could perform phagocytosis to clear such substances and obtain information about functioning neurons and other glial cells [73]. However, upon encountering a substance that potentially could harm the cells, microglia are activated and switched from surveillance mode to directed mode. The switch from surveillance and directed motility is an important step for initiating immune responses.

The molecular mechanisms for switching from surveillance mode to directed mode and, vice-versa, are complex and established by the involvement of the cytoskeleton [74]. Cytoskeleton, one of the most conserved structures during evolution [75], provides support for the cells with the three main structures: (1) microfilament (actin), (2) microtubules (tubulin), and (3) intermediate filaments [76]. With these structures, a cell is capable of undergoing differentiation and apoptosis, maintaining and modifying the cellular shape, being motile, and performing signaling transduction as well as many other cellular functions that provide cells with the necessary flexibility to be dynamic [77].

1.3.1.1 First Requirement: Changing Cellular Shape

In the brain, for microglia to perform its functions such as phagocytosis, surveilling the environment, and responding to cues; the first requirement is the ability to change their shape. This flexibility is provided by creating membrane protrusions by the rearrangement of the cytoskeleton, specifically, the actin filaments. This rearrangement is possible due to actin-binding proteins (ABP). As the name suggests, these proteins bind to actin to create two distinct actin structures: actin bundles generated by cross-linking actin filaments to form parallel and densely packed actin polymers, and actin networks produced by branching the actin filaments [77]. In motility, since protrusions cannot be generated without actin network assembly and disassembly, these ABPs have vital importance as extensively reviewed in Blanchoin et al [78].

Lamellipodia and filopodia are two distinct cellular protrusions formed by actin bundles and branched actin networks, respectively. They are structures required for motility and phagocytosis [79][80]. Filopodia formation is accomplished by cross-linking protein, allograft inflammatory factor 1 (Aif1), also known as ionized calcium-binding adapter molecule (Iba1). Aif1 is found only in the macrophages, and microglia [81]. Hence, in the studies focusing on the brain, Aif1 has been used to label microglia [82][83].

On the other hand, lamellipodia formation depends on an actin-binding protein, Actin Related Protein 2/3 (Arp2/3) complex, to create actin branches [79]. Furthermore, actin branches, formed by the Arp2/3 complex, provide a scaffold for lamellipodia and filopodia. The Arp2/3 complexes belong to ABPs, and generate actin branches by binding to minus ends of actin filaments and nucleating actin polymerization with 70° angle which is constant [84]. This sophisticated and unique branching ability capacitates cells to be motile since branching pushes the membrane forward. Arp2/3 complex is composed of 7 subunits: actin-related proteins that are the major subunits of this complex (Arp2 and Arp3), Arpc1b (p41-Arc), Arpc2 (p34-Arc), Arpc3 (p21-Arc), Arpc4 (p20-Arc) and Arpc5 (p16-Arc) [85] (**Figure 1.5**).

One of the regulators of the Arp2/3 complex is conserved coronin containing 5 WD (Trp-Asp) repeats which enables the binding of coronin with other proteins. The regulation is performed by directly interacting with both Arp2/3 complex and F-actin via WD repeats, contributing to the branch formation required for motility [86][87]. The efficiency of phagocytosis is directly related to coronin presence. For instance, yeast cells without coronin lose about two-thirds of their phagocytic ability [88].

In 1998, it was revealed that coronin is conserved in mice and humans, and there are at least 4 proteins belonging to the coronin family. These were named coronin 1, -2, -3, and -4 [89]. Now, there are known six members of the coronin family in the mammals [90]. The expression levels of each member varies across different tissues. The cells expressing Coronin 1,such as microglia, were shown to originate from hematopoietic stem cells. Even though expression of Coronin 1 in the brain is lower compared to other coronin members, Coronin 1 has been used as a microglial marker due to its specificity to these cells [89][91]. These seemingly contradictory results may be explained by the discrepancies between the total microglial percentage in the whole brain (up to 12%) and regional heterogeneity, which could increase the microglial population up to 14-15% [92].

Aif1, Arp2/3 complexes and Coronin1a are influenced by aging. Aif1 is significantly increased during aging in the gerbil spinal cord [93] and hippocampus [94] and rat CNS [95]. Additionally, humans without dementia have increased levels of IBA1 as well [96]. It has been thought that the increase in IBA1 expression indicates an increased number of immunoreactive microglia. On the other hand, four subunits of the Arp2/3 complex (Arp2, Arp3, Arpc2, and Arpc5) are downregulated in the mice hippocampus [97]. Moreover, the transcriptomic studies performed on human microglial cells discovered that ARPC1B showed a decreased expression along with several genes that have been shown to interact with the actin cytoskeleton during aging. This study also revealed that Gene Ontology (GO) terms of the enriched genes during aging are “immune response”, “cytoskeleton” and “motility” [98].

Another study focusing on mortality and gene expression levels collected blood

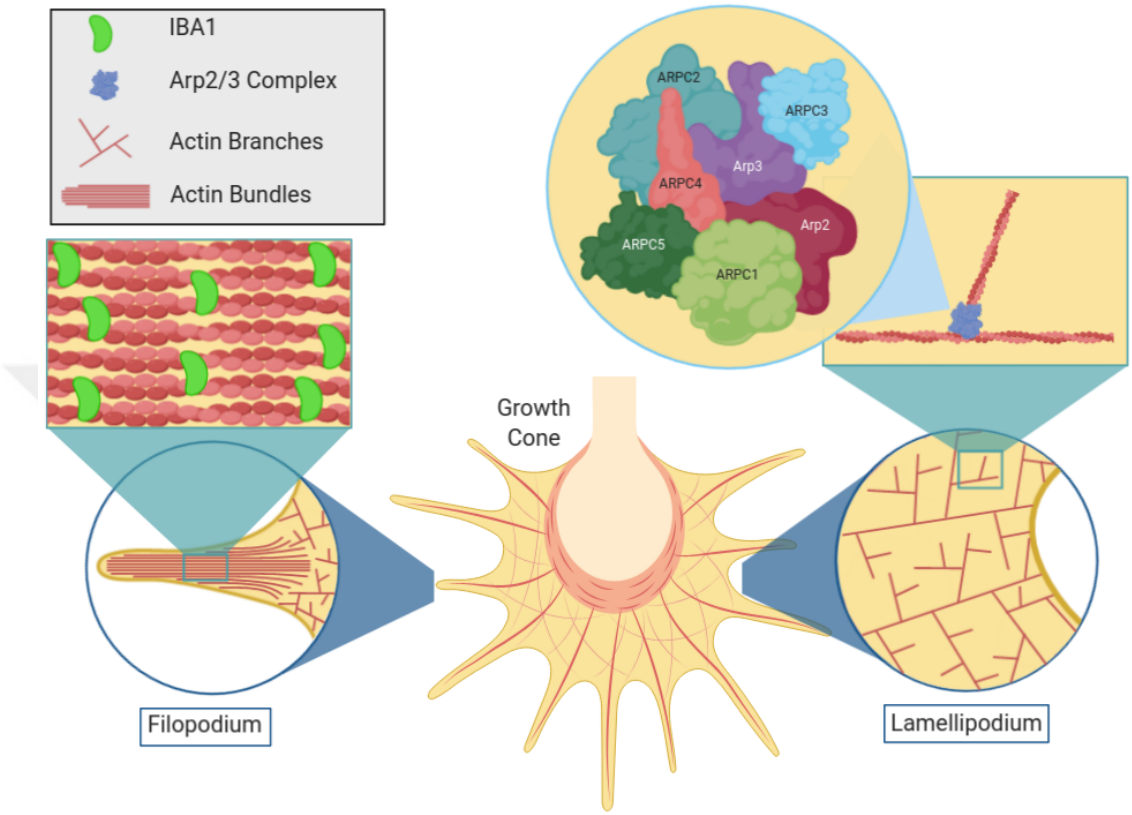


Figure 1.5: The formation of filopodia and lamellipodia depends on actin bundles and actin branches. While actin bundles are created by IBA1 (Aif1), the Arp2/3 complex is responsible for creating actin branches. This image is created with BioRender.com

from elderly, middle-aged, and young participants who are related. The result was CORO1A shows decreased expression during the aging process, and there is a negative correlation between expression level and mortality, meaning mortality is increased [99]. Additionally, microglia isolated from human post mortem brains revealed that expression levels of coronin 1a is reduced significantly during aging [98], supporting previous findings in the brain. Furthermore, Coronin 1a has been thought to be involved in NDs such as AD. Activation of Coronin 1a showed a decreased amount of Aβ load in the SAMP8 mice brains [100]. Taken together, these motility-related proteins may have key roles in the neurobiological processes that are affected during aging.

1.3.1.2 Second Requirement: Sensing Environmental Cues

The second requirement for microglia is to be able to sense the environmental cues so that it can migrate toward a specific direction and initiate inflammatory responses. One of these cues is the ATP released from other cells in the brain to the extracellular matrix due to physiological abnormalities. Under normal circumstances, ATP is found inside the cell and extracellular ATP could be considered as a danger signal since it can trigger inflammatory responses [101]. On the other hand, ATP can also function as a signaling molecule and neurotransmitter [102]. Purinergic receptors found on the surface of microglia detect and direct the microglia to the source of the ATP, which eventually induces inflammation. Among the purinergic receptors, ATP-gated ionotropic purinergic receptor P2X 7 (P2X7R) is one of the most interesting receptors.

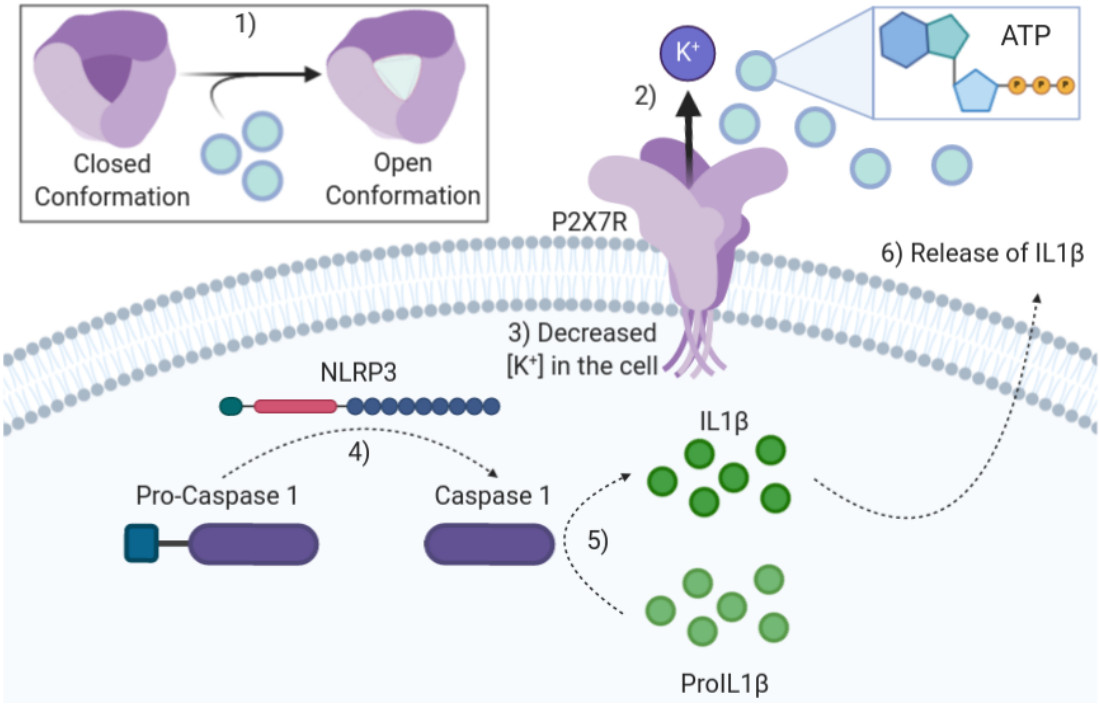


Figure 1.6: Involvement of P2X7R in IL1β secretion. 1) When P2X7R encounters with ATP, a conformational change occurs, 2) increasing its permeability to K+. 3) Efflux of K+ leads to a decrease in its concentration in the cell and 4) causing NLRP3 activation. Activated NLRP3 in return activates caspase 1. 5) Caspase 1 cleaves proIL1β and generates mature IL1β. 6) Then IL1β is released from the cell. This image is created with BioRender.com

As mentioned previously, purine nucleotides can initiate inflammatory response but also they can function as signaling molecules and neurotransmitters in the healthy cells found in the brain [102]. The detection of purine nucleotides is accomplished by purinergic receptor families that have 15 members in total. These receptors are found mostly in microglia and are the sensors of purine nucleotides. Results from an RNA sequencing study performed with human and mouse microglia revealed that P2X4R, P2X7R and P2Y6R proteins -belonging to purinergic receptor families- are highly expressed in microglia as compared to astrocytes [103].

In 2001, a mice line that does not express the P2x7 gene was generated. Studies conducted with this line indicated that even though the absence of P2x7 gene does not cause any significant developmental abnormalities, following ATP addition to the environment, this mice line failed to release cytokines belonging to the IL1 family such as IL1β. In other words, mechanisms for producing cytokines are impaired without the P2x7 gene [104].

One of the hypothesized mechanisms for IL1 secretion is that upon encountering ATP, P2X7R provides microglia with enhanced permeability to certain cations such as K+, Ca+, and Na+ [102]. Lowered K+ concentration in the cell activates caspase 1, which subsequently induces IL1 maturation [105]. The activation of caspase 1, on the other hand, has been proposed to be accomplished by glial NLRP3 inflammasome [106] (**Figure 1.6**).

Elevation of inflammatory responses in the brain during aging has been thought to contribute to the decline in cognitive functioning. Furthermore, the link between NDs and inflammation has been a debate for decades. In a 2011 study focusing on P2x7 receptor and its antagonist, GSK1370319A, it has been revealed that blockage of P2x7 receptor could be potentially protective. According to this study, GSK1370319A successfully prevented the NLRP3 inflammasome assembly and IL1β maturation. Furthermore, it provided a neuroprotective effect and decreased long term potentiation in aged animals [107]. Therefore, P2X7R

has been considered as the contributor to inflammatory response leading to age-related NDs [102]. Consequently, many studies that focus mainly on the therapeutic effect of abolishing the normal functioning of P2X7 receptors in NDs such as AD [108], HD [109], and PD [110] emerged.

In light of these findings, it can be inferred that the microglial motility and phagocytosis depend on many different proteins, complexes, and receptors. The involvement of many proteins in the orchestrated regulation of the actin cytoskeleton increases the risk of altered functioning due to their susceptibility to aging. This complex structure of the actin cytoskeleton therefore lowers the possibility of compensating the mutations with other regulatory proteins involved in actin organization [111]. Taken together, changes in the motility of microglia during aging could depend on cytoskeletal reorganizations. The disruptions of proteins involving actin reorganization could potentially be a target for anti-aging studies. Therefore, Iba1 (Aif1), Arpc1b- a subunit of the Arp2/3 complex-, Coro1a and P2x7 has pivotal importance that cannot be neglected, and worth examining.

1.3.2 Cytoskeleton Dictates Morphology

Microglia continue to be active under normal physiological conditions, which are called resting-state, or surveillance mode. The processes of resting-state microglia adopt a ramified morphology, thus it is also called ramified microglia. Resting-state microglia inspect the environment with its processes, constantly extending and retracting to detect a substance that could damage the other cells and be a sign of inflammation. Via this inspection, microglia could perform phagocytosis to clear such substances and obtain information about the functioning neurons and other glial cells. Therefore, microglia is active even in the absence of infections and injuries [73]. Upon encountering a substance that could potentially harm the cells, microglia are activated and switched from surveillance mode to directed mode by adopting an amoeboid morphology, which provides the ability to perform phagocytosis. Additionally, during this activation, microglia start to produce and release inflammatory molecules by changing gene expressions. Furthermore, its processes become less in number and much ticker

while the cell body enlarges [112]. These switches between surveillance mode to directed mode are required for the correct functioning of microglia.

The pattern recognition receptors such as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) alert the cells for possible insults [113]. This recognition results in microglia to adopt "activated" morphology and initiates the release of proinflammatory cytokines, which are molecules secreted by immune cells, and used for the initiation of inflammation to protect the neighboring cells. This is also known as classically activated or M1 microglia. Normally, M1 microglia is cytotoxic and useful for the clearance of dangerous substances and balancing the homeostasis in the brain. After the clearance, the damage caused by substances or the actions of M1 microglia should be healed so that the correct functioning of the cells in the brain can be preserved. After the completion of the clearance, healing of the damages caused by cytokines and substances initiating inflammation, or the actions of M1 microglia should take place. Initiating the healing process can be achieved by the release of anti-inflammatory cytokines. As a result, inflammation decreases. Thus, microglia switch to M2 state, protective microglia, and release anti-inflammatory cytokines [114][115]. Since both M1 and M2 microglia release different types of cytokines, they have unique gene expression patterns. Therefore, detecting and differentiating M1 and M2 stages based on specific gene expression levels is possible (**Figure 1.7**).

Beside ramified and activated microglia, there are many morphologies adopted by microglia. Their functions are changing according to their morphologies, proving that morphology has vital importance for healthy functioning, as mentioned previously [116]. Therefore, it is important to understand how morphology defines the function, to better interpret the inflammatory responses given by microglia.

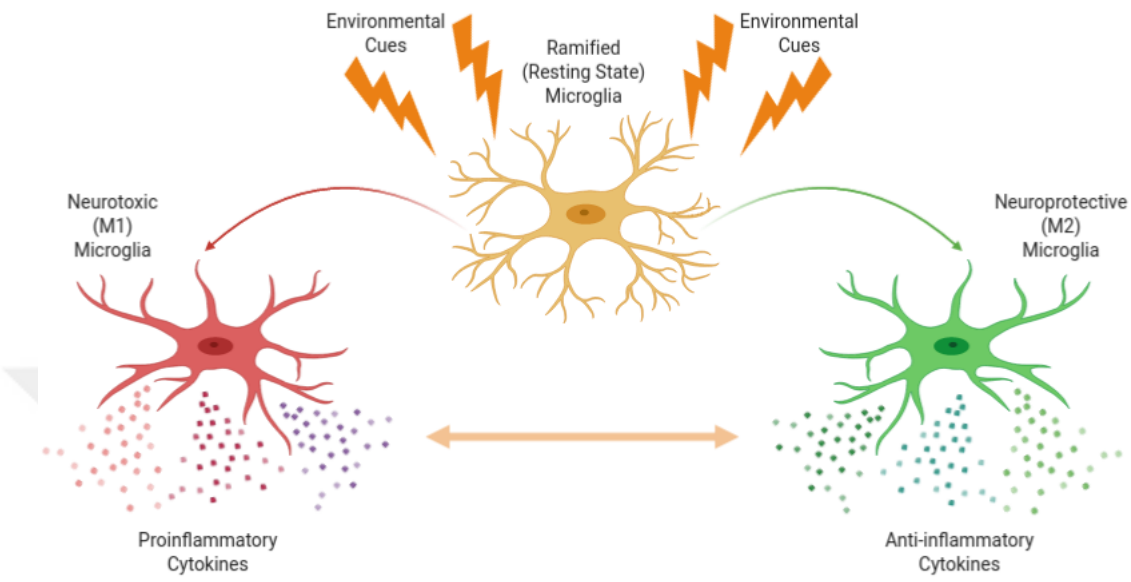


Figure 1.7: Microglial Morphologies and Activation States. Ramified microglia have many branches to survey the environment better. During surveillance, if microglia receive danger signals indicating inflammation, they become activated (i.e. M1-type activation) and start releasing proinflammatory cytokines. After the danger is eliminated, to be able to heal the environment, they switch to an M2 activation state and release anti-inflammatory cytokines. This image is created with BioRender.com

1.3.3 Morphology Dictates Function

1.3.3.1 M1 Microglial Markers

As previously mentioned, M1 microglia release proinflammatory cytokines as a response to injuries (e.g. presence of apoptotic cells) and infection to fight against them to protect the homeostasis [114]. Therefore, when microglia are in the M1 stage, the expression levels of these cytokines are increased. These proinflammatory cytokines which are going to be discussed below are IL1β and tumor necrosis factor alpha (TNFα).

1.3.3.1.1 IL1β

Interleukin-1 beta (IL1β) is one of the proinflammatory cytokines that are produced by microglia. IL1β belongs to the IL1 family consisting of 11 members as extensively reviewed by Charles A. Dinarello in 2009 [105] and was discovered around the early 1970s [117]. All members of the IL1 family have an ability to start inflammation, both systemic and local, to fight against microbes, viruses, and the substances that disrupt brain homeostasis. Both types of inflammation are required for the destruction of the cause of inflammation. Later, the members of IL1 family assist in the healing process [105]. Furthermore, it has been demonstrated that synaptic plasticity can be modulated by IL1β [118].

