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Chapter 1

Zebrafish Aging Models and Possible Interventions

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http://dx.doi.org/10.5772/intechopen.75554

Abstract

Across the world, the aging population is expanding due to an increasing average life expectancy. The percentage of elderly over the age of 65 is expected to be more than 15% of the total world population by 2025. As the lifespan increases, there will be a need for maintaining a healthy state for these individuals. Our current knowledge on types and durations of potential anti-aging therapies is quite limited. Recently the zebrafish has emerged as a promising model for understanding the cognitive and neurobiological changes during aging, as well as its use with potential anti-aging interventions. Like humans this model organism ages gradually, displays similar behavioral properties and social characteristics, and in addition, there is a wealth of molecular and genetic tools to uncover the cellular mechanism that contribute to age-related cognitive declines. Drug effect and toxicity can be easily tested in the zebrafish. Therefore, this animal model can provide information about potential therapies that could be translated directly into human populations or provide a more focused treatment direction for testing in other mammalian animal models. The zebrafish will be a powerful tool for uncovering the mysteries of the aging brain.

Keywords: aging, behavior, neurobiology, dietary regimens, rapamycin, morpholino, zebrafish aging models

1. Introduction: the zebrafish as a model organism for brain aging

Throughout history, humans have tried to find ways to delay or reverse aging and cure age-related diseases. Whether it was a dream of finding the "fountain of youth" or use of modern applications such as drugs with alleged "anti-aging" properties; to date, there are no

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interventions that met the expectations of the humankind. Therefore, we should first understand the complex multifactorial nature of aging and its underlying mechanisms before we aim to intervene. Both genetic and epigenetic factors are involved in aging [1, 2] and this holds true across different species.

Extensive investigations have focused on the mechanisms of aging by using both vertebrate and invertebrate animal models to provide an insight into age-related physiological, cognitive and neurobiological changes, with each giving a piece of the puzzle. Some of the animal models that have been studied in the context of aging are worms (e.g., *Caenorhabditis elegans*) [3], fruit flies (e.g., *Drosophila melanogaster*) [4], mice (*Mus musculus*) [5], and non-human primates (e.g., rhesus monkeys) [6–9]. Rhesus monkeys provided valuable information about the mechanisms that underlie physiological and cognitive changes related to age [9], whereas genetic screens on invertebrate models yielded a list of genes involved in the regulation of life span [6, 7]. All these animal models have played important roles in understanding mammalian aging and age-related diseases but having only one animal model might be limiting. For example, invertebrate models have a short lifespan, which might not be correctly translated to humans, and genes that are associated with longevity in vertebrates with longer lifespans might not be revealed by studies on these models. Non-human primates, on the other hand, have a lifespan of about 25 years, making longitudinal studies very difficult. Mice are nocturnal animals while humans are diurnal and their diverse circadian rhythms may affect the aging process differentially, which might complicate the translation of the knowledge obtained from mice studies into humans. Thus, recently the zebrafish (*Danio rerio*) has emerged as a novel model for vertebrate aging research [10–15]. While it has been used in numerous fields previously due to its optical transparency during early development, cost-effectiveness, high fecundity, detailed characterization of its genome, diverse mutant or transgenic strains that have been made available; it was the emergence of evidence for the gradual aging phenotype in zebrafish and its diurnal nature that drew attention from researchers studying normal aging.

The zebrafish has on average a maximum lifespan of 3 to 5 years in laboratory conditions. The gradual senescence phenotype allowed researchers to identify age-related changes in gene expression [16], endocrine [14] and neuroendocrine system [17], musculoskeletal [10], visual function and morphology [18], cognitive functions [14], and sleep [13, 19]. In addition to studies monitoring changes in zebrafish from early development to old age [13, 19, 20], zebrafish has become a model for assessment of behavior and cognitive functions. There are several cognitive tests available for use on zebrafish to index learning and memory [21–23], anxiety and stress response [24]. The use of zebrafish in cognitive and neurobiological aging research increased with the accumulation of knowledge about the neuroanatomy of the zebrafish brain. The standard neuroanatomical, neuropharmacological and immunohistochemical techniques have been applied to characterize the organization of the zebrafish brain [25, 26]. These studies demonstrated similarities of zebrafish sensory and motor systems, and central nervous system circuits with other vertebrates. In addition to homology at systemic level, homologous structures such as zebrafish lateral pallium and mammalian hippocampus have been shown based on electrophysiological [27] and neurochemical data [28]. Lateral pallium is particularly important due to its suggested role in learning and memory in zebrafish [29], processes that are deeply affected by age. While understanding the age-related physiological