Upon recognition of DAMPs and PAMPs through pattern recognition receptors, inflammasome complex assembly is initiated. Under normal circumstances, IL1β is produced as an inactive precursor in the cells, and obtaining fully functional IL1β from the precursor is achieved through the cleavage of IL1β precursor by caspase 1 enzyme. NLRP3 inflammasome provides a scaffold for caspase 1 to cleave precursor IL1β so that active and fully functional IL1β can be obtained [16].

1.3.3.1.2 TNFα

Following the discovery of TNFα in 1975 [119], its characterization was completed in 1984 [120]. The name refers to its ability to induce cell death by apoptosis and necrosis in tumor cells [119]. Microglia is one of the main sources of TNFα production [121]. When the cell receives a production stimulus for TNFα, it starts to produce the inactive precursors of TNFα. The inactive precursor is cleaved by tumour necrosis factor-alpha converting enzyme, which results in activated TNFα. After activation, TNFα starts to accumulate in triplicates generating active homotrimers. These homotrimers bind to their corresponding surface receptors, TNF receptor 1 and 2 (TNFR1 and TNFR2, respectively), found both in neurons and glia [122] and stimulating multiple pathways, including the nuclear factor kappa B (NFϰB) pathway.

This pathway is responsible for inducing genes related to inflammation, such as TNFα [123]. This creates a positive feedback loop, amplifying the effects exerted by TNFα [124]. Besides, TNFα can initiate neuroprotective responses through NFϰB. Under normal conditions, hippocampal synaptic plasticity, the fundamental cellular processes of learning and memory, is regulated by TNFα through NFϰB [125]. Furthermore, TNFα can synergize with interferons such as interferon-gamma to exert its cytotoxic and inflammatory effect [126].

1.3.3.2 M2 Microglial Markers

As mentioned previously, M2 microglia is known as protective microglia and responsible for the production of anti-inflammatory cytokines after the dangers are eliminated. Therefore, during the healing process, the shift from M1 to M2 microglia requires changes in gene expression levels of both pro- and anti-inflammatory markers. While the expression levels of pro-inflammatory markers decrease, those of anti-inflammatory markers increase. The markers that will be discussed below are transforming growth factor beta (TGFβ) and interleukin 10 (IL10).

1.3.3.2.1 TGFβ

In 1978, a pioneering study on malignant cell transformation was published. Mouse fibroblasts infected with Murine Sarcoma Virus were found to release growth factors that are polypeptides. These polypeptides "transform" fibroblast cells to grow progressively, unlike normal fibroblast cells. These growth factors compete with the epidermal growth factor to bound their receptors (EGFR). Since these newly identified polypeptides stimulated growth in these cells, they were named Sarcoma Growth Factors (SGF). Partial purification of SGFs paved the way for other studies [127]. Later, in the early 1980s, purification and characterization of SGF were performed, and it was discovered that SGF composed of 2 polypeptides: TGF α and β [128]. In the late 1980s, it was discovered that TGFβ has three isoforms in mammalian tissues: TGFβ1, TGFβ2, and TGFβ3.

The three TGFβ isoforms are inactive precursors [129].

The active TGFβ can initiate two TGFβ signaling pathways: canonical and noncanonical. Both pathways start with the activated ligand, TGFβ, binding to its receptor dimer (type I and type II TGFβ receptors, TβRI and TβRII respectively), cross phosphorylation of the receptors. The canonical pathway continues with the phosphorylation of mothers against decapentaplegic homolog (SMAD) by the receptors, which attracts other SMAD proteins, forming a complex. Later, this complex goes into the nucleus and initiates the transcription of TGFβ target genes [129].

On the other hand, the noncanonical TGFβ pathway could interact with other signaling pathways such as mitogen-activated protein kinase (MAPK), NFkB, phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), and mechanistic target of rapamycin (mTOR) without the SMAD involvement. The noncanonical pathway can produce a variety of responses ranging from differentiation to uncontrolled proliferation [130]. The deficiency of TGFβ1 has been implicated in defective synaptic plasticity and a decrease in dendritic spines of hippocampal pyramidal cells residing in the CA1 region. Furthermore, TGFβ1 deficiency causes brain weight reductions and increased astrogliosis [131]. Additionally, a previous study indicated that loss of TGFβ1 in the mouse brain causes increases in both neuron loss and microgliosis [132].

1.3.3.2.2 IL10

Interleukin-10 (IL10) is a cytokine that was purified and characterized in 1989. IL10 was named as cytokine synthesis inhibitory factor because of inhibiting the production of cytokines by the Th1 cells and Th1 cell activation [133]. Later, an independent study indicated that IL10 was produced by B cells and Lyl+ B cell lymphomas, not Th2 cells [134]. Now it is known that other than Th2 cells and B cell lymphomas, several cell types found in the immune system such as subtypes of T cells (CD4+ and CD8+ T), macrophages, dendritic cells, Natural Killer cells, and microglia can produce IL10 [135][136][137].

IL10 superfamily is composed of several cytokines along with the main member IL10, including IL19, IL20, IL22, IL24, IL26, IL28, and IL29 [138]. IL10 forms homodimers via two disulfide bonds provided by four cysteine residues [139]. Binding to its heterodimer receptor (IL10RI and IL10RII) initiates the Janus Kinase Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway. The signaling cascade results in the STAT3 translocating to the nucleus and reducing the gene expression levels of cytokines and major histocompatibility complex II [140][141].

The deficiency of IL10 in the mice leads to retardation in growth and chronic enterocolitis. Moreover, the subjects developed out-of-control inflammation which resulted in inflammatory bowel disease [142]. Additionally, it was shown that proinflammatory cytokines (TNFα, IL1β, and interferon gamma) produced due to bacterial infection can be inhibited by IL10, supporting its anti-inflammatory role [143]. Besides, it has been proven that insufficient IL10 in the body could be the potential cause of neurodegeneration and autoimmune diseases [144]. Moreover, when the IL10 receptors of rat hippocampal neurons are knocked down, microglia-induced synaptic formation disappears as well. This indicates the importance of the microglial role and IL10 in synaptic formation. In zebrafish (*Danio rerio*) gills, loss of IL10 results in an exaggerated inflammatory response, indicating both IL10 is indeed an anti-inflammatory cytokine, and zebrafish have a paralog of IL10 [145].

1.3.3.3 Inflammation-Related Marker

E74-like factor 2 (ELF2), is a transcription factor regulating T- and B-cell-specific genes, and expressed in fetal and adult tissues. It was characterized in 1996 as a new Ets-related factor (NERF) and its three distinct isoforms were discovered [146]. Even though characterization was completed almost 30 years ago, the functions of ELF2 remain to be elucidated. According to the Uniprot Database, the GO terms associated with ELF2 are regulation of transcription by RNA polymerase II (GO:0006357), cell differentiation (GO:0030154), negative and positive regulation of transcription, DNA-templated transcription

(GO:0045892, GO:0045893, GO:0006355), and regulation of B cell receptor signaling pathway (GO:0050855) [147]. Mostly, cancer-related research areas focus on ELF2. For example, in one study, two of ELF2 isoforms, ELF2A and ELF2B were investigated for their roles in primary and cancer cell lines. Overexpression of ELF2B or ELF2A resulted in different responses in the cells. While ELF2B overexpression resulted in a significantly decreased cell proliferation and increased apoptosis, ELF2A overexpression showed more marginal effects on proliferation and apoptosis [148].

It was also proposed that ELF2 was probably involved in the early development of T and B cells together with myeloid cells [148]. Yet another study using the human liver cancer cell line (HepG2) discovered that overexpression of ELF2 induces tumor cell proliferation, and depletion of it results in slowing down tumor growth [149]. Collectively, these two studies refer to the importance of ELF2 in cell proliferation. Furthermore, ELF2 target valosin-containing protein (VCP), a protein that participates in NFκB activation in B cell lines [150], has been shown to regulate survival and proliferation in a breast cancer cell line while under cytokine stress [151]. More specifically, oxidative stress caused by neuronal injury makes the hippocampal VCP more vulnerable, indicating its significance in inflammatory conditions. Furthermore, according to PathCards, which is an online database for human biological pathways, there are two pathways that ELF2 is involved in: Immune response IL23 signaling pathway and adenosine diphosphate signaling through P2Y purinoceptor 12 [152]. It is important to note that, P2Y12 receptor is involved in chemotaxis [153] and can be used for resting-state microglia identification [154]. Therefore, there is a link between ELF2 and P2Y12 receptors, emphasizing the relation of ELF2 and inflammation.

As discussed previously, still little is known about ELF2 and most of the known functions emerged from cancer-related research. Furthermore, research related to aging and ELF2 has only been conducted in a limited number of organisms. From previous discussions, it can be concluded that ELF2 is involved in proliferation and apoptosis; the important processes that also occur during inflammatory response in the brain. Therefore, Elf2 functions should be examined in the context of aging as well.

1.3.3.4 Aging and Inflammation

IL1β has been considered as one of the most complicated cytokines since the regulation of IL1β could be specific to both tissues and age. Furthermore, IL1β plays a pivotal role in several signaling pathways, making it harder to comprehend the age-related effects by just focusing on IL1β alone. Therefore, there are many contradictory results about its expression level changes during aging. According to Gee et al., IL1β shows altered mRNA expression only in the rat cortex, not in the striatum and hippocampus. However, there were no significant changes in the protein level in those three areas [155]. On the other hand, a microarray experiment performed on the mouse hippocampus indicated that there is an increase of IL1β expression level in the aged hippocampus compared to young counterparts [156]. In the same study, it was indicated that besides IL1β, TNFα showed an increased expression level in the same brain region of the mouse, along with a total of 128 differentially expressed genes.

In Sierra et al., microglia derived from young and old mice were used for assessing the level of proinflammatory and anti-inflammatory cytokines. It was discovered that proinflammatory cytokines such as IL1β and TNFα, and one anti-inflammatory cytokine (TGFβ) were found to be elevated in the old aged group. On the other hand, IL10 had not shown any change between those groups. Additionally, there was no effect of gender in these groups in terms of the level of these cytokines. The authors explained why there are increases in both pro- and anti-inflammatory cytokines as balancing both types of cytokines prevent over activation and perverse homeostasis [157]. Several years after this publication, Norden and Godbout critically reviewed the literature and suggested that since aging causes an increase in inflammation, the secretion of proinflammatory cytokines increases as well. However, contrary to Sierra et al., a decrease in anti-inflammatory cytokines was observed. Because of this disruption of the balance between cytokines, the number of microglia adopting the activated morphology is expanded. Furthermore, it was stated that microglia become less sensitive to regulation and resist switching from pro- to anti-inflammatory phenotypes due to increases in proinflammatory cytokines [158].

In conclusion, there are opposing ideas and contradictory results on the gene expression level changes of cytokines and growth factors during aging. Therefore, the relation among pro- and anti-inflammatory cytokines as well as growth factors should be examined carefully in the context of aging.

1.4 Zebrafish as a Gerontological Model

Zebrafish has become a popular model organism in many different research areas. They are small, space- and cost-efficient organisms, giving extraordinary ease to researchers of handling during experiments and maintaining them in relatively small spaces [159]. Developmental studies benefit from ex uterus development and transparency of embryos. Combined with producing many offspring in one breeding, high throughput developmental research that cannot be achieved on rats or mice can be accomplished by using zebrafish. Other than developmental studies, drug screening and development of new drugs draw advantage from this feature of zebrafish as well [160]. Being a vertebrate,zebrafish share a significant amount of similarity in terms of physiology and genetics to mammals, especially to humans [159].

All the major organs of zebrafish such as the liver, kidney, muscles, eyes, and especially the brain show molecular, cellular, and morphological similarities with mammals. For example, the zebrafish brain has a simpler organization but is similar compared to higher vertebrates, and shows homology in the brain regions [161]. More specifically, ventral pallium in the zebrafish brain corresponds to the hippocampus in the mammalian brain. Furthermore, it was shown that adult neurogenesis occurs in the zebrafish even though it is not restricted as in the mammalian brain [162].

The zebrafish genome is fully sequenced and thus, introducing a specific mutation in the gene of interest or tagging a specific gene with a fluorescent dye is possible. Due to duplication in the zebrafish genome, 71.4% of the genes including the ones related to diseases found in humans have at least one corresponding gene (ortholog) in zebrafish [163]. With the advancement in genetic methods,

knocking out, silencing, or overexpressing a specific gene as well as generation of mutant strains and transgenic lines can be accomplished to examine the nature of diseases.

Moreover, age-related changes similar to humans can also be detected in zebrafish. According to the results of microarray and quantitative polymerase chain reaction (qPCR) performed on young and old zebrafish brains, there are differentially expressed genes involved in pathways such as development of CNS, neurogenesis and replicative senescence [164]. In terms of senescence, the aging biomarker ß-galactosidase associated with senescence has been shown to correlate positively with increasing age both in humans and zebrafish. In other words, gradual senescence during aging can be detected by increasing ß-galactosidase in zebrafish as well [165]. Considering these advantages, zebrafish is becoming one of the most prominent models in gerontological studies.

1.5 Aim of the Study

To be able to develop appropriate genetic or non-genetic interventions against age-related deficits, comprehending the changes in the healthy aging brain has crucial importance. Previous studies focusing on other model organisms produced a significant amount of information regarding the gene expression level changes, however, the genes described above have not been studied on zebrafish in the context of aging. Those genes can be divided into three different yet related categories: (i) cytoskeletal changes, (ii) microglial changes, and (iii) inflammation-related changes. As explained previously, cytoskeletal changes that emerged during aging could affect the morphology of the cells, especially motile cells such as microglia (from resting state to activated state). The changed cell morphology could influence the function of the cells (protective or cytotoxic). As a result of these functional changes, the type of cytokines and growth factors released from those cells have an influence on the neighboring cells via their receptors and forcing them to change their cytoskeletal structure. Therefore, it can be concluded as these changes are related to each other in the context of aging, and examining

their gene expression level changes provide a great opportunity to understand the described cycle.

In this study, the aim was to understand the relation among the gene expression levels of cytoskeletal, microglial and inflammatory markers in zebrafish aging brain. To this aim, first, *in silico* analysis was performed to select differentially expressed genes in mouse hippocampus and brain. Later, decided genes were tested in young and old zebrafish brains by using qPCR.

Chapter 2

Materials and Methods

2.1 *In Silico* Analyses

In this section, two mouse microarray datasets with the Gene Expression Omnibus (GEO) accession numbers GSE9909 and GSE34378 were analyzed. For the analysis and construction of the graphics, R was used. For both datasets, regardless of the significance levels, IL1β, TNFα, IL10, and TGFβ genes were considered for the quantitative polymerase chain reaction (qPCR) experiments, simply because these are the well-established markers related to either pro- or anti-inflammation. Additionally, activated microglia characterized by releasing proinflammatory cytokines to protect the balance in the brain has been considered as one of the reasons causing neuroinflammation during aging. Coro1a has been used as the marker of microglia in most of the studies using zebrafish as a model organism [166][167][168][169], whereas IBA1 (Aif1) has been used as the microglial marker, in other species [81][82][83]. An ortholog of IBA1 in zebrafish is *aif1l* and one study used *aif1l* as the activated microglial marker [170]. Therefore, both *coro1a* and *aif1l* in were included in further experiments to investigate the overall microglial population and activated microglia in the zebrafish brain, respectively.

2.1.1 AGEMAP

This microarray data consists of 11 different tissues and 5 different regions of brain tissues extracted from different age groups of C57BL/6 strain mice. These age groups are very young (1 month old), young (6 months old), middle-aged (16 months old), and old (24 months old). All age groups were treated with either *ad libitum* diet or short-term caloric restriction. The previously normalized data (log2 transformation followed by the Z-score method) can be found in the Geo Database as GSE9909. The platform used for RNA hybridizations is called NIA Mouse 17K cDNA arrays. However, in this study, the analysis was performed on only hippocampus tissues from 3 age groups (young, middle-aged, and old) and 2 gender groups (female and male) treated with *ad libitum* diet. The very young group was removed from the study since that age group is more related to developmental studies rather than aging [171][172].

In this study, the data used for further analysis were extracted from https://statweb.stanford.edu/~owen/data/AGEMAP/. The data provided in this site is processed more than the data provided in Geo Datasets. All probes used in the platform are merged in a specific way if they represent the same gene. Hippocampal data have 5 samples in each of the groups, resulting in a total of 30 samples. The data was initially analyzed with the Linear Models for Microarray Data (limma) package [173] from R that was developed to analyze microarray data [173], followed by a two-way analysis of variance (ANOVA) to complement the obtained results from limma analysis.

2.1.1.1 Limma Analysis

Limma is used for finding differentially expressed genes (DEGs) among the given samples. To select DEGs, p-value adjustment was not used because in the documentation of the topTable function it is stated that "if the smallest p-value is bigger than or equal to 1/(number of genes with p values), all adjusted p-values could be equal to 1" [173]. Since the used data has approximately 9000 genes, and the smallest p-value is higher than 1/9000, raw p-values were used.

Limma analyzes the data according to the provided comparisons. After the analysis, to pinpoint the genes that are differentially expressed in specific comparison groups, each comparison were analyzed separately. The genes were selected according to their significance level of the performed tests.

2.1.1.2 Two-way ANOVA

Since limma analysis provides DEGs, two-way ANOVA (factors: age and gender) was performed on the same dataset to complement the results obtained previously. After performing ANOVA, the Tukey test was applied as a *post hoc* test. The genes with significant interaction had been chosen to investigate for their possible involvement in inflammation.

2.1.2 Female Mouse Whole Brain

Previous data investigated the effect of aging on several different regions of the brain,including the hippocampus. The data obtained from the hippocampus was selected for limma and ANOVA analyses. However, since changes that occur in the whole brain was insterested, another dataset that used whole-brain measurements from mice samples was selected [174].

The selected microarray data consisted of 5 tissues of C57BL/6J female mice including whole brain tissues. The platform used in this microarray is [Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array (mm4302daiplusv5cdf). The age groups that were investigated in the original study were 13, 26, 52, 78, 104, and 130 weeks. Each age group has 3 biological replicates. The raw data can be found in the Geo Database as GSE34378. However, in this study, 13-26 weeks old mice were grouped as young, 52-78 weeks as middle-aged, and lastly, 104- 130 weeks as old. By doing so, the biological replicates in each age group were increased to 6, increasing the statistical power. Additionally, for this study, the middle-aged group was removed from further analysis since middle-age group was not included in the qPCR experiments. The one downside of this dataset is

that all subjects were female.

The data normalization and annotation were done by using the R script of GEO2R [175]. Limma package was used to detect DEGs between young and old female mice brains [173]. Since in the previous data, raw p values were considered instead of adjusted p values, the adjusted p values were also not considered for this dataset to overcome the mismatch that could be created due to selecting different p values.

2.2 *In Vivo* Experiments

2.2.1 Animals

ZebTec housing system (Tecniplast, Italy) was used for maintenance and the housing of the fish. This system keeps the temperature and pH of the recirculating water at 28°C and 7.0 - 7.5, respectively. The quality of the water is checked by readings of the two filters: mechanical and carbon. The fish have been housed in either 3L or 8L tanks with a light cycle of 14D:10L. Their daily diet is composed of dry flakes (Sera, Germany) twice a day and artemia (Sera, Germany) once a day.

There were 12 wild type fish (with AB background) used in this study. There were two age (young -5 months old- and old -28 to 29 months old-) and gender (male and female) groups. All groups have three subjects. Euthanization of animals was performed by adding ice to the plexiglass holding tank filled with system water. Before the dissections, the tail-pinch reflex test was employed to determine whether zebrafish lost the reflex ability, and they were observed until their gills stopped moving. The procedure was approved by İhsan Doğramacı Bilkent University Local Animal Ethics Committee (HADYEK) on March 7, 2019. The decision number is 2019/14.

2.2.2 Dissections

Following euthanasia, dry weight and length were measured and recorded. Decapitation of the head was performed with a scalpel blade. The peritoneal cavity was opened and checked for the existence of eggs (female) or testicles (male) to determine the gender of the animal. Following gender determination, the ventral side of the head is placed under the stereomicroscope. First, the eyes were removed carefully without injuring the optic nerves. Then, connective tissues right under the brain and gills were removed, leading to the ventral side of the skull being exposed. After carefully breaking the skull, the brain was removed and checked whether every part of the brain is intact. Right after the removal of the body, eyes, and brain, respectively, the tissues were placed into a 1.5ml tube, weighed, and snapped frozen with liquid nitrogen. All the tissues had been kept in the -80°C freezer until further experiments.

2.2.3 RNA Isolation from the Whole Brain

There were a total of 3 sets of RNA isolation and each set had 4 subjects from each group. Each dissected brain was lysed and homogenized with 250 µL TRIzol (Thermo Fisher Scientific, USA, 15596018) and centrifugation was performed at 12,000×g for 5 minutes at 2–3°C. The clear supernatant was transferred to another tube and 5 minutes of incubation at room temperature was performed to allow nucleoprotein complexes to dissociate. Afterwards, 50 µL Chloroform was added to each tube. Following 2-3 minutes of incubation, samples were centrifuged at 12,000×g for 15 minutes at 4°C. Then, from 3 distinct layers, the first layer, the aqueous phase containing RNA, was transferred into a new tube. For precipitating RNA, 125 µL of isopropanol was added into each new tube containing the aqueous phase. Prior to centrifugation at 12,000 × g and 4°C for 10 minutes, incubation of the samples for 10 minutes was performed at room temperature. After centrifugation, since RNA precipitated as a white pellet at the bottom of the tubes, supernatants from each tube were discarded. To wash the pellets, 250 µL of 75% ethanol was added to each tube and mixed

gently. Following brief a vortexing of samples, they were centrifuged at 12,000 × g and 4°C for 5 minutes. After the removal of excess ethanol, RNA pellets were air-dried. Later, each pellet was resuspended with 20 µL nuclease-free water (Thermo Fisher Scientific, USA, AM9937) and incubated for 10 minutes at 55°C in the heat block. Finally, the concentration of each RNA was determined by NanoDrop™ 2000. Isolated RNA sample had been kept in the -80°C freezer until further experiments.

2.2.4 DNase Treatment and cDNA Synthesis

After the quick spin down of the 12 RNA samples, 10 µL of RNA was taken from each and put into separate tubes. Sequentially, 7 µL nuclease-free water, 2 µL of 10x Buffer, and 1 µL of DNase enzyme from TURBO DNA-free™ Kit (Thermo Fisher Scientific, USA, AM1907) was added into each tube. Following the incubation of the mixture for 30 minutes at 37°C, 2 µL DNase inactivation buffer from TURBO DNA-free™ Kit (Thermo Fisher Scientific, USA, AM1907) was added. After incubating the samples for 5 minutes at room temperature, centrifugation at 11000xg for 2 minutes was performed. Since the inactivation buffer precipitated with DNase enzyme at the bottom of the tubes, supernatants were collected from the tubes and put into their new corresponding tubes. Finally, concentration was measured by NanoDrop™ 2000.

Following the DNase treatment to prevent DNA contamination, cDNA synthesis was performed for 12 DNase-treated RNA samples. According to the measured concentrations, the calculation was performed to obtain 500ng of cDNA from each sample. TTo be able to obtain 15 µL 500ng of DNase-treated RNA, samples are diluted with RNase/DNase-free water from iScript™ cDNA Synthesis Kit (Bio-Rad, USA, 1708891). After obtaining 15 µL of diluted DNase treated RNAs, 4 µL 5X Buffer and 1 µL reverse transcriptase were added in each tube. Following a quick spin at room temperature for 10 seconds, the samples were placed into a Bio-Rad C1000 Thermal Cycler and incubated for 5 minutes at 5°C, 20 minutes of 46°C, 1 minute at 95°C and the machine was stabilized at 4°C in the end. Finally, the samples were kept at -20°C.

2.2.5 qPCR

2.2.5.1 Primer Design

2.2.5.1.1 Designed Primers

The *tgfb1a*, *elf2b*, *erbb4a*, and *arpc1b* primers were designed by using NCBI Primer Blast [176]. Based on the melting temperature (Tm) differences in primer pairs, length of the product, length of the primers, GC% content, self-complementarity, self-3'-complementarity and whether there are "unintended" targets for given primers (any region that they are complementing in zebrafish RNA and DNA), most suitable 4 primer pairs for each gene were selected. Later, these 4 primer pairs were analyzed in more detail by using the OligoAnalyzer™ Tool [177]. Each forward and reverse primer were controlled for the formation of the homodimer, heterodimer, and hairpin structures. Furthermore, the MFEprimer3.1 tool [178] was used to investigate whether there are any sequences that these primers can replicate. In this tool, both RNA and genomic sequences of a given organism can be selected. The tool gives a histogram of the possible products of the primers together with their length. Oligo Analysis Tool by Eurofins Genomics was used for providing an overview of the possible complementarity of both homodimers and heterodimers. Finally, one primer pair corresponding to each gene was selected for qPCR experiments.

2.2.5.1.2 The primers that were taken from the literature

Annealing temperature or the length of the primer product may not be specified in the article. Therefore, to be able to find these, Primer Blast was used. Afterward, the three tools mentioned above (OligoAnalyzer™ Tool, MFEprimer3.1, and Oligo Analysis Tool) were used for previously specified reasons. In the literature, there was only one *aif1l* primer pair that had been shown to work in qPCR. However, for both *coro1a* and *p2x7r* primer pairs, there were two options.

2.2.5.2 Dilutions of Samples and Primers

After the arrival of the primers, since they were in lyophilized form, dissolving them with nuclease-free water (Thermo Fisher Scientific, USA, AM9937) was required. Following this step, primers should be kept at -20°C. Each primer had been tested on 4 different dilutions (undiluted, 1:2, 1:4, and 1:8 diluted) of samples (young and old zebrafish brain) and one dilution of primer pairs (1:10) in qPCR. According to the gel results, the best sample dilutions that primer pairs had been shown to work were selected for qPCR experiments.

2.2.5.3 qPCR protocol

For both optimization and actual experiments, total reaction volume in each well was 20 µL composed of 10 50 µL LightCycler® 480 SYBR Green I Master (Roche, Switzerland, 04707516001), 6 µL nuclease-free water, 2 µL 1:10 diluted primer pair, and 2 µL cDNA. First, SYBR Green I Master and nuclease-free water was mixed in a tube (MasterMix). Secondly, according to primer dilutions, 5 µL of forward, 5 µL of reverse primers mixed with 90 µL nuclease-free water to obtain 1:10 dilution. Lastly, from 1:10 dilution of primer pair (final concentrations can be found in **Table 2.1**), the respective volume was taken and mixed with MasterMix (PrimerMasterMix). Each of LightCycler® 480 Multiwell Plate 96 (Roche, Switzerland, 04729692001) well was filled with 18 µL of PrimerMasterMix. After completion, 2 µL of cDNA samples were added to each well containing PrimerMasterMix. The sealed plate was centrifuged for 2 minutes at 1500 rpm. Following centrifugation, the plate was placed into LightCycler ® 480 instrument (Roche).

In the qPCR experiments, four samples from three groups produced unreliable Ct values for *il10*. Removing these samples causes a decrease in the sample size of the groups. While some groups left with two samples, one group (young female) had only one sample left. Therefore, *il10* was removed from the study.

2.2.5.4 Statistical Analysis

Fold change calculations were performed by using the $2^{-\Delta\Delta CT}$ method. Average relative expression values of all samples were used to calculate the fold change. Housekeeping *rpl13a* gene was used as a reference gene, and relative expression levels were calculated with respect to *rpl13a*. Diagnostic tests were applied to fold change data before performing ANOVA. Shapiro Wilk and Levene's tests were used to validate normality and homogeneity of variance assumptions, respectively. Even though the majority of the genes violated the normality assumption, log transformation was not performed. This is because transformed data do not always preserve the relations that were found in the original data. Therefore, interpreting the actual relations the log transformed data could be harder.

A two-way ANOVA was performed for the fold change of the genes following a normal distribution to have an idea about the factors and their interaction. Then, if any significance could not be detected in the factors or interaction, one-way ANOVA was used to investigate each factor individually. For the genes that do not follow a normal distribution, non-parametric Kruskal Wallis tests were applied. However, one-way ANOVA or its non-parametric equivalent Kruskal Wallis tests provide the information related to individual gene expression levels among age or gender groups. Therefore, one-way multivariate analysis of variance (MANOVA) was performed to see whether there are any significant differences between the age or gender groups on the genes related to inflammation, inflammation-related processes, microglia and cytoskeleton. Following the statistical tests, principal component analysis (PCA) was performed to have an insight into how samples were distributed and clustered with respect to age or gender. Additionally, to have a better idea about how genes and samples clustered, heatmap was constructed. Furthermore, to be able to understand the relation of the selected genes with each other, the correlation calculation was performed by using Spearman's Rank-Order Correlation. All statistical tests [179][180] and data visualization [181][182][183][184][185][186] was performed by using R [187].

Table 2.1: Detailed information regarding the primer pairs targeting genes of interest for quantitative reverse transcription polymerase chain reaction analysis. *: final concentration in the reaction.

Gene Symbol	**Forward Primer (5' → 3')**	**Reverse Primer (5' → 3')**	**Product Length**	**Sample Dilutions**	**Final Conc.***
arpc1b	CCACCACCGAAAGCAAAGAAG	ATGCCTCCATCCATTCCAGTG	127	1:2	1 µM
p2x7r [188]	CTGCCGAGACCTTACAGGAG	AAAGCGTTCTCCAGGACAGA	115	1:4	1 µM
coro1a [189]	GTGGAGGAGCCTTCATTG	GTTGTCATTATGTGGGCAAA	122	1:4	1 µM
aif1l [170]	CAACATGGACTTACAAGGCG	TCCTCTTCGTCTCTGTACTTCTG	117	1:2	1 µM
elf2b	CTGCACCTCTGGACGAAAC	CACAAACACCTCTGGGCTTCT	196	1:4	1 µM
erbb4a	GATACCTCGTCATACAGGGCG	GCATCCATTGTTGTGCGTGA	181	1:8	1 µM
tgfb1a	TGTACCCGCAATCCTTGACC	CCGACTGAGAAATCGAGCCA	169	1:4	1 µM
il1b	GATCCGCTTGCAATGAGCTAC	TCAGGGCGATGATGACGTTC	109	1:2	1 µM
tnfa[190]	AGGAACAAGTGCTTATGAGCCATGC	AAATGGAAGGCAGCGCCGAG	157	1:2	1 µM
igf1[164]	GGGCATTGGTGTGATGTCTT	CCAGTGAGAGGGTGTGGGTA	67	1:2	0.5 µM
rpl13a[191]	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	148	1:4	1 µM

Chapter 3

Results

3.1 *In Silico*

3.1.1 AgeMap Data

3.1.1.1 Limma Analysis

When only age groups are considered regardless of the gender, the number of differentially expressed genes (DEGs) is the highest between middle-aged and young animals, and is the lowest between old and young animals (**Table 3.1**). If the age groups together with gender is considered, then, the number of DEGs is higher in the females between old and young, and middle-aged and young, whereas the male group has a higher number of DEGs in old vs middle-aged animals (**Table 3.2**).

Table 3.1: Differentially expressed genes in the AgeMap (mouse hippocampus) data according to age groups ($p \leq 0.05$)

	Old vs Young	Old vs Middle Aged	Middle Aged vs Young
Upregulated	162	168	544
Downregulated	90	295	491
Significant	**252**	**463**	**1045**

Table 3.2: Differentially expressed genes in the AgeMap (mouse hippocampus) data according to age groups with respect to gender ($p \leq 0.05$)

	Old vs Young		Old vs Middle Aged		Middle Aged vs Young	
	Female	**Male**	**Female**	**Male**	**Female**	**Male**
Upregulated	263	48	31	403	209	137
Downregulated	263	48	31	403	209	137
Significant	**481**	**160**	**66**	**887**	**384**	**280**

The genes were selected according to their significance level of the performed tests. These genes were: Arpc1b, Erbb4 and Elf2 (**Figure 3.2 and 3.3**). On the other hand, Coro1a and Aif1 genes were not included in the data, therefore, they could not be analyzed.

3.1.1.2 Two-Way ANOVA

According to the two-way ANOVA results, while Erbb4 and Arpc1b showed significant main effects of age, the main effect of gender was observed in Elf2. Comparisons between ANOVA and limma revealed that they generated more or less similar results. Even though it seems limma generated more results than ANOVA (**Figure 3.1**), this could be caused by the inability of limma to perform p-value adjustment in this specific data.

For Errb4 gene expression levels, all significant comparisons were found in both ANOVA and limma. The main effect of age was observed in comparisons

between young and middle age, and young and old age groups. Other significant comparisons were young and middle age in both females and males, old and middle age in males, and young and old in females.

Figure 3.1: Intersection of two analysis. The Venn Diagram indicating the significantly different genes in old and young group comparison with limma and Two-Way ANOVA with Tukey test.

In terms of the expression level of Arpc1b, three significant comparisons were found with ANOVA: old and middle age in both males and merged gender, and young and middle age. However, three more comparisons were found with limma in addition to previously found significant comparisons: young and middle age in both males and females, and female and male in the old age group.

For Elf2, while the main effect of gender was found with ANOVA, this main effect could not be found with limma. In both analyses, female and male in the old age group comparison was found to be significantly different. However, according to the limma analysis, there was also another comparison found to be significant: old and middle age in males.

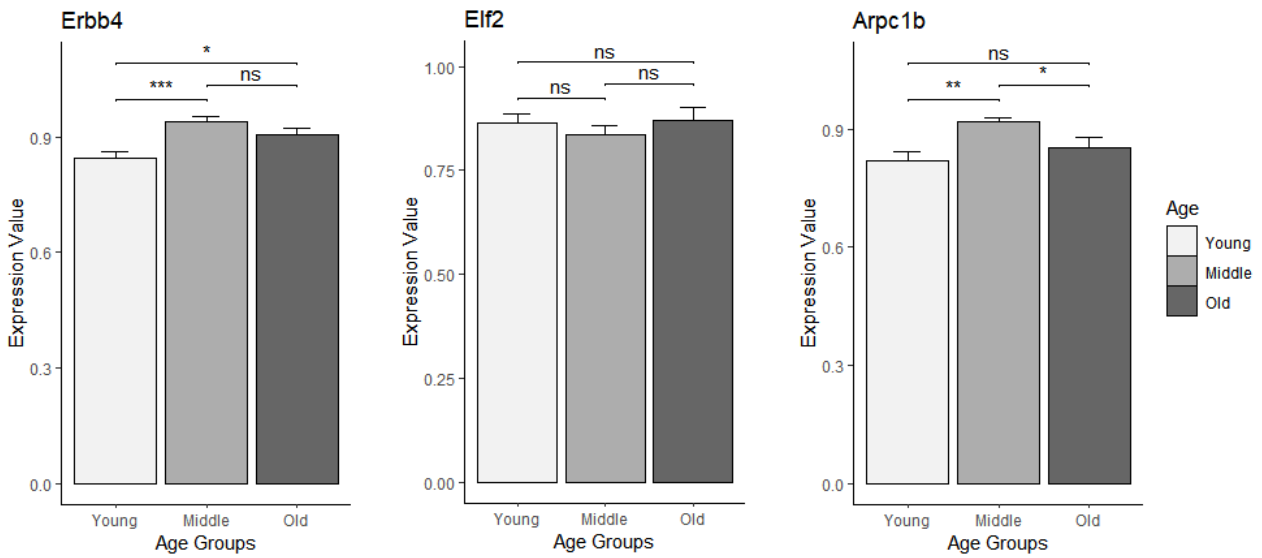


Figure 3.2: Expression levels of Erbb4, Elf2 and Arpc1b in AgeMap. The error bars represent +Standard Error (SE). (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)

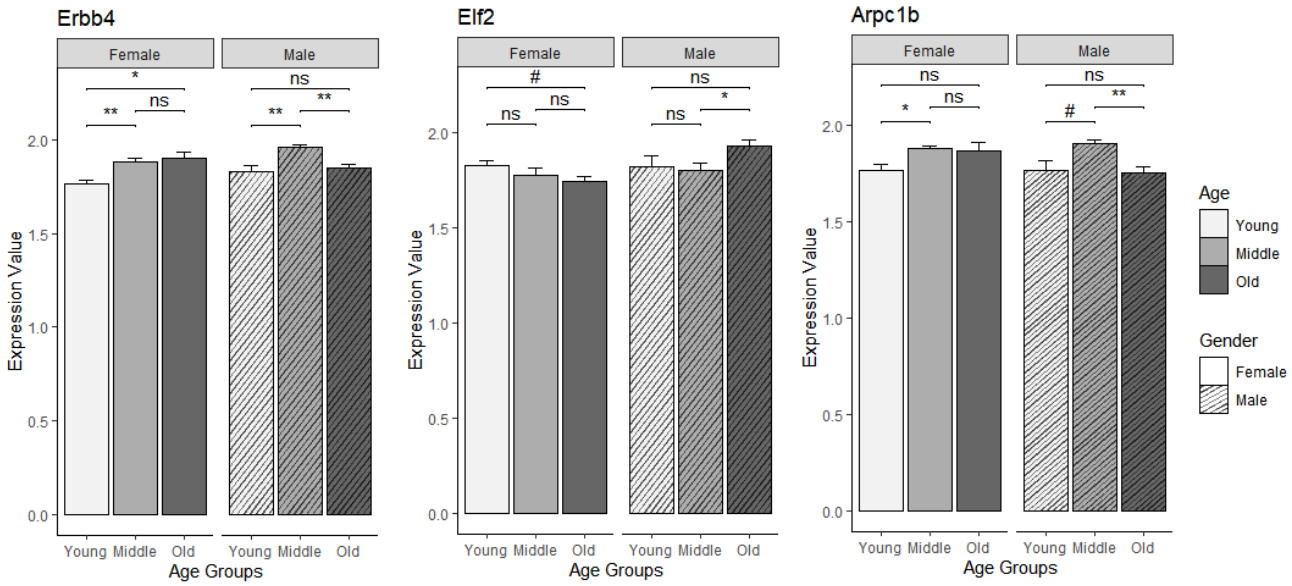


Figure 3.3: Expression levels of Erbb4, Elf2 and Arpc1b in AgeMap according to age with respect to gender. The error bars represent +Standard Error (SE). (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)

3.1.2 Female Mice Whole Brain (FMWB)

From the previous analysis, Arpc1b, Erbb4, and Elf2 genes were selected because of their significant changes across groups and relation with inflammation. Therefore, these three genes were analyzed first, to see whether there is a similarity of differentially expressed genes between the hippocampus and the whole brain in female mice in the data. However, since the Arpc1b gene does not exist in FMWB data, it is not included in the analysis.

In the AgeMap data, Erbb4 was differentially expressed between young and old female mice. However, this significance was not replicated in this data (**Figure 3.4d**). Furthermore the similar findings in Elf2 can be observed. While Elf2 was marginally significant in AgeMap data (**Figure 3.3**), no significance found in this data (**Figure 3.4c**), if the comparison between female old and young groups are considered only.

The other genes that showed statistical significance with aging were P2x7r and Il1β. As previously mentioned, P2X7R is responsible for IL1β secretion, and therefore, it was added to further experiments. Other than P2x7r and Il1β, an anti-inflammatory cytokine receptor Il10rb but not IL10, and tumor necrosis alpha-induced proteins **Tnfaip2** and **Tnfaip3** showed a significant difference between young and old whole mice brains (data not shown). Since the expression levels of Aif1 and Coro1a genes represent the microglial population, and Tgfb1 has been considered to have anti inflammatory properties, they were analyzed in this data. However, there were no significant differences in the comparisons (**Figure 3.4a, 3.4b, and 3.4h**).