and cognitive changes remain crucial, studies involving interventions that might delay or reverse these changes run parallel. Zebrafish is an outstanding model for investigating drug toxicity and/or effect throughout lifespan, identification of age-associated biomarkers that could become predictors of premature aging phenotypes, and screening mutants for identification of strains with accelerated or decelerated aging phenotypes. In this chapter, we will review age-related behavioral and neurobiological changes in zebrafish and continue with existing models that have delayed or accelerated aging phenotype.

2. Age-related changes in behavior and biology

2.1. Behavioral changes

2.1.1. Age-related changes in human perceptual and cognitive performance

Vision is the most informative of our senses and hence, essential for survival in a dynamic world. By relying on vision, we are able to recognize visual objects in the environment, judge the trajectories of fast approaching objects and cruise through morning traffic. Therefore, most of the aging studies on human perception focused on visual perception and cognition. A number of studies have shown that visual functioning is significantly altered throughout aging. Older adults have typically impaired visual sensitivity and altered perception of different visual features such as motion [30]. As opposed to the traditional view focusing on structural changes in the eye and retina, accumulating evidence suggests that impairments in neural circuitry and functioning in the cortex have important contributions to the age-related changes in visual sensitivity and perception [31]. For instance, behavioral studies have shown that older adults are less accurate in discriminating motion direction and speed compared to younger adults [32, 33]. Moreover, older adults typically need more time to make visual judgments. This suggests that older population have also slowed visual processing speed. In line with these changes in perceptual performance, neurophysiological studies on different species have shown that neurons located in visual area V1 and MT have less direction and speed sensitivity due to aging [34, 35]. These changes in the cortical neurons have been mostly explained by the deterioration in synaptic connections and integrity, and hence increase in the noise level of the local cortical network.

Normal aging is most notably accompanied by declines in cognitive functions and processes. Accumulating evidence suggests that age-related decline exists in both low-level and high-level cognitive processes [36]. Previous studies have pointed out a general deceleration in cognitive processes and a decline in attentional resources due to aging [37, 38]. Moreover, there exist age-correlated deficits in learning, memory and cognitive control. Age-related declines in cognitive function are typically reflected as significant losses of learning and memory abilities [39]. Though decrements in implicit and short-term memory tasks are mostly slight, age-related performance declines in working, episodic and prospective memory tasks are substantial [40]. In older adults, the reduction in memory context and false recollections are also commonly found. Another common observation is that older adults have difficulty in associating different aspects of an event. It should be also noted that some

cognitive functions can remain intact and may even improve (e.g., semantic knowledge) throughout aging. It was initially thought that age-related cognitive decline was due to massive loss of neurons. However, current research mostly supports the view that subtle changes at the cellular and subcellular level, and in synaptic connectivity play major roles in the age-related decline in cognitive performance.

2.1.2. Age-related changes in zebrafish perceptual and cognitive performance

Zebrafish display a rich repertoire of behaviors, which depends on perceptual and cognitive processes [41]. As in other vertebrates, zebrafish have basic sensory systems and pathways for low-level sensory processing. For instance, the basic components and pathways of zebrafish visual system and visual processing hierarchy are similar to those commonly found in other species [42]. Of particular note, zebrafish can discriminate visual objects differing in color, shape and motion direction [43, 44]. There is a growing interest to assess motion perception acuity of zebrafish through optomotor responses or eye movements. Recent studies have shown that zebrafish perceive first- and second-order motions and also experience motion adaptations and illusions which are even thought to be seen only by humans [45, 46]. In addition, motion acuity and contrast sensitivity function have been found to be qualitatively similar to those of humans [47, 48]. These findings support the view that zebrafish visual system and perception (in particular, motion perception) rely on similar principles commonly found in humans. On the other hand, although there are studies comparing larval responses to visual stimulation (e.g., motion) with that of adult zebrafish [49], there is almost no systematic investigation on perceptual changes during aging. Future studies examining age-related changes