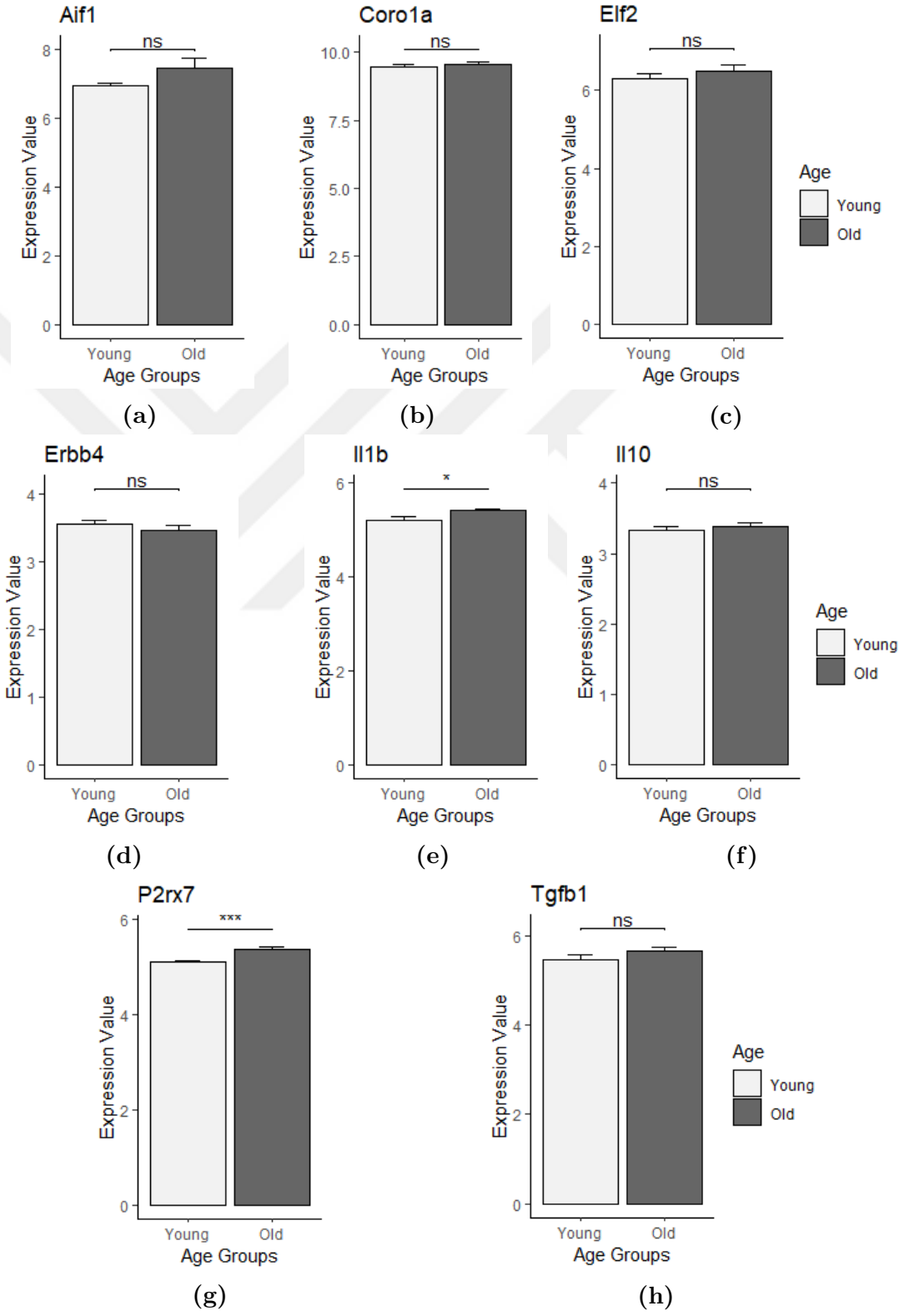
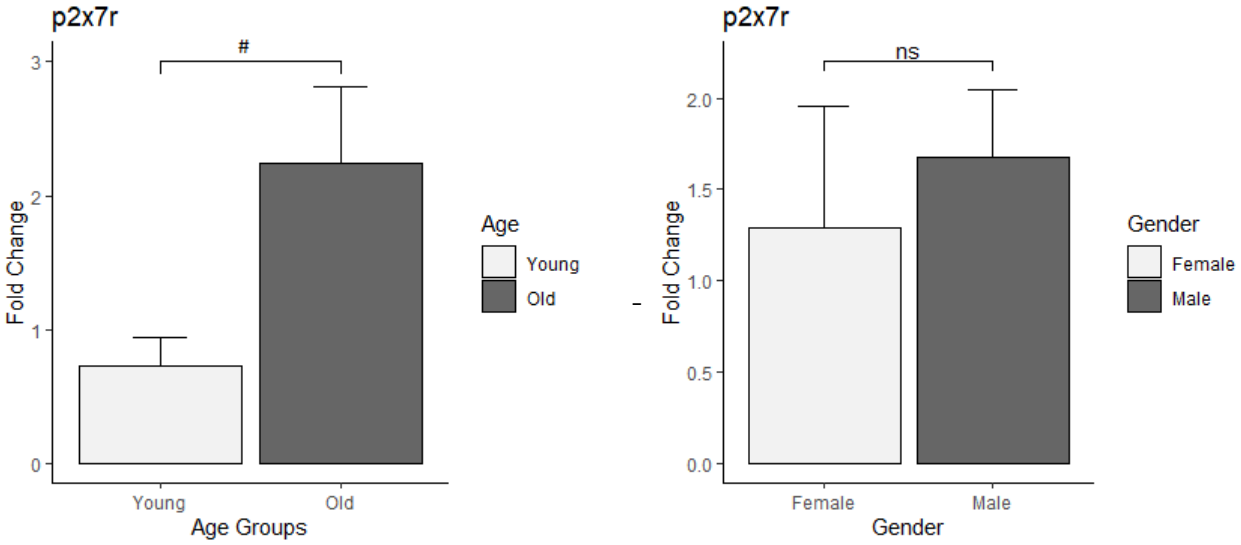


Figure 3.4: Expression levels of different genes in FMWB. The error bars represent +Standard Error (SE). (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)

3.2 *In Vivo*

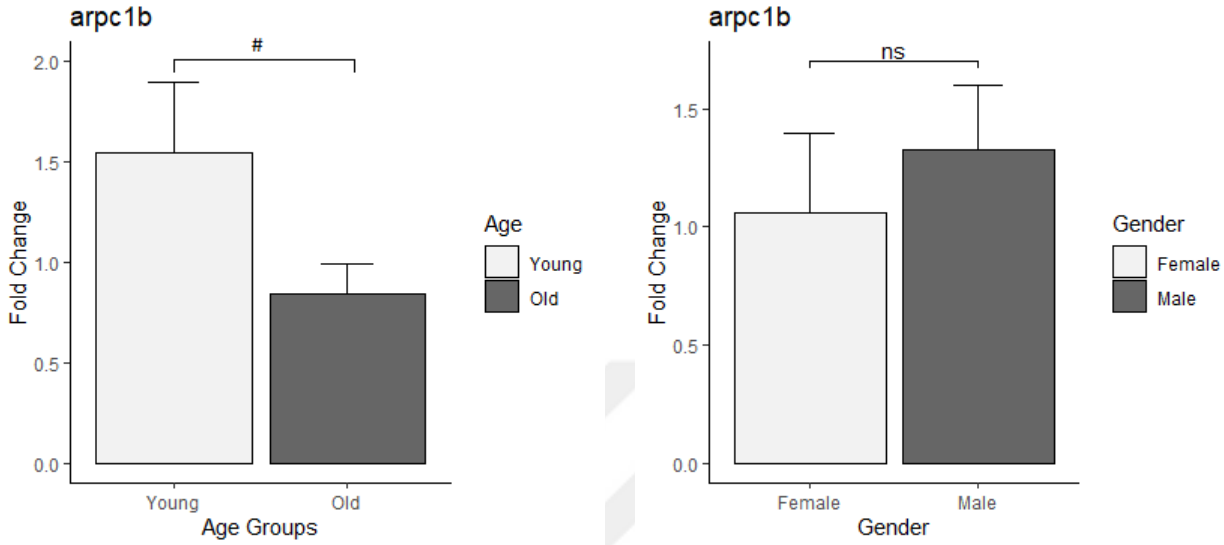
3.2.1 qPCR

According to the tests, *p2x7r* and *arpc1b* genes showed marginally significant increase ($\chi^2(1) = 3.690$, p=0.055) and decrease ($\chi^2(1) = 3.103$, p=0.078), respectively in old animals (**Figures 3.5a** and **3.6a**). These two results are similar to previous findings [98][107]. Additionally, *igf1* showed no significance in gender ($F(1,10)$=2.122, p=0.176) and in age groups ($F(1,10)$=0.905, p=0.364) (**Figure 3.15**), contradicting with previous results [164]. Moreover, the rest of the genes included in the analysis did not show a statistically significant difference in age or gender groups (**Figures 3.11-3.18**). To further investigate the age effect, one-way MANOVA was performed on multiple genes that belong to same group of markers. There were no significant changes between age groups on the inflammation, inflammation-related, microglial and cytoskeletal markers.



(a) **(b)**

Figure 3.5: Fold change of *p2x7r* gene with respect to a) age, and b) gender. The error bars represent +Standard Error (SE). (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)



(a) **(b)**

Figure 3.6: Fold change of *arpc1b* gene with respect to a) age, and b) gender. The error bars represent +Standard Error (SE). (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)

The expression patterns of these genes were further investigated with principal component analysis (PCA). The sample clustering was performed either by age (**Figure 3.7**) or gender (**Figure 3.8**). In both approaches, the first two components (PC1 and PC2) were used for the construction of the PCA plot. While 36.8% of the total variances in the data was explained by the first component (PC1) with eigenvalue of 3.68, 18.9% was explained by the second component (PC2) with eigenvalue of 1.89. In other words, a total of 55.7% of the variability in the data was captured or can be explained by these two components. However, the addition of PC3 increased explained variability to 72%. The main contributors of PC1 were *il1b* and *aif1l* with loading scores of 0.423 and 0.414 respectively. Furthermore, *elf2b* and *ifg1* were the main contributors of PC2 with loading scores -0.581 and 0.487 respectively. In both components, the cutoff for loading score was 0.4. However, since PCA explained only 55.7% of the variability in the data, to have a better idea about how samples are clustered, heatmap was generated (**Figure 3.9**).

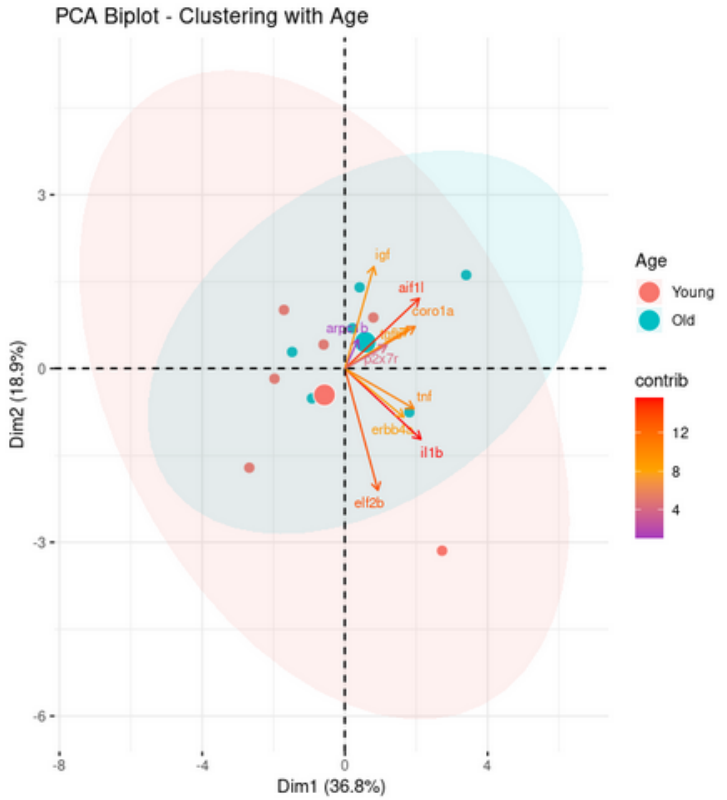


Figure 3.7: The graphical representation of PCA clustered with age. Dim1: PC1, Dim2: PC2.

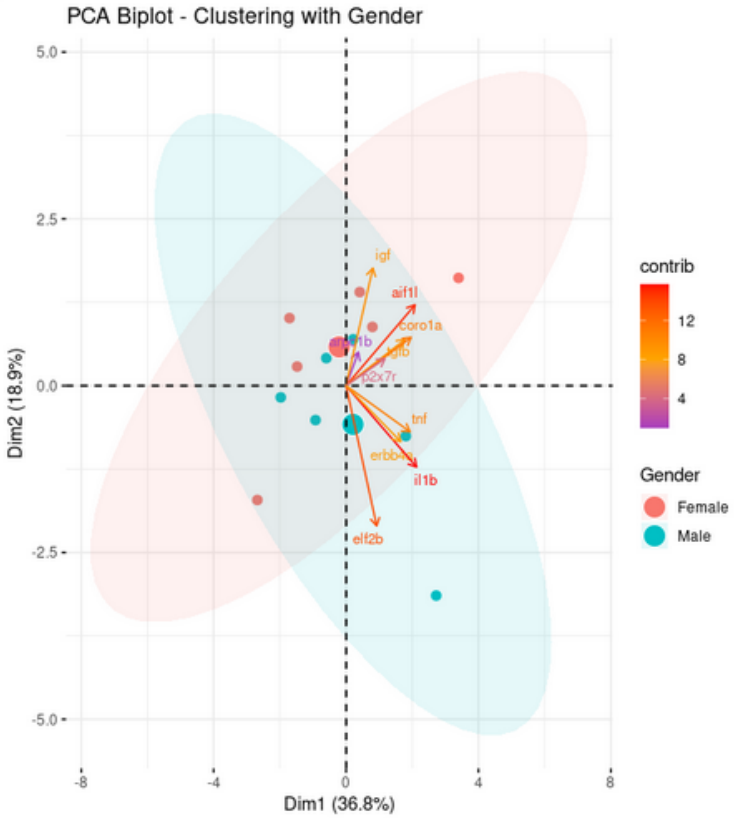


Figure 3.8: The graphical representation of PCA clustered with gender. Dim1: PC1, Dim2: PC2.

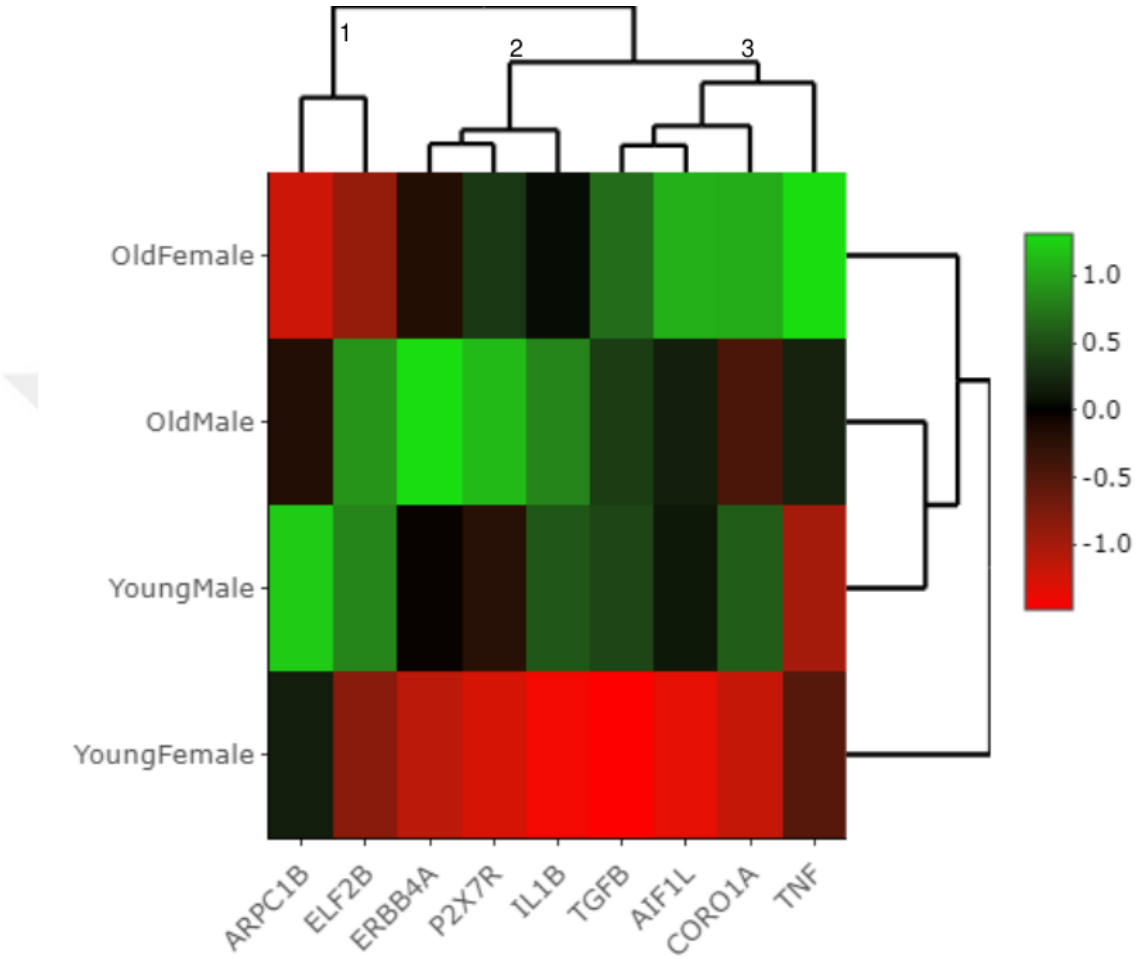


Figure 3.9: Heatmap of the *in vivo* data. Rows represent samples, columns represent genes.

As can be seen in **Figure 3.10**, all significant correlations were strong (coefficient value r: $\pm$ 0.50 and $\pm$ 1) and positive. The correlation between *coro1a* and *aif1l* was expected because both genes are found in microglia and used as microglial markers. As previously explained, there is a strong relation between microglia and inflammation. Therefore, both *coro1a* and *aif1l* were expected to correlate with other inflammatory markers: *il1b*, *tnfa*, and *tgfb1*. Even though this expectation was met for *il1b* for both microglia markers, there was no correlation between *aif1l* and *tnfa*, and among microglial markers and *tnfa*. Furthermore, the expected correlation between proinflammatory markers could not be found. Another interesting outcome was *p2x7r* to correlate with *tgfb1* but not with *il1b* due to the previously explained relation between *p2x7r* and *il1b*. A significant

correlation between *erbb4a* and *elf2b* was expected since they are both involved in several closely related cellular processes such as apoptosis and cell proliferation. Additionally, there was an unexpected correlation between *il1b* and *erbb4a*, and *p2x7r* and *erbb4a*.

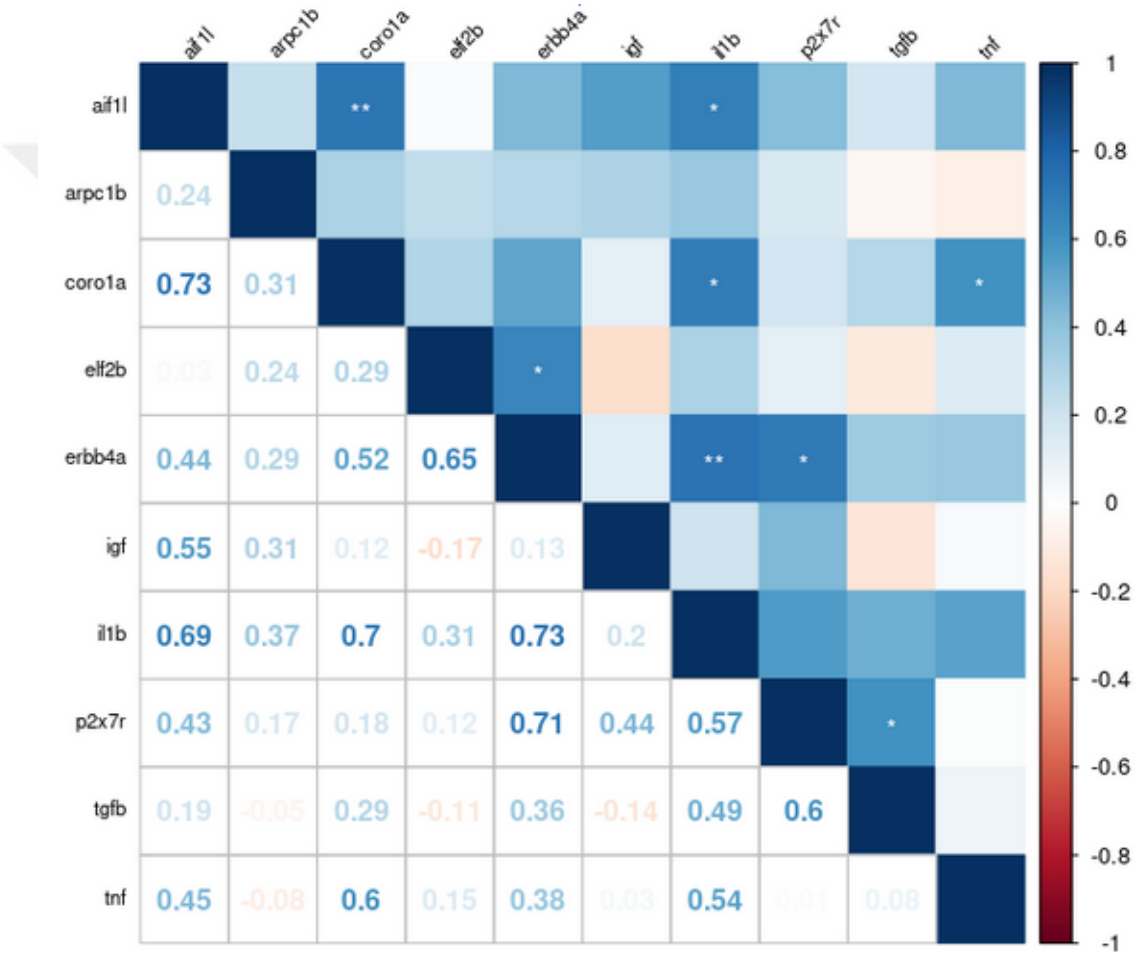
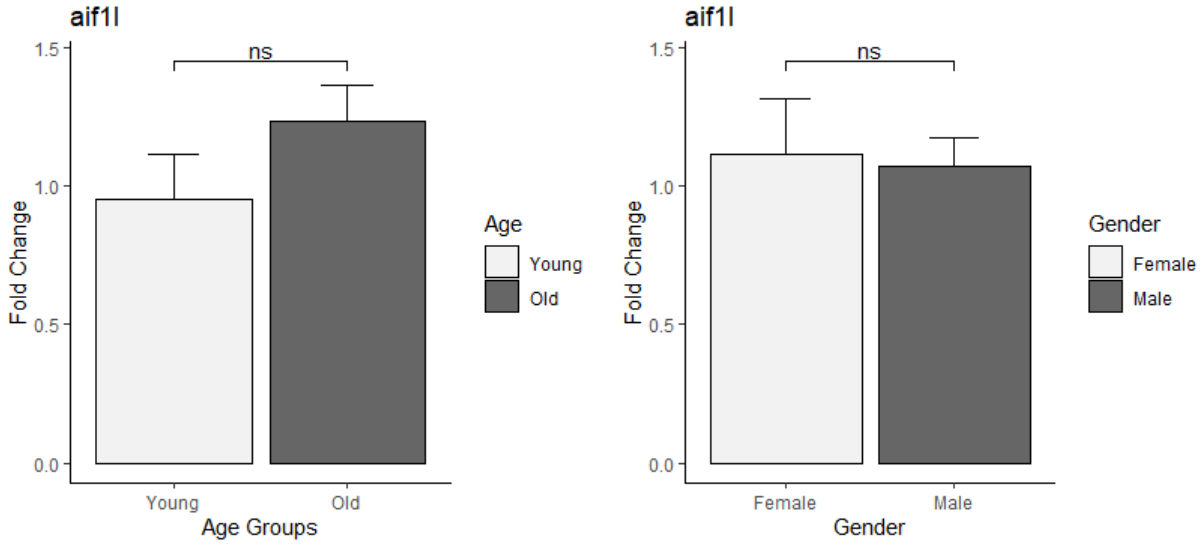


Figure 3.10: Correlation plot for the genes in qPCR. Correlation coefficients are given inside the lower triangle, and significance levels of the correlations are given inside the upper triangle (**:p≤0.01, *:p≤0.05, : p>0.1)

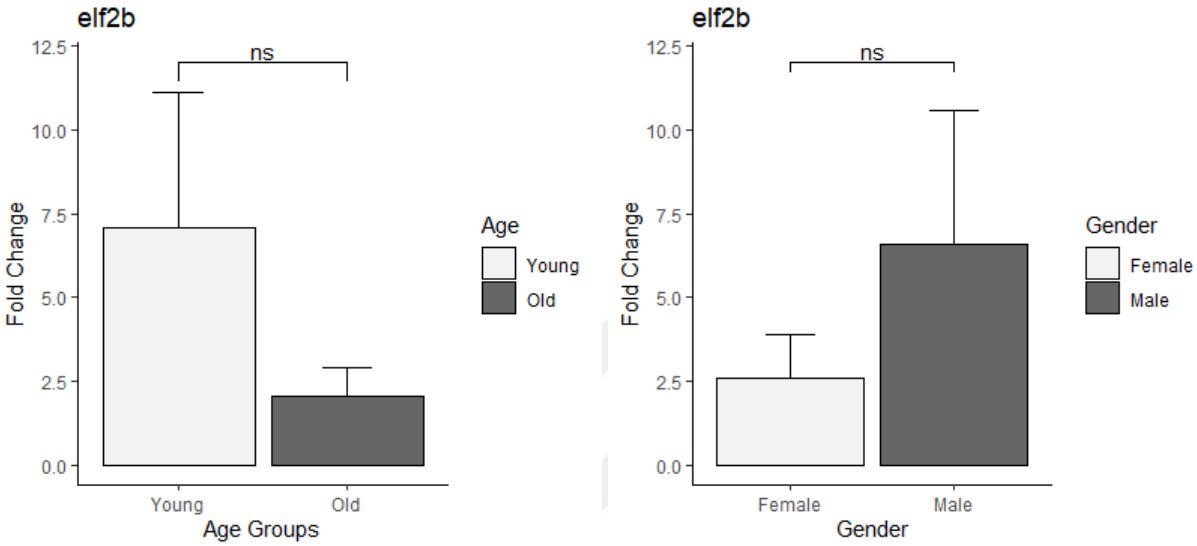
(a) (b)

Figure 3.12: Fold change of *coro1a* gene with respect to a) age and b) gender. The error bars represent +Standard Error (SE). (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)



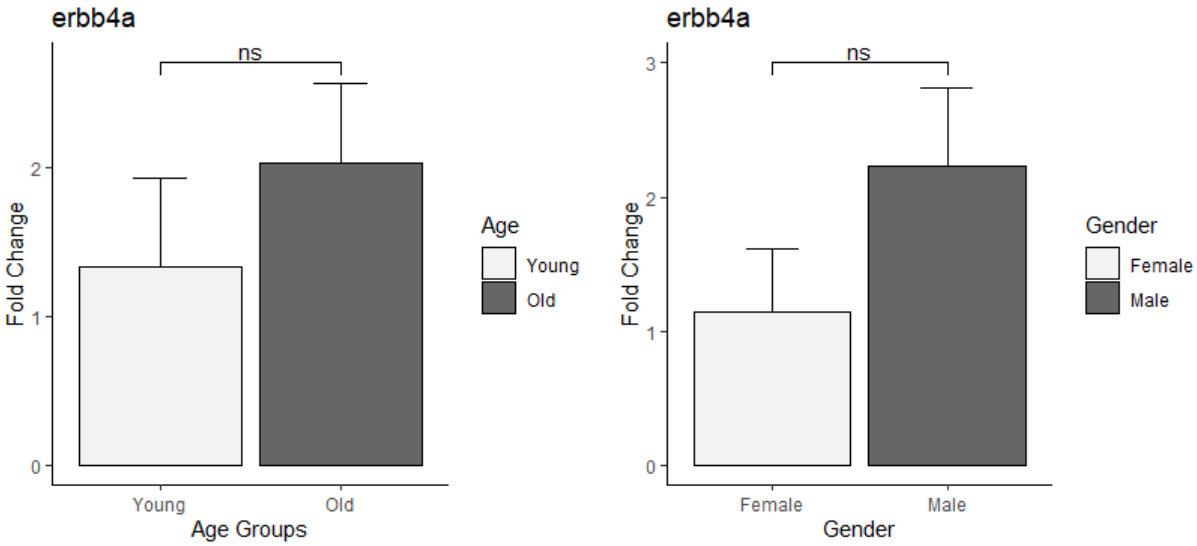
(a) (b)

Figure 3.11: Fold change of *aif1l* gene with respect to a) age and b) gender. The error bars represent +Standard Error (SE). (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)



(a) **(b)**

Figure 3.13: Fold change of *elf2b* gene with respect to a) age and b) gender. The error bars represent +Standard Error (SE). (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)

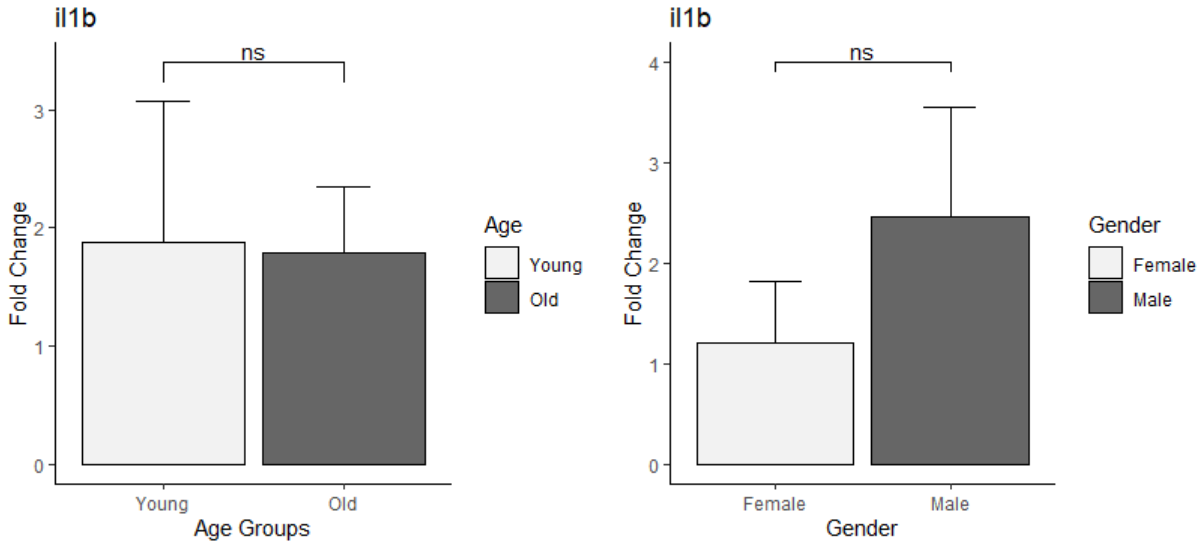


(a) **(b)**

Figure 3.14: Fold change of *erbb4a* gene with respect to a) age and b) gender. (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)

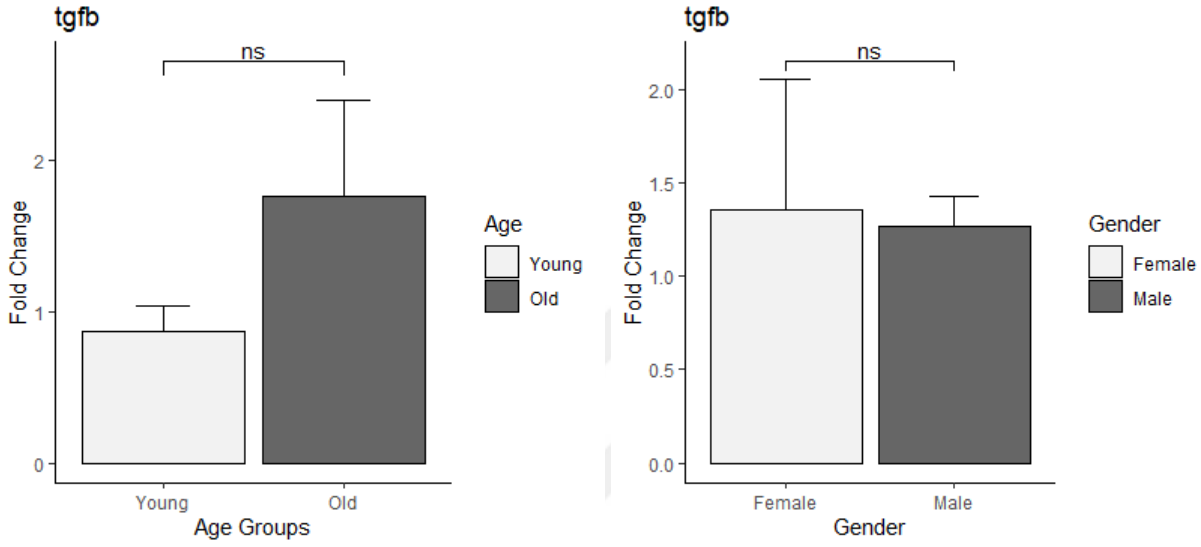
(a) (b)

Figure 3.15: Fold change of *igf1* gene with respect to a) age and b) gender. (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)



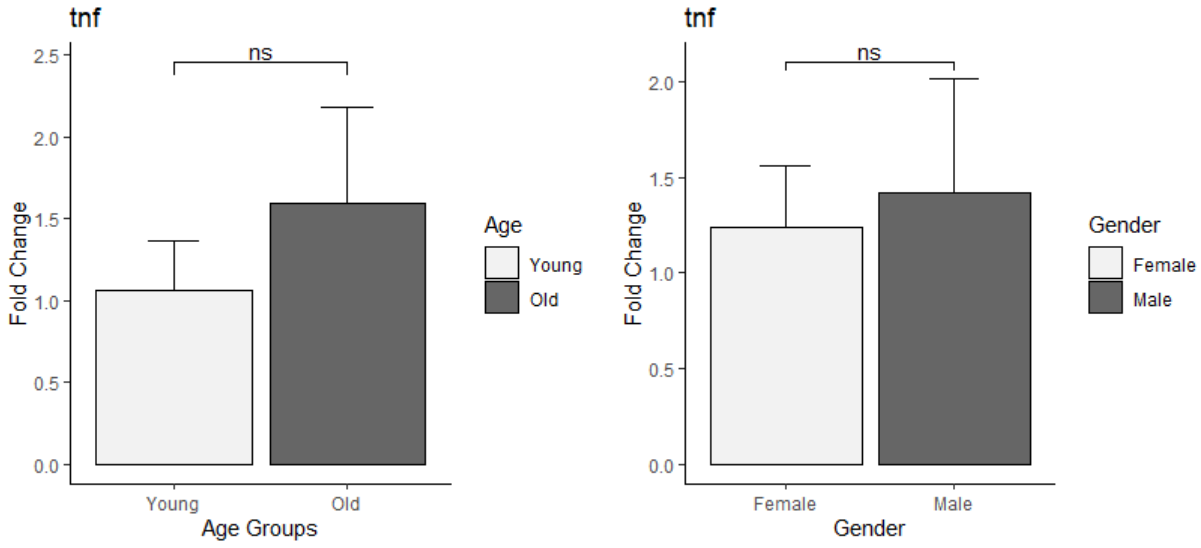
(a) (b)

Figure 3.16: Fold change of *il1b* gene with respect to a) age and b) gender. (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)



(a) (b)

Figure 3.17: Fold change of *tgfb* gene with respect to a) age and b) gender. (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)



(a) (b)

Figure 3.18: Fold change of *tnfa* gene with respect to a) age and b) gender. (***:p≤0.001, **:p≤0.01, *:p≤0.05, #:p≤0.1, ns: p>0.1)

Chapter 4

Discussion

The present study investigated the effect of aging on genes related to cytoskeleton, microglia, and inflammation in the zebrafish brain. By using *in silico* approach, the genes involved in the pathways of interest were selected according to their expression level differences in different age and gender groups. To further examine the expression levels of the selected genes in the zebrafish brain, qPCR was used as an *in vivo* approach.

In silico analysis revealed that the expression levels of the genes involved in neurogenesis (Erbb4), actin cytoskeleton organization (Arpc1b), and inflammation-related processes (Elf2 and P2x7r) were significantly affected by age. Besides age, Elf2's expression was affected by gender too. Furthermore, inflammatory (Il1b, Il10, and Tgfb1) and microglial markers (Aif1 and Coro1a) were not affected significantly by age except Il1b. To further investigate whether the results obtained from *in silico* analysis could be confirmed, qPCR experiments were performed with the following genes: *arpc1b*, *coro1a*, *aif1l*, *p2x7r*, *il1b*, *tnfa*, *tfgb1a*, *il10*, *erbb4a*, and *elf2b*.

Arpc1b is the subunit of the Arp2/3 complex responsible for branched formation [85]. Branched actin creates a base for filopodia and lamellipodia, the

structures required for cellular motility [79].The recycling of the Arp2/3 complex inside the cell has crucial importance for the motile cells such as microglia. For microglia to move forward, formation and destruction of the branching should continue, and this model is called the treadmill model [192]. The Arp2/3 complex should be removed from the mother actin filament to destroy branches [193].

The statistically significant change was not observed only between young and old organisms in AgeMap data. There is a significant increase from young to middle age, which later in the lifespan turns to decrease from middle age to old if age is considered only. Furthermore, the inclusion of gender did not change this trend tremendously. However, in the qPCR data, there was a marginally significant decrease in the old zebrafish brain. In the aging rhesus and rat hippocampus, the gene expression level of Arpc1b was upregulated [194]. However, the transcriptomic study performed on human cortical microglia indicated a decrease in the ARPC1B expression level due to aging [98]. Decreased expression of *aprc1b* in the old zebrafish brains could mean that the Arp2/3 complex could not be formed due to declined expression of its subunits, leading to the decreased branched formation. Therefore, the decreased expression could lead microglia to a partial loss of their motility because of aging.

Aif1 and Coro1a showed no statistically significant change due to aging in the FMWB data, consistent with zebrafish whole brain data. As previously explained, Aif1 [81][82][83] and Coro1a [89][91] represent the microglia population in the brain. In the previous studies, Aif1 (Iba1) was shown to increase [93][94][95][96] while Coro1a was shown to decrease due to aging [98][99]. In the present study, the similar gene expression levels of microglial markers in the age groups might indicate the total microglia population does not change during aging. However, the ratio of resting-state and activated microglia or M1 and M2 microglia might change. Besides, the localization of the protein product of these genes inside the microglia could change without significant expression level differences due to aging. Immunohistochemistry (IHC) can be used for obtaining information regarding where protein products are, in a broader sense. To be able to understand exact locational differences, cell fractionation can be used. With cell fractionation, the comparisons regarding the expression level differences among

the individual cellular compartments can be established.

IL1β and were the only genes that showed changes among young and old female mice brains. The expression levels of these genes were increased during aging. The involvement of P2x7r in IL1β secretion [102][105] was explained in a detailed manner in the introduction section (1.3.1.2). Therefore, it could have been concluded as during the course of aging, gene expression levels of IL1β, a proinflammation marker, and P2X7R, its secretory receptor, increases in the brains of female mice. These increases could have also meant the brain might tend to exhibit a more inflammatory phenotype. However, this relation was not observed in the zebrafish brain. Even though *p2x7r* showed similar trend as in FMWB data with marginal significance, *il1b* did not show statistically significant difference between young and old zebrafish brains. In the study examining NLRP3 inflammasome components, Il1b and P2x7r were shown to increase during aging [195]. However, in this study, while there was an increase in *p2x7r* expression level, there was not in *il1b*. Furthermore, studies examining the effect of aging on IL1β expression level indicated increased expression levels [155][156][157]. Potential interventions employing drug treatments can target *p2x7r* increase in the zebrafish whole brain. Since the P2X7R is a homotrimeric receptor, designing drugs (antagonists) is less complicated than the receptors composed of different subunits. For example, GSK1370319A is an antagonist of the P2x7 receptor, and it was shown that blockage of the receptor prevents NLRP3 inflammasome assembly and IL1β maturation. Therefore, it has been thought to have a protective effect on aged animals [107].

Since Il10 and Tgfb1 genes were present only in FMWB data, the expression level changes in the hippocampus during aging could not be examined. According to the analysis, these genes showed no significant changes between young and old female mice. This data (Tgfb1) is consistent with the zebrafish whole brain.In both AgeMap and FMWB data, Tnfa was not present. Therefore, there is only one result coming from the zebrafish whole brain, and comparative discussion cannot be held. In zebrafish, *tnfa* gene showed no statistically significant difference between young and old organisms. There are inconsistencies in the literature regarding the expression levels of IL10 and TGFβ. While Sierra et al.

indicated an increased expression of TGFβ and no significant difference in IL10 in microglia derived from the old organisms [157], decreased anti-inflammatory cytokines expression was observed in another study [158]. On the other hand, TNFα expression levels levels have been shown to be increased with age [156][157][158].

According to qPCR data analysis, there is no significant difference in both types of inflammatory markers (pro- and anti-inflammatory) during aging in the whole zebrafish brain. In other words, the mRNA levels of the cytokines or growth factors do not change during aging. However, this does not mean there is no change in the protein levels, which provides a more accurate estimation. After all, proteins are the structures involved in signaling cascades rather than mRNA. Additionally, it is also known that correlation coefficient between mRNA and protein levels could be as low as 0.4. This discovery was unexpected at first. However, it was found that due to further processing of mRNA (post-transcriptional modifications) and its protein (post translational modifications) are the main reasons behind this low correlation as extensively reviewed by Vogel and Marcotte in 2012 [196].

The most common and studied post-translational modifications are phosphorylation, acetylation, methylation, and ubiquitination. The addition/removal of these groups leads to changes in the protein expression levels. For example, a phosphate group added to the protein causes activity state changes by introducing conformational changes in protein structure. Even though there are no significant changes in the protein levels, their activity states might be changing. To be able to detect these changes in the activity state, there are several methods can be employed. For example, specific antibodies recognizing the site of phosphorylation can be generated to detect the phosphorylation state [197]. Therefore, further studies investigating the age-related changes in the protein expression levels are required.

Erbb4 and Elf2 genes were present in both data. Therefore, the comparison of the results generated from AgeMap (hippocampus) and FMWB data analysis revealed specific differences in the gene expression levels between the hippocampus and the whole brain in females. For example, gene expression of Erbb4 is

significantly different among female organisms belonging to young and old besides young and middle age groups. However, this significance is not present in the whole brain of females. These contradictory results between the hippocampus and the whole brain could be explained by the relation of Erbb4 with adult neurogenesis [40][41][42].

As previously discussed, even though neuronal death increases during aging, the number of neurons does not change due to the continuation of neurogenesis in adulthood [27]. However, the rate of neurogenesis decreases during aging [34]. This trend can be observed in the hippocampus data where genders are merged. There is a significant increase in the expression level of Erbb4 from young to middle age. This increment could be due to the increased requirement of neurogenesis caused by increased apoptosis during aging [198]. However, there is no significant difference between young and old zebrafish brains as expected, supporting previously discussed relations.

Another example is the Elf2 gene. Expression level of Elf2 gene in AgeMap data showed no statistically significant change. However, dividing age groups into gender revealed that while there is a marginally significant decrease in middle-age females, a significant increase is observed in old males. Since the middle age group was removed from FMWB data, the significance detected among females could not be tested. However, in both of the data, there is no significant change among young and old females. This trend was observed in young and old zebrafish as well. As previously discussed in the Introduction section (1.3.3.3), there is still a lot to discover about ELF2B in the context of aging.

Besides one-way ANOVA and Kruskal Wallis tests, one-way MANOVA was performed to examine whether there is a significant difference of grouped markers among the age or gender groups. Since ANOVA or corresponding non-parametric test provide expression level difference of only one gene among the age or gender groups, MANOVA can provide the differences in the combination of several genes among the same groups. However, the results indicated that there is no significant change. In other words, even though the related genes were tested together, the expression levels were similar among age or gender groups. Since MANOVA is

more stringent than ANOVA, significancies might be lost. The categorization of the markers might be changed to obtain significant results.

The only 55.7% of the variability total variability in the *in vivo* data was explained by the first two components. Therefore, to observe the patterns in the different groups of samples, a heatmap was constructed. According to the heatmap of the *in vivo* data, different expression patterns were observed among the samples. For example, the young female group is the most different group since most of the genes exhibit reduced expression levels. Additionally, old and young male zebrafish are the most closely related samples in terms of expression levels of the examined genes. Different gene clusters exhibit different patterns among the sample groups. For example, higher expression levels of the second cluster (2) were observed in old male zebrafish, while expression levels of the third cluster of genes (3) were observed in old female zebrafish. It is important to note that *elf2b* gene, thought to be differentially expressed among gender groups, exhibited different patterns among the gender groups. Therefore, the heatmap provided more information regarding the clustering of the samples and genes.

The correlation between *coro1a* and *aif1l* was expected because both genes are found in microglia and used as microglial markers [89][91][81][82][83]. However, if they were expressed in the same microglia populations, the correlation coefficient would be more than obtained. Even though both genes label microglia, the type of microglia that they label could be different. For example, while activated microglia express *aif1l*, all microglia populations express *coro1a*. If this is the case, the expected outcome was both microglia markers should correlate with proinflammatory cytokines while only *coro1a* should correlate with anti-inflammatory cytokines. Even though this theory holds for *il1b*, it is invalid for *tnfa* and *tgfb1a*. Also, it was unexpected that proinflammatory markers did not correlate with each other. These could be resulting from their insignificant expression level changes.

Another surprising outcome was the gene expression levels of *p2x7r* to correlate with *tgfb*'s, but not with *il1b*'s. Since *p2x7r* has been thought to mediate the secretion of *il1b* [102][105], a significant correlation between *p2x7r* and *il1b* was expected. On the other hand, in one study, it was shown that P2x7r stimulated

Tgfb1 mRNA expression which involved in the PKC/MAPK signaling pathway by using an astrocyte cell line derived from rat brain [199]. Furthermore, *il1b* correlated with *erbb4a*. The reason for this correlation could lie in the previously mentioned link between neurogenesis and neuroinflammation [35]. However, more experiments should be performed to understand this link. As explained above, if the gene expression levels of *il1b* and *erbb4a* correlate, then *p2x7r* is expected to correlate with *erbb4a*. This expectation occurs due to the previously mentioned relation between *il1b* and *p2x7r* [102][105]. Moreover, there was an expected significant correlation between *erbb4a* and *elf2b*. This correlation could be explained by their involvement in the same cellular processes such as apoptosis and cell proliferation [37][148].

igf1 gene was included in this study to investigate the similarity of the results obtained previously [164]. The previous study showed that there was a significant difference among female and male zebrafish brains in *igf1* expression levels. However, this significance could not be found in this study. One reason for this inconsistency could be the differences in sample dilutions. While cDNA dilution was 1:3 in the previous study, the sample dilution in this study was 1:2. Another reason could be the amount of cDNA loaded into wells. Previously, the amount of cDNA in each reaction was 3 µL. However, in this study, 2 µL of cDNA was used. The last and more relevant reason could be the differences in the age groups between these two studies. Compared to the previous study, both the ages of young and old zebrafish were different from the present study. Although there is no explicit age cutoff for which age range belongs to which age group, the old zebrafish generally is between 25 and 30 months old[200][201]. According to this age categorization, old zebrafish in the present study (28-29 months old) may fall into the same category. On the other hand, the old zebrafish in the previous study was 31-36 months old. Therefore, the absence of expected significant difference could be related to the different ages of old groups in the study. These could lead to this particular inconsistency observed in the gene expression levels of *igf1*.

According to the results, it is hypothesized that the formation of actin branches, the structures providing motility to microglia, disturbed during aging. Therefore, decreased expression of the *arpc1b* gene could lead microglia to lose their ability to move partially. On the other hand, increased *p2x7r* expression in the old zebrafish brain could lead to increased *il1b* secretion. Under normal circumstances, expression levels of proinflammatory markers were expected to increase during aging, which was not the case. This contradiction could be explained via the involvement of multiple proteins in the secretion of inflammation related cytokines [202]. The proinflammatory effect of P2X7R could be compensated via different mechanisms. Further experiments should be done in order to understand this relation.

The unexpected results of these experiments could be related to having a small sample size. Even though there were a total of 12 subjects, each group had three biological replicas. Having a small sample size could lead to abnormally higher variations in the groups caused by biological differences among subjects and technical errors. This issue could result in any possible significance to be masked. On the contrary, the sample size of *in vivo* experiments, FMWB, and AgeMap data have six and five biological replicates in each age group, respectively. Furthermore, AgeMap data consists of several brain regions but not the whole brain. Therefore, using hippocampal tissues only could potentially create problems when *in vivo* experiments are performed on the whole brain of zebrafish. Another reason could be bioinformatic analysis was performed on mice, whereas in the qPCR experiments, zebrafish was used as a model organism. Therefore, unexpected results could be driven by organism-related differences. The last reason could be the imbalance of gender in FMWB data. In the AgeMap, different numbers of DEGs with respect to age and gender, suggesting the importance of gender balance in experiment designs. Therefore, gender-biased results could be obtained in FMWB data.

Chapter 5

Conclusion and Future Prospects

The development of treatments for age-related cognitive deficits depends on understanding how a healthy brain ages. To serve this aim, markers related to (i) cytoskeleton, (ii) microglia, and (iii) inflammation were examined in the brain of zebrafish during aging. These markers were selected based on microarray data analysis performed on mice hippocampus and whole brain. According to qPCR, most of the gene expression levels in the brain showed consistent results with the result of bioinformatics analysis.

By using *in silico* approaches, several markers involved in actin cytoskeletal organization (Arpc1b), inflammation (Il1b, Il10, Tgfb1) and inflammation-related processes (P2x7r, Elf2) and neurogenesis (Erbb4a) were observed to be changed in the course of aging. Expression levels of Elf2 was dependent gender as well. These results were further investigated with animal experiments, and controversial results with literature and/or *in silico* analysis were obtained in inflammatory (*il1b*, *tgfb1a*, *tnfa*) and microglial markers (*coro1a*, *aif1l*). This may be due to the difference of the animal model being used in experiments, the studied tissue being a whole brain or a specific region, or the expression level (mRNA or protein) being looked at.

With the experiments performed in this study, it became clear that bioinformatics analyses are the reliable indicators of the *in vivo* experimental results. The preliminary *in silico* analysis could ease selection of the target genes which will be further investigated through *in vivo* approaches.The *in vivo* experiments provided evidence for zebrafish being a reliable gerontological model like mice since most of the expected results were obtained.

There are some limitations of this study that should be noted. First of all, each group has three samples due to ethical constraints. To extrapolate the results obtained from this study to the population, the required sample size in each group should be more than 30. Therefore, the sample size should be increased at least to six samples per group for extrapolating these results to the population. Secondly, significant changes do not occur only in young and old subjects as shown in the results section. The addition of the third age group could provide a better understanding of how and when these changes occur. Therefore, the middle-aged group could be included in further studies. In terms of selected genes, expression levels of some genes could be region-dependent, such as *erbb4a*. This region dependency could have led to limitations in the study. Since there is a link between *erbb4a* and neurogenesis, the brain region where Erbb4 is expressed mostly is the hippocampus region. Therefore, the region corresponding to the hippocampus in the zebrafish brain (i.e. pallium) should be examined to detect significant changes in the expression levels of *erbb4a* during aging.

Another limitation of this study is to conduct bioinformatics analysis with only two data. For example, the expression levels of Tnfa gene could not be examined *in silico* because it was not present in both AgeMap and FMWB data. By including more data in the analysis, these types of incidence could be eliminated. However, the most important limitation was investigating the expression level of the genes at mRNA level. It is known that mRNA level does not always correlate with protein level. Therefore, first, the Western blot technique could be used to detect changes caused by aging at the protein level. Isolating microglia from the brain could even produce more specific results about the inflammatory stage of the brain. Then, IHC can be performed to identify and count microglia populations to support further the obtained results from Western blot.

Bibliography

[1] U. Nations, World Population Ageing 2019. United Nations Publications, 2020.

[2] TurkStat, "İstatistiklerle yaşlılar," Haber Bülteni, 2018.

[3] K. Zainabadi, "A brief history of modern aging research," Experimental gerontology, vol. 104, pp. 35–42, 2018.

[4] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The hallmarks of aging," Cell, vol. 153, no. 6, pp. 1194–1217, 2013.

[5] D. Harraan, "Aging: a theory based on free radical and radiation chemistry," Journal of Gerontology, 1955.

[6] B. D'Autréaux and M. B. Toledano, "Ros as signalling molecules: mechanisms that generate specificity in ros homeostasis," Nature reviews Molecular cell biology, vol. 8, no. 10, pp. 813–824, 2007.

[7] E. Cadenas, "Basic mechanisms of antioxidant activity," Biofactors, vol. 6, no. 4, pp. 391–397, 1997.

[8] B. Halliwell, "Oxidative stress and neurodegeneration: where are we now?," Journal of neurochemistry, vol. 97, no. 6, pp. 1634–1658, 2006.

[9] B. Halliwell, "Oxidants and the central nervous system: some fundamental questions. is oxidant damage relevant to parkinson's disease, alzheimer's disease, traumatic injury or stroke?," Acta Neurologica Scandinavica, vol. 80, pp. 23–33, 1989.

[10] J. Cadet and J. R. Wagner, "Dna base damage by reactive oxygen species, oxidizing agents, and uv radiation," Cold Spring Harbor perspectives in biology, vol. 5, no. 2, p. a012559, 2013.

[11] A. Linnane, T. Ozawa, S. Marzuki, and M. Tanaka, "Mitochondrial dna mutations as an important contributor to ageing and degenerative diseases," The Lancet, vol. 333, no. 8639, pp. 642–645, 1989.

[12] M. Neiman and D. R. Taylor, "The causes of mutation accumulation in mitochondrial genomes," Proceedings of the Royal Society B: Biological Sciences, vol. 276, no. 1660, pp. 1201–1209, 2009.

[13] J. H. Hoeijmakers, "Dna damage, aging, and cancer," New England Journal of Medicine, vol. 361, no. 15, pp. 1475–1485, 2009.

[14] G. Giglia-Mari, A. Zotter, and W. Vermeulen, "Dna damage response," Cold Spring Harbor perspectives in biology, vol. 3, no. 1, p. a000745, 2011.

[15] A. Sancar, L. A. Lindsey-Boltz, K. Ünsal-Kaçmaz, and S. Linn, "Molecular mechanisms of mammalian dna repair and the dna damage checkpoints," Annual review of biochemistry, vol. 73, no. 1, pp. 39–85, 2004.

[16] E.-K. Jo, J. K. Kim, D.-M. Shin, and C. Sasakawa, "Molecular mechanisms regulating nlrp3 inflammasome activation," Cellular & molecular immunology, vol. 13, no. 2, pp. 148–159, 2016.

[17] M. Letteria, P. Domenico, R. Mariagrazia, I. Natasha, M. Herbert, A. Vincenzo, B. Alessandra, C. Giovanni, P. Antonina, and S. Francesco, "Ros-mediated nlrp3 inflammasome activation in brain, heart, kidney, and testis ischemia/reperfusion injury. oxid med cell longev," 2016.

[18] A. Semmler, T. Okulla, M. Sastre, L. Dumitrescu-Ozimek, and M. T. Heneka, "Systemic inflammation induces apoptosis with variable vulnerability of different brain regions," Journal of chemical neuroanatomy, vol. 30, no. 2-3, pp. 144–157, 2005.

[19] D. Rossi, "Bryder d, seita j, nussenzweig a, hoeijmakers j, weissman il," Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature, vol. 447, pp. 725–729, 2007.

[20] A. C. Sartori, D. E. Vance, L. Z. Slater, and M. Crowe, "The impact of inflammation on cognitive function in older adults: Implications for health care practice and research," The Journal of Neuroscience Nursing, vol. 44, no. 4, p. 206, 2012.

[21] G. Ricci, "Social aspects of dementia prevention from a worldwide to national perspective: A review on the international situation and the example of italy," Behavioural neurology, vol. 2019, 2019.

[22] D. Purves, "Neuroscience," 2004.

[23] H. Lodish, A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell, "Neurotransmitters, synapses, and impulse transmission," in Molecular Cell Biology. 4th edition, WH Freeman, 2000.

[24] J. Darnell, H. Lodish, A. Berk, L. Zipursky, P. Matsudaira, and D. Baltimore, "Overview of neuron structure and function," in Molecular cell biology, WH Freeman and Company, 2000.

[25] H. Brody, "Organization of the cerebral cortex. iii. a study of aging in the human cerebral cortex," Journal of Comparative Neurology, vol. 102, no. 2, pp. 511–556, 1955.

[26] M. West, L. Slomianka, and H. J. G. Gundersen, "Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator," The Anatomical Record, vol. 231, no. 4, pp. 482–497, 1991.

[27] P. R. Rapp and M. Gallagher, "Preserved neuron number in the hippocampus of aged rats with spatial learning deficits," Proceedings of the National Academy of Sciences, vol. 93, no. 18, pp. 9926–9930, 1996.

[28] T. Rasmussen, T. Schliemann, J. C. Sørensen, J. Zimmer, and M. J. West, "Memory impaired aged rats: no loss of principal hippocampal and subicular neurons," Neurobiology of aging, vol. 17, no. 1, pp. 143–147, 1996.

[29] A. Peters, J. Morrison, D. L. Rosene, and B. T. Hyman, "Are neurons lost from the primate cerebral cortex during normal aging?," Cerebral cortex (New York, NY: 1991), vol. 8, no. 4, pp. 295–300, 1998.

[30] J. Burianová, L. Ouda, and J. Syka, "The influence of aging on the number of neurons and levels of non-phosporylated neurofilament proteins in the central auditory system of rats," Frontiers in aging neuroscience, vol. 7, p. 27, 2015.

[31] M. Götz and W. B. Huttner, "The cell biology of neurogenesis," Nature reviews Molecular cell biology, vol. 6, no. 10, pp. 777–788, 2005.

[32] G. Kempermann and F. H. Gage, "New nerve cells for the adult brain," Scientific American, vol. 280, no. 5, pp. 48–53, 1999.

[33] C. G. Gross, "Neurogenesis in the adult brain: death of a dogma," Nature Reviews Neuroscience, vol. 1, no. 1, pp. 67–73, 2000.

[34] C. Dennis, L. Suh, M. Rodriguez, J. Kril, and G. Sutherland, "Human adult neurogenesis across the ages: an immunohistochemical study," Neuropathology and applied neurobiology, vol. 42, no. 7, pp. 621–638, 2016.

[35] I. Russo, S. Barlati, and F. Bosetti, "Effects of neuroinflammation on the regenerative capacity of brain stem cells," Journal of neurochemistry, vol. 116, no. 6, pp. 947–956, 2011.

[36] C. Kizil, N. Kyritsis, and M. Brand, "Effects of inflammation on stem cells: together they strive?," EMBO reports, vol. 16, no. 4, pp. 416–426, 2015.

[37] Z. Cai, P. Grover, Z. Zhu, M. I. Greene, and H. Zhang, "Signaling of the erbb receptor family in carcinogenesis and the development of targeted therapies," in Predictive Biomarkers in Oncology, pp. 231–242, Springer, 2019.

[38] Y. Liu, B. D. Ford, M. A. Mann, and G. D. Fischbach, "Neuregulin-1 increases the proliferation of neuronal progenitors from embryonic neural stem cells," Developmental biology, vol. 283, no. 2, pp. 437–445, 2005.

[39] T. Sato, F. Sato, A. Kamezaki, K. Sakaguchi, R. Tanigome, K. Kawakami, and A. Sehara-Fujisawa, "Neuregulin 1 type ii-erbb signaling promotes cell divisions generating neurons from neural progenitor cells in the developing zebrafish brain," PLoS One, vol. 10, no. 5, p. e0127360, 2015.

[40] E. Anton, H. Ghashghaei, J. L. Weber, C. McCann, T. M. Fischer, I. D. Cheung, M. Gassmann, A. Messing, R. Klein, M. H. Schwab, et al., "Receptor tyrosine kinase erbb4 modulates neuroblast migration and placement in the adult forebrain," Nature neuroscience, vol. 7, no. 12, pp. 1319–1328, 2004.

[41] I. Mahar, A. MacIsaac, J. J. Kim, C. Qiang, M. A. Davoli, G. Turecki, and N. Mechawar, "Effects of neuregulin-1 administration on neurogenesis in the adult mouse hippocampus and characterization of immature neurons along the septotemporal axis," Scientific reports, vol. 6, no. 1, pp. 1–14, 2016.

[42] H. Ghashghaei, J. Weber, L. Pevny, R. Schmid, M. H. Schwab, K. K. Lloyd, D. D. Eisenstat, C. Lai, and E. Anton, "The role of neuregulin–erbb4 interactions on the proliferation and organization of cells in the subventricular zone," Proceedings of the National Academy of Sciences, vol. 103, no. 6, pp. 1930–1935, 2006.

[43] H. Zhang, X. He, Y. Mei, and Q. Ling, "Ablation of erbb4 in parvalbumin-positive interneurons inhibits adult hippocampal neurogenesis through down-regulating bdnf/trkb expression," Journal of Comparative Neurology, vol. 526, no. 15, pp. 2482–2492, 2018.

[44] G. Corfas, K. Roy, and J. D. Buxbaum, "Neuregulin 1-erbb signaling and the molecular/cellular basis of schizophrenia," Nature neuroscience, vol. 7, no. 6, pp. 575–580, 2004.

[45] A. R. Chaudhury, K. M. Gerecke, J. M. Wyss, D. G. Morgan, M. N. Gordon, and S. L. Carroll, "Neuregulin-1 and erbb4 immunoreactivity is associated with neuritic plaques in alzheimer disease brain and in a transgenic model of alzheimer disease," Journal of Neuropathology & Experimental Neurology, vol. 62, no. 1, pp. 42–54, 2003.

[46] Y. Takahashi, A. Uchino, A. Shioya, T. Sano, C. Matsumoto, Y. Numata-Uematsu, S. Nagano, T. Araki, S. Murayama, and Y. Saito, "Altered immunoreactivity of erbb4, a causative gene product for als19, in the spinal cord of patients with sporadic als," Neuropathology, vol. 39, no. 4, pp. 268–278, 2019.

[47] M. Sundvall, K. Iljin, S. Kilpinen, H. Sara, O.-P. Kallioniemi, and K. Elenius, "Role of erbb4 in breast cancer," Journal of mammary gland biology and neoplasia, vol. 13, no. 2, pp. 259–268, 2008.

[48] Z. Yang, Q. Jiang, S.-X. Chen, C.-L. Hu, H.-F. Shen, P.-Z. Huang, J.-P. Xu, J.-P. Mei, B.-P. Zhang, and W.-J. Zhao, "Differential changes in neuregulin-1 signaling in major brain regions in a lipopolysaccharide-induced neuroinflammation mouse model," Molecular medicine reports, vol. 14, no. 1, pp. 790–796, 2016.

[49] A. S. Alsegiani and Z. A. Shah, "The role of cofilin in age-related neuroinflammation," Neural Regeneration Research, vol. 15, no. 8, p. 1451, 2020.

[50] L. Soreq, J. Rose, E. Soreq, J. Hardy, D. Trabzuni, M. R. Cookson, C. Smith, M. Ryten, R. Patani, J. Ule, et al., "Major shifts in glial regional identity are a transcriptional hallmark of human brain aging," Cell reports, vol. 18, no. 2, pp. 557–570, 2017.

[51] D. U. Silverthorn, W. C. Ober, C. W. Garrison, A. C. Silverthorn, and B. R. Johnson, Human physiology: an integrated approach. Pearson/Benjamin Cummings San Francisco, 2013.

[52] I. H. Salas, J. Burgado, and N. J. Allen, "Glia: victims or villains of the aging brain?," Neurobiology of Disease, p. 105008, 2020.

[53] A. Wilkins, S. Chandran, and A. Compston, "A role for oligodendrocyte-derived igf-1 in trophic support of cortical neurons," Glia, vol. 36, no. 1, pp. 48–57, 2001.

[54] Y. Du and C. F. Dreyfus, "Oligodendrocytes as providers of growth factors," Journal of neuroscience research, vol. 68, no. 6, pp. 647–654, 2002.

[55] K. D. Dougherty, C. F. Dreyfus, and I. B. Black, "Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury," Neurobiology of disease, vol. 7, no. 6, pp. 574–585, 2000.

[56] A. Wilkins, H. Majed, R. Layfield, A. Compston, and S. Chandran, "Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor," Journal of Neuroscience, vol. 23, no. 12, pp. 4967–4974, 2003.

[57] T. Philips, J. D. Rothstein, et al., "Oligodendroglia: metabolic supporters of neurons," The Journal of clinical investigation, vol. 127, no. 9, pp. 3271–3280, 2017.

[58] D. M. McTigue and R. B. Tripathi, "The life, death, and replacement of oligodendrocytes in the adult cns," Journal of neurochemistry, vol. 107, no. 1, pp. 1–19, 2008.

[59] D. Uberti, E. Yavin, S. Gil, K.-R. Ayasola, N. Goldfinger, and V. Rotter, "Hydrogen peroxide induces nuclear translocation of p53 and apoptosis in cells of oligodendroglia origin," Molecular brain research, vol. 65, no. 2, pp. 167–175, 1999.

[60] S. K. Thorburne and B. H. Juurlink, "Low glutathione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress," Journal of neurochemistry, vol. 67, no. 3, pp. 1014–1022, 1996.

[61] N. A. Oberheim, T. Takano, X. Han, W. He, J. H. Lin, F. Wang, Q. Xu, J. D. Wyatt, W. Pilcher, J. G. Ojemann, et al., "Uniquely hominid features of adult human astrocytes," Journal of Neuroscience, vol. 29, no. 10, pp. 3276–3287, 2009.

[62] O. Gonzalez-Perez, V. Lopez-Virgen, and A. Quiñones-Hinojosa, "Astrocytes: everything but the glue," Neuroimmunology and neuroinflammation, vol. 2, no. 2, p. 115, 2015.

[63] M. V. Sofroniew and H. V. Vinters, "Astrocytes: biology and pathology," Acta neuropathologica, vol. 119, no. 1, pp. 7–35, 2010.

[64] A. Castiglioni, M. Legare, D. Busbee, and E. Tiffany-Castiglioni, "Morphological changes in astrocytes of aging mice fed normal or caloric restricted diets," Age, vol. 14, no. 4, pp. 102–106, 1991.

[65] N. M. Kanaan, J. H. Kordower, and T. J. Collier, "Age-related changes in glial cells of dopamine midbrain subregions in rhesus monkeys," Neurobiology of aging, vol. 31, no. 6, pp. 937–952, 2010.

[66] H. Jyothi, D. Vidyadhara, A. Mahadevan, M. Philip, S. K. Parmar, S. G. Manohari, S. Shankar, T. R. Raju, and P. A. Alladi, "Aging causes morphological alterations in astrocytes and microglia in human substantia nigra pars compacta," Neurobiology of aging, vol. 36, no. 12, pp. 3321–3333, 2015.

[67] A. Grosche, J. Grosche, M. Tackenberg, D. Scheller, G. Gerstner, A. Gumprecht, T. Pannicke, P. G. Hirrlinger, U. Wilhelmsson, K. Hüttmann, et al., "Versatile and simple approach to determine astrocyte territories in mouse neocortex and hippocampus," PLoS One, vol. 8, no. 7, p. e69143, 2013.

[68] J. L. Zamanian, L. Xu, L. C. Foo, N. Nouri, L. Zhou, R. G. Giffard, and B. A. Barres, "Genomic analysis of reactive astrogliosis," Journal of neuroscience, vol. 32, no. 18, pp. 6391–6410, 2012.

[69] S. A. Liddelow, K. A. Guttenplan, L. E. Clarke, F. C. Bennett, C. J. Bohlen, L. Schirmer, M. L. Bennett, A. E. Münch, W.-S. Chung, T. C. Peterson,

et al., "Neurotoxic reactive astrocytes are induced by activated microglia," Nature, vol. 541, no. 7638, pp. 481–487, 2017.

[70] M. Prinz and J. Priller, "Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease," Nature Reviews Neuroscience, vol. 15, no. 5, pp. 300–312, 2014.

[71] B. Spittau, "Aging microglia—phenotypes, functions and implications for age-related neurodegenerative diseases," Frontiers in aging neuroscience, vol. 9, p. 194, 2017.

[72] W. Sun, K. Suzuki, D. Toptunov, S. Stoyanov, M. Yuzaki, L. Khiroug, and A. Dityatev, "In vivo two-photon imaging of anesthesia-specific alterations in microglial surveillance and photodamage-directed motility in mouse cortex," Frontiers in neuroscience, vol. 13, p. 421, 2019.

[73] A. Nimmerjahn, F. Kirchhoff, and F. Helmchen, "Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo," Science, vol. 308, no. 5726, pp. 1314–1318, 2005.

[74] E. Abd-El-Basset, J. Prashanth, and K. A. Lakshmi, "Up-regulation of cytoskeletal proteins in activated microglia," Medical Principles and Practice, vol. 13, no. 6, pp. 325–333, 2004.

[75] B. Wickstead and K. Gull, "The evolution of the cytoskeleton," Journal of Cell Biology, vol. 194, no. 4, pp. 1,513–525, 2011.

[76] A. D. Bershadsky and J. M. Vasiliev, Cytoskeleton. Springer Science & Business Media, 2012.

[77] D. A. Fletcher and R. D. Mullins, "Cell mechanics and the cytoskeleton," Nature, vol. 463, no. 7280, pp. 485–492, 2010.

[78] L. Blanchoin, R. Boujemaa-Paterski, C. Sykes, and J. Plastino, "Actin dynamics, architecture, and mechanics in cell motility," Physiological reviews, vol. 94, no. 1, pp. 235–263, 2014.

[79] F. Korobova and T. Svitkina, "Arp2/3 complex is important for filopodia formation, growth cone motility, and neuritogenesis in neuronal cells," Molecular biology of the cell, vol. 19, no. 4, pp. 1561–1574, 2008.

[80] K. Ohsawa, Y. Imai, H. Kanazawa, Y. Sasaki, and S. Kohsaka, "Involvement of iba1 in membrane ruffling and phagocytosis of macrophages/microglia," Journal of cell science, vol. 113, no. 17, pp. 3073–3084, 2000.

[81] Y. Sasaki, K. Ohsawa, H. Kanazawa, S. Kohsaka, and Y. Imai, "Iba1 is an actin-cross-linking protein in macrophages/microglia," Biochemical and biophysical research communications, vol. 286, no. 2, pp. 292–297, 2001.

[82] Y. Imai, I. Ibata, D. Ito, K. Ohsawa, and S. Kohsaka, "A novel geneiba1in the major histocompatibility complex class iii region encoding an ef hand protein expressed in a monocytic lineage," Biochemical and biophysical research communications, vol. 224, no. 3, pp. 855–862, 1996.

[83] D. Ito, Y. Imai, K. Ohsawa, K. Nakajima, Y. Fukuuchi, and S. Kohsaka, "Microglia-specific localisation of a novel calcium binding protein, iba1," Molecular brain research, vol. 57, no. 1, pp. 1–9, 1998.

[84] R. D. Mullins, J. A. Heuser, and T. D. Pollard, "The interaction of arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments," Proceedings of the National Academy of Sciences, vol. 95, no. 11, pp. 6181–6186, 1998.

[85] L. M. Machesky and K. L. Gould, "The arp2/3 complex: a multifunctional actin organizer," Current opinion in cell biology, vol. 11, no. 1, pp. 117–121, 1999.

[86] C. L. Humphries, H. I. Balcer, J. L. D'Agostino, B. Winsor, D. G. Drubin, G. Barnes, B. J. Andrews, and B. L. Goode, "Direct regulation of arp2/3 complex activity and function by the actin binding protein coronin," The Journal of cell biology, vol. 159, no. 6, pp. 993–1004, 2002.

[87] E. L. de Hostos, "The coronin family of actin-associated proteins," Trends in cell biology, vol. 9, no. 9, pp. 345–350, 1999.

[88] M. Maniak, R. Rauchenberger, R. Albrecht, J. Murphy, and G. Gerisch, "Coronin involved in phagocytosis: dynamics of particle-induced relocalization visualized by a green fluorescent protein tag," Cell, vol. 83, no. 6, pp. 915–924, 1995.

[89] M. Okumura, C. Kung, S. Wong, M. Rodgers, and M. L. Thomas, "Definition of family of coronin-related proteins conserved between humans and mice: close genetic linkage between coronin-2 and cd45-associated protein," DNA and cell biology, vol. 17, no. 9, pp. 779–787, 1998.

[90] A. C. Uetrecht and J. E. Bear, "Coronins: the return of the crown," Trends in cell biology, vol. 16, no. 8, pp. 421–426, 2006.

[91] Z. Ahmed, G. Shaw, V. P. Sharma, C. Yang, E. McGowan, and D. W. Dickson, "Actin-binding proteins coronin-1a and iba-1 are effective microglial markers for immunohistochemistry," Journal of Histochemistry & Cytochemistry, vol. 55, no. 7, pp. 687–700, 2007.

[92] L. J. Lawson, V. H. Perry, P. Dri, and S. Gordon, "Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain," Neuroscience, vol. 39, no. 1, pp. 151–170, 1990.

[93] K. Y. Lee, J. Y. Kang, J. I. Yun, J. Y. Chung, I. K. Hwang, M. H. Won, and J. H. Choi, "Age-related change of iba-1 immunoreactivity in the adult and aged gerbil spinal cord," Anatomy & cell biology, vol. 50, no. 2, pp. 135–142, 2017.

[94] J. H. Choi, C. H. Lee, I. K. Hwang, M. H. Won, J. K. Seong, Y. S. Yoon, H. S. Lee, and I. S. Lee, "Age-related changes in ionized calcium-binding adapter molecule 1 immunoreactivity and protein level in the gerbil hippocampal ca1 region," Journal of Veterinary Medical Science, vol. 69, no. 11, pp. 1131–1136, 2007.

[95] F. Xie, J.-C. Zhang, H. Fu, and J. Chen, "Age-related decline of myelin proteins is highly correlated with activation of astrocytes and microglia in the rat cns," International journal of molecular medicine, vol. 32, no. 5, pp. 1021–1028, 2013.

[96] D. Raj, Z. Yin, M. Breur, J. Doorduin, I. R. Holtman, M. Olah, I. J. Mantingh-Otter, D. Van Dam, P. P. De Deyn, W. den Dunnen, et al., "Increased white matter inflammation in aging-and alzheimer's disease brain," Frontiers in molecular neuroscience, vol. 10, p. 206, 2017.

[97] M. J. Schafer, I. Dolgalev, M. J. Alldred, A. Heguy, and S. D. Ginsberg, "Calorie restriction suppresses age-dependent hippocampal transcriptional signatures," PLoS One, vol. 10, no. 7, p. e0133923, 2015.

[98] T. F. Galatro, I. R. Holtman, A. M. Lerario, I. D. Vainchtein, N. Brouwer, P. R. Sola, M. M. Veras, T. F. Pereira, R. E. Leite, T. Möller, et al., "Transcriptomic analysis of purified human cortical microglia reveals age-associated changes," Nature neuroscience, vol. 20, no. 8, p. 1162, 2017.

[99] R. A. Kerber, E. O'Brien, and R. M. Cawthon, "Gene expression profiles associated with aging and mortality in humans," Aging Cell, vol. 8, no. 3, pp. 239–250, 2009.

[100] H. F. Poon, S. A. Farr, W. A. Banks, W. M. Pierce, J. B. Klein, J. E. Morley, and D. A. Butterfield, "Proteomic identification of less oxidized brain proteins in aged senescence-accelerated mice following administration of antisense oligonucleotide directed at the aβ region of amyloid precursor protein," Molecular brain research, vol. 138, no. 1, pp. 8–16, 2005.

[101] S. Gallucci and P. Matzinger, "Danger signals: Sos to the immune system," Current opinion in immunology, vol. 13, no. 1, pp. 114–119, 2001.

[102] S. D. Skaper, P. Debetto, and P. Giusti, "The p2x7 purinergic receptor: from physiology to neurological disorders," The FASEB Journal, vol. 24, no. 2, pp. 337–345, 2010.

[103] Y. He, N. Taylor, L. Fourgeaud, and A. Bhattacharya, "The role of microglial p2x7: modulation of cell death and cytokine release," Journal of neuroinflammation, vol. 14, no. 1, p. 135, 2017.

[104] M. Solle, J. Labasi, D. G. Perregaux, E. Stam, N. Petrushova, B. H. Koller, R. J. Griffiths, and C. A. Gabel, "Altered cytokine production in mice

lacking p2x7receptors," Journal of Biological Chemistry, vol. 276, no. 1, pp. 125–132, 2001.

[105] C. A. Dinarello, "Immunological and inflammatory functions of the interleukin-1 family," Annual review of immunology, vol. 27, pp. 519–550, 2009.

[106] N. Yue, H. Huang, X. Zhu, Q. Han, Y. Wang, B. Li, Q. Liu, G. Wu, Y. Zhang, and J. Yu, "Activation of p2x7 receptor and nlrp3 inflammasome assembly in hippocampal glial cells mediates chronic stress-induced depressive-like behaviors," Journal of neuroinflammation, vol. 14, no. 1, p. 102, 2017.

[107] N. Murphy, T. R. Cowley, J. C. Richardson, D. Virley, N. Upton, D. Walter, and M. A. Lynch, "The neuroprotective effect of a specific p2x7 receptor antagonist derives from its ability to inhibit assembly of the nlrp3 inflammasome in glial cells," Brain Pathology, vol. 22, no. 3, pp. 295–306, 2012.

[108] J. K. Ryu and J. G. McLarnon, "Block of purinergic p2x7 receptor is neuroprotective in an animal model of alzheimer's disease," Neuroreport, vol. 19, no. 17, pp. 1715–1719, 2008.

[109] M. Díaz-Hernández, M. Díez-Zaera, J. Sánchez-Nogueiro, R. Gómez-Villafuertes, J. M. Canals, J. Alberch, M. T. Miras-Portugal, and J. J. Lucas, "Altered p2x7-receptor level and function in mouse models of huntington's disease and therapeutic efficacy of antagonist administration," The FASEB Journal, vol. 23, no. 6, pp. 1893–1906, 2009.

[110] X.-H. Wang, X. Xie, X.-G. Luo, H. Shang, and Z.-Y. He, "Inhibiting purinergic p2x7 receptors with the antagonist brilliant blue g is neuroprotective in an intranigral lipopolysaccharide animal model of parkinson's disease," Molecular medicine reports, vol. 15, no. 2, pp. 768–776, 2017.

[111] D. K. Franco-Bocanegra, C. McAuley, J. A. Nicoll, and D. Boche, "Molecular mechanisms of microglial motility: changes in ageing and alzheimer's disease," Cells, vol. 8, no. 6, p. 639, 2019.

[112] H. Kettenmann and A. Verkhratsky, "Neuroglia–living nerve glue," Fortschritte der Neurologie-Psychiatrie, vol. 79, no. 10, p. 588, 2011.

[113] O. Takeuchi and S. Akira, "Pattern recognition receptors and inflammation," Cell, vol. 140, no. 6, pp. 805–820, 2010.

[114] J. D. Cherry, J. A. Olschowka, and M. K. O'Banion, "Neuroinflammation and m2 microglia: the good, the bad, and the inflamed," Journal of neuroinflammation, vol. 11, no. 1, p. 98, 2014.

[115] M. Colonna and O. Butovsky, "Microglia function in the central nervous system during health and neurodegeneration," Annual review of immunology, vol. 35, pp. 441–468, 2017.

[116] D. D. Raj, B. J. Eggen, and H. W. Boddeke, "Ageing and the immune response in the cns," Neuroinflammation and CNS Disorders, pp. 59–88, 2014.

[117] I. Gery and B. H. Waksman, "Potentiation of the t-lymphocyte response to mitogens: Ii. the cellular source of potentiating mediator (s)," The Journal of experimental medicine, vol. 136, no. 1, pp. 143–155, 1972.

[118] S. L. Patterson, "Immune dysregulation and cognitive vulnerability in the aging brain: Interactions of microglia, il-1β, bdnf and synaptic plasticity," Neuropharmacology, vol. 96, pp. 11–18, 2015.

[119] E. Carswell, L. J. Old, R. Kassel, S. Green, N. Fiore, and B. Williamson, "An endotoxin-induced serum factor that causes necrosis of tumors," Proceedings of the National Academy of Sciences, vol. 72, no. 9, pp. 3666–3670, 1975.

[120] B. B. Aggarwal, B. Moffat, and R. N. Harkins, "Human lymphotoxin. production by a lymphoblastoid cell line, purification, and initial characterization.," Journal of Biological Chemistry, vol. 259, no. 1, pp. 686–691, 1984.

[121] U.-K. Hanisch, "Microglia as a source and target of cytokines," Glia, vol. 40, no. 2, pp. 140–155, 2002.

[122] I. Figiel, "Pro-inflammatory cytokine tnf-alpha as a neuroprotective agent in the brain," Acta Neurobiol Exp (Wars), vol. 68, no. 4, pp. 526–534, 2008.

[123] J. Aronowski and X. Zhao, "Molecular pathophysiology of cerebral hemorrhage: secondary brain injury," Stroke, vol. 42, no. 6, pp. 1781–1786, 2011.

[124] P. J. Barnes and M. Karin, "Nuclear factor-κb—a pivotal transcription factor in chronic inflammatory diseases," New England journal of medicine, vol. 336, no. 15, pp. 1066–1071, 1997.

[125] B. C. Albensi and M. P. Mattson, "Evidence for the involvement of tnf and nf-κb in hippocampal synaptic plasticity," Synapse, vol. 35, no. 2, pp. 151–159, 2000.

[126] B. D. Williamson, E. A. Carswell, B. Y. Rubin, J. S. Prendergast, and L. J. Old, "Human tumor necrosis factor produced by human b-cell lines: synergistic cytotoxic interaction with human interferon," Proceedings of the National Academy of Sciences, vol. 80, no. 17, pp. 5397–5401, 1983.

[127] J. E. De Larco and G. J. Todaro, "Growth factors from murine sarcoma virus-transformed cells," Proceedings of the National Academy of Sciences, vol. 75, no. 8, pp. 4001–4005, 1978.

[128] A. B. Roberts, M. A. Anzano, L. C. Lamb, J. M. Smith, and M. B. Sporn, "New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues," Proceedings of the National Academy of Sciences, vol. 78, no. 9, pp. 5339–5343, 1981.

[129] X.-m. Meng, D. J. Nikolic-Paterson, and H. Y. Lan, "Tgf-β: the master regulator of fibrosis," Nature Reviews Nephrology, vol. 12, no. 6, p. 325, 2016.

[130] Y. E. Zhang, "Non-smad pathways in tgf-β signaling," Cell research, vol. 19, no. 1, pp. 128–139, 2009.

[131] T. Koeglsperger, S. Li, C. Brenneis, J. L. Saulnier, L. Mayo, Y. Carrier, D. J. Selkoe, and H. L. Weiner, "Impaired glutamate recycling and glun2b-mediated neuronal calcium overload in mice lacking tgf-β1 in the cns," Glia, vol. 61, no. 6, pp. 985–1002, 2013.

[132] T. C. Brionne, I. Tesseur, E. Masliah, and T. Wyss-Coray, "Loss of tgf-β1 leads to increased neuronal cell death and microgliosis in mouse brain," Neuron, vol. 40, no. 6, pp. 1133–1145, 2003.

[133] D. F. Fiorentino, M. W. Bond, and T. Mosmann, "Two types of mouse t helper cell. iv. th2 clones secrete a factor that inhibits cytokine production by th1 clones.," The Journal of experimental medicine, vol. 170, no. 6, pp. 2081–2095, 1989.

[134] R. Yang, M. Zhan, M. Guo, H. Yuan, Y. Wang, Y. Zhang, W. Zhang, S. Chen, H. de The, Z. Chen, et al., "Yolk sac-derived pdcd11-positive cells modulate zebrafish microglia differentiation through the nf-ϰb-tgfβ1 pathway," Cell Death & Differentiation, pp. 1–14, 2020.

[135] C. M. Hedrich and J. H. Bream, "Cell type-specific regulation of il-10 expression in inflammation and disease," Immunologic research, vol. 47, no. 1-3, pp. 185–206, 2010.

[136] K. W. Moore, R. de Waal Malefyt, R. L. Coffman, and A. O'Garra, "Interleukin-10 and the interleukin-10 receptor," Annual review of immunology, vol. 19, no. 1, pp. 683–765, 2001.

[137] C. L. Maynard and C. T. Weaver, "Diversity in the contribution of interleukin-10 to t-cell-mediated immune regulation," Immunological reviews, vol. 226, no. 1, pp. 219–233, 2008.

[138] S. Commins, J. W. Steinke, and L. Borish, "The extended il-10 superfamily: Il-10, il-19, il-20, il-22, il-24, il-26, il-28, and il-29," Journal of Allergy and Clinical Immunology, vol. 121, no. 5, pp. 1108–1111, 2008.

[139] A. Zdanov, C. Schalk-Hihi, A. Gustchina, M. Tsang, J. Weatherbee, and A. Wlodawer, "Crystal structure of interleukin-10 reveals the functional

dimer with an unexpected topological similarity to interferon γ," Structure, vol. 3, no. 6, pp. 591–601, 1995.

[140] P. J. Murray, "Understanding and exploiting the endogenous interleukin-10/stat3-mediated anti-inflammatory response," Current opinion in pharmacology, vol. 6, no. 4, pp. 379–386, 2006.

[141] D. Lobo-Silva, G. M. Carriche, A. G. Castro, S. Roque, and M. Saraiva, "Balancing the immune response in the brain: Il-10 and its regulation," Journal of neuroinflammation, vol. 13, no. 1, pp. 1–10, 2016.

[142] R. Kühn, J. Löhler, D. Rennick, K. Rajewsky, and W. Müller, "Interleukin-10-deficient mice develop chronic enterocolitis," Cell, vol. 75, no. 2, pp. 263–274, 1993.

[143] T. K. Varma, T. E. Toliver-Kinsky, C. Y. Lin, A. P. Koutrouvelis, J. E. Nichols, and E. R. Sherwood, "Cellular mechanisms that cause suppressed gamma interferon secretion in endotoxin-tolerant mice," Infection and immunity, vol. 69, no. 9, pp. 5249–5263, 2001.

[144] A. Kwilasz, P. Grace, P. Serbedzija, S. Maier, and L. Watkins, "The therapeutic potential of interleukin-10 in neuroimmune diseases," Neuropharmacology, vol. 96, pp. 55–69, 2015.

[145] A. F. Hurlstone, F. Bottiglione, C. T. Dee, R. Lea, L. Zeef, A. P. Badrock, M. Wane, L. Bugeon, M. J. Dallman, and J. E. Allen, "Zebrafish il-4-like cytokines and il-10 suppress inflammation but only il-10 is essential for gill homeostasis.," bioRxiv, 2020.

[146] P. Oettgen, Y. Akbarali, J. Boltax, J. Best, C. Kunsch, and T. A. Libermann, "Characterization of nerf, a novel transcription factor related to the ets factor elf-1.," Molecular and cellular biology, vol. 16, no. 9, pp. 5091–5106, 1996.

[147] T. U. Consortium, "UniProt: a worldwide hub of protein knowledge," Nucleic Acids Research, vol. 47, pp. D506–D515, 11 2018.

[148] F. H. Guan, C. G. Bailey, C. Metierre, P. O'Young, D. Gao, T. L. Khoo, J. Holst, and J. E. Rasko, "The antiproliferative elf2 isoform, elf2b, induces apoptosis in vitro and perturbs early lymphocytic development in vivo," Journal of hematology & oncology, vol. 10, no. 1, pp. 1–17, 2017.

[149] I.-H. Chung, H. Liu, Y.-H. Lin, H.-C. Chi, Y.-H. Huang, C.-C. Yang, C.-T. Yeh, B. C.-M. Tan, and K.-H. Lin, "Chip-on-chip analysis of thyroid hormone-regulated genes and their physiological significance," Oncotarget, vol. 7, no. 16, p. 22448, 2016.

[150] R.-M. Dai, E. Chen, D. L. Longo, C. M. Gorbea, and C.-C. H. Li, "Involvement of valosin-containing protein, an atpase co-purified with iκbα and 26 s proteasome, in ubiquitin-proteasome-mediated degradation of iκbα," Journal of Biological Chemistry, vol. 273, no. 6, pp. 3562–3573, 1998.

[151] B. Zhang, Y. Tomita, Y. Qiu, J. He, E. Morii, S. Noguchi, and K. Aozasa, "E74-like factor 2 regulates valosin-containing protein expression," Biochemical and biophysical research communications, vol. 356, no. 3, pp. 536–541, 2007.

[152] F. Belinky, N. Nativ, G. Stelzer, S. Zimmerman, T. Iny Stein, M. Safran, and D. Lancet, "PathCards: multi-source consolidation of human biological pathways," Database, vol. 2015, 02 2015. bav006.

[153] S. E. Haynes, G. Hollopeter, G. Yang, D. Kurpius, M. E. Dailey, W.-B. Gan, and D. Julius, "The p2y 12 receptor regulates microglial activation by extracellular nucleotides," Nature neuroscience, vol. 9, no. 12, pp. 1512–1519, 2006.

[154] O. Butovsky, M. P. Jedrychowski, C. S. Moore, R. Cialic, A. J. Lanser, G. Gabriely, T. Koeglsperger, B. Dake, P. M. Wu, C. E. Doykan, et al., "Identification of a unique tgf-β–dependent molecular and functional signature in microglia," Nature neuroscience, vol. 17, no. 1, pp. 131–143, 2014.

[155] J. R. Gee, Q. Ding, and J. N. Keller, "Age-related alterations of apolipoprotein e and interleukin-1β in the aging brain," Biogerontology, vol. 7, no. 2, pp. 69–79, 2006.

[156] Y. Kajiwara, S. Franciosi, N. Takahashi, L. Krug, J. Schmeidler, K. Taddei, V. Haroutunian, U. Fried, M. Ehrlich, R. N. Martins, et al., "Extensive proteomic screening identifies the obesity-related nyggf4 protein as a novel lrp1-interactor, showing reduced expression in early alzheimer's disease," Molecular neurodegeneration, vol. 5, no. 1, p. 1, 2010.

[157] A. Sierra, A. C. Gottfried-Blackmore, B. S. McEwen, and K. Bulloch, "Microglia derived from aging mice exhibit an altered inflammatory profile," Glia, vol. 55, no. 4, pp. 412–424, 2007.

[158] D. M. Norden and J. P. Godbout, "Microglia of the aged brain: primed to be activated and resistant to regulation," Neuropathology and applied neurobiology, vol. 39, no. 1, pp. 19–34, 2013.

[159] A. M. Stewart, O. Braubach, J. Spitsbergen, R. Gerlai, and A. V. Kalueff, "Zebrafish models for translational neuroscience research: from tank to bedside," Trends in Neurosciences, vol. 37, no. 5, pp. 264 – 278, 2014.

[160] J. Ablain and L. I. Zon, "Of fish and men: using zebrafish to fight human diseases," Trends in Cell Biology, vol. 23, no. 12, pp. 584 – 586, 2013. Special Issue: Translational Cell Biology.

[161] M. F. Wulliman, B. Rupp, and H. Reichert, Neuroanatomy of the zebrafish brain: a topological atlas. Birkhäuser, 2012.

[162] C. Kizil, J. Kaslin, V. Kroehne, and M. Brand, "Adult neurogenesis and brain regeneration in zebrafish," Developmental neurobiology, vol. 72, no. 3, pp. 429–461, 2012.

[163] A. J. Vilella, J. Severin, A. Ureta-Vidal, L. Heng, R. Durbin, and E. Birney, "Ensemblcompara genetrees: Complete, duplication-aware phylogenetic trees in vertebrates," Genome research, vol. 19, no. 2, pp. 327–335, 2009.

[164] A. Arslan-Ergul and M. M. Adams, "Gene expression changes in aging zebrafish (danio rerio) brains are sexually dimorphic," BMC neuroscience, vol. 15, no. 1, p. 29, 2014.

[165] S. Kishi, J. Uchiyama, A. M. Baughman, T. Goto, M. C. Lin, and S. B. Tsai, "The zebrafish as a vertebrate model of functional aging and very gradual senescence," Experimental gerontology, vol. 38, no. 7, pp. 777–786, 2003.

[166] J. Xu, L. Zhu, S. He, Y. Wu, W. Jin, T. Yu, J. Y. Qu, and Z. Wen, "Temporal-spatial resolution fate mapping reveals distinct origins for embryonic and adult microglia in zebrafish," Developmental Cell, vol. 34, no. 6, pp. 632–641, 2015.

[167] Y. Zhang, B. Xu, Q. Chen, Y. Yan, J. Du, and X. Du, "Apoptosis of endothelial cells contributes to brain vessel pruning of zebrafish during development," Frontiers in Molecular Neuroscience, vol. 11, p. 222, 2018.

[168] T. Wang, B. Yan, L. Lou, X. Lin, T. Yu, S. Wu, Q. Lu, W. Liu, Z. Huang, M. Zhang, et al., "Nlrc3-like is required for microglia maintenance in zebrafish," Journal of Genetics and Genomics, vol. 46, no. 6, pp. 291–299, 2019.

[169] D. Gan, S. Wu, B. Chen, and J. Zhang, "Application of the zebrafish traumatic brain injury model in assessing cerebral inflammation," Zebrafish, vol. 17, no. 2, pp. 73–82, 2020.

[170] K. N. Veth, J. R. Willer, R. F. Collery, M. P. Gray, G. B. Willer, D. S. Wagner, M. C. Mullins, A. J. Udvadia, R. S. Smith, S. W. John, et al., "Mutations in zebrafish lrp2 result in adult-onset ocular pathogenesis that models myopia and other risk factors for glaucoma," PLoS Genet, vol. 7, no. 2, p. e1001310, 2011.

[171] J. M. Zahn, S. Poosala, A. B. Owen, D. K. Ingram, A. Lustig, A. Carter, A. T. Weeraratna, D. D. Taub, M. Gorospe, K. Mazan-Mamczarz, et al., "Agemap: a gene expression database for aging in mice," PLoS Genet, vol. 3, no. 11, p. e201, 2007.

[172] X. Xu, M. Zhan, W. Duan, V. Prabhu, R. Brenneman, W. Wood, J. Firman, H. Li, P. Zhang, C. Ibe, et al., "Gene expression atlas of the mouse central

nervous system: impact and interactions of age, energy intake and gender," Genome biology, vol. 8, no. 11, p. R234, 2007.

[173] M. E. Ritchie, B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi, and G. K. Smyth, "limma powers differential expression analyses for rna-sequencing and microarray studies," Nucleic acids research, vol. 43, no. 7, pp. e47–e47, 2015.

[174] M. J. Jonker, J. P. Melis, R. V. Kuiper, T. V. van der Hoeven, P. F. Wackers, J. Robinson, G. T. van der Horst, M. E. Dollé, J. Vijg, T. M. Breit, et al., "Life spanning murine gene expression profiles in relation to chronological and pathological aging in multiple organs," Aging cell, vol. 12, no. 5, pp. 901–909, 2013.

[175] T. Barrett, S. E. Wilhite, P. Ledoux, C. Evangelista, I. F. Kim, M. Tomashevsky, K. A. Marshall, K. H. Phillippy, P. M. Sherman, M. Holko, et al., "Ncbi geo: archive for functional genomics data sets—update," Nucleic acids research, vol. 41, no. D1, pp. D991–D995, 2012.

[176] J. Ye, G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden, "Primer-blast: a tool to design target-specific primers for polymerase chain reaction," BMC bioinformatics, vol. 13, no. 1, p. 134, 2012.

[177] R. Owczarzy, A. V. Tataurov, Y. Wu, J. A. Manthey, K. A. McQuisten, H. G. Almabrazi, K. F. Pedersen, Y. Lin, J. Garretson, N. O. McEntaggart, et al., "Idt scitools: a suite for analysis and design of nucleic acid oligomers," Nucleic acids research, vol. 36, no. suppl_2, pp. W163–W169, 2008.

[178] K. Wang, H. Li, Y. Xu, Q. Shao, J. Yi, R. Wang, W. Cai, X. Hang, C. Zhang, H. Cai, et al., "Mfeprimer-3.0: quality control for pcr primers," Nucleic acids research, vol. 47, no. W1, pp. W610–W613, 2019.

[179] W. W. Burchett, A. R. Ellis, S. W. Harrar, and A. C. Bathke, "Nonparametric inference for multivariate data: The R package npmv," Journal of Statistical Software, vol. 76, no. 4, pp. 1–18, 2017.

[180] F. E. Harrell Jr and C. Dupont, "Hmisc: Harrell miscellaneous. r package version 3.12-2," Computer software]. Available from http://cran. R-project. Org/web/packages/Hmisc, 2013.

[181] H. Wickham, ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.

[182] A. Kassambara, ggpubr: 'ggplot2' Based Publication Ready Plots, 2020. R package version 0.4.0.

[183] T. Wei and V. Simko, R package "corrplot": Visualization of a Correlation Matrix, 2017. (Version 0.84).

[184] A. Kassambara and F. Mundt, factoextra: Extract and Visualize the Results of Multivariate Data Analyses, 2020. R package version 1.0.7.

[185] M. FC, ggpattern: Geoms with Patterns, 2020. http://github.com/coolbutuseless/ggpattern, https://coolbutuseless.github.io/package/ggpattern/index.html.

[186] Galili, Tal, O'Callaghan, Alan, Sidi, Jonathan, Sievert, and Carson, "heatmaply: an r package for creating interactive cluster heatmaps for online publishing," Bioinformatics, 2017.

[187] R Core Team, R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2020.

[188] M.-Y. Chang, J.-K. Lu, Y.-C. Tian, Y.-C. Chen, C.-C. Hung, Y.-H. Huang, Y.-H. Chen, M.-S. Wu, C.-W. Yang, and Y.-C. Cheng, "Inhibition of the p2x7 receptor reduces cystogenesis in pkd," Journal of the American Society of Nephrology, vol. 22, no. 9, pp. 1696–1706, 2011.

[189] S. Wang, Q. He, D. Ma, Y. Xue, and F. Liu, "Irf4 regulates the choice between t lymphoid-primed progenitor and myeloid lineage fates during embryogenesis," Developmental cell, vol. 34, no. 6, pp. 621–631, 2015.

[190] N. Wang, Z. Luo, M. Jin, W. Sheng, H.-T. Wang, X. Long, Y. Wu, P. Hu, H. Xu, and X. Zhang, "Exploration of age-related mitochondrial dysfunction and the anti-aging effects of resveratrol in zebrafish retina," Aging (Albany NY), vol. 11, no. 10, p. 3117, 2019.

[191] G. M. T. de Oliveira, L. W. Kist, T. C. B. Pereira, J. W. Bortolotto, F. L. Paquete, E. M. N. de Oliveira, C. E. Leite, C. D. Bonan, N. R. de Souza Basso, R. M. Papaleo, et al., "Transient modulation of acetylcholinesterase activity caused by exposure to dextran-coated iron oxide nanoparticles in brain of adult zebrafish," Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, vol. 162, pp. 77–84, 2014.

[192] S. A. Koestler, A. Steffen, M. Nemethova, M. Winterhoff, N. Luo, J. M. Holleboom, J. Krupp, S. Jacob, M. Vinzenz, F. Schur, et al., "Arp2/3 complex is essential for actin network treadmilling as well as for targeting of capping protein and cofilin," Molecular biology of the cell, vol. 24, no. 18, pp. 2861–2875, 2013.

[193] T. D. Pollard, "Regulation of actin filament assembly by arp2/3 complex and formins," Annu. Rev. Biophys. Biomol. Struct., vol. 36, pp. 451–477, 2007.

[194] E. M. Blalock, R. Grondin, K.-c. Chen, O. Thibault, V. Thibault, J. D. Pandya, A. Dowling, Z. Zhang, P. Sullivan, N. M. Porter, et al., "Aging-related gene expression in hippocampus proper compared with dentate gyrus is selectively associated with metabolic syndrome variables in rhesus monkeys," Journal of Neuroscience, vol. 30, no. 17, pp. 6058–6071, 2010.

[195] L. J. Mawhinney, J. P. de Rivero Vaccari, G. A. Dale, R. W. Keane, and H. M. Bramlett, "Heightened inflammasome activation is linked to age-related cognitive impairment in fischer 344 rats," BMC neuroscience, vol. 12, no. 1, pp. 1–10, 2011.

[196] C. Vogel and E. M. Marcotte, "Insights into the regulation of protein abundance from proteomic and transcriptomic analyses," Nature reviews genetics, vol. 13, no. 4, pp. 227–232, 2012.

[197] M. Carter and J. Shieh, "Chapter 15 - biochemical assays and intracellular signaling," in Guide to Research Techniques in Neuroscience (Second Edition) (M. Carter and J. Shieh, eds.), pp. 311 – 343, San Diego: Academic Press, second edition ed., 2015.

[198] J. Adams, S. Mukherjee, L. Klaidman, M. Chang, and R. Yasharel, "Apoptosis and oxidative stress in the aging brain.," Annals of the New York Academy of Sciences, vol. 786, pp. 135–151, 1996.

[199] C.-M. Wang, Y.-Y. Chang, and S. H. Sun, "Activation of p2x7 purinoceptor-stimulated tgf-β1 mrna expression involves pkc/mapk signalling pathway in a rat brain-derived type-2 astrocyte cell line, rba-2," Cellular signalling, vol. 15, no. 12, pp. 1129–1137, 2003.

[200] M. J. Gilbert, T. C. Zerulla, and K. B. Tierney, "Zebrafish (danio rerio) as a model for the study of aging and exercise: physical ability and trainability decrease with age," Experimental gerontology, vol. 50, pp. 106–113, 2014.

[201] E. T. Karoglu, D. O. Halim, B. Erkaya, F. Altaytas, A. Arslan-Ergul, O. Konu, and M. M. Adams, "Aging alters the molecular dynamics of synapses in a sexually dimorphic pattern in zebrafish (danio rerio)," Neurobiology of Aging, vol. 54, pp. 10–21, 2017.

[202] R. Medzhitov, "Inflammation 2010: new adventures of an old flame," Cell, vol. 140, no. 6, pp. 771–776, 2010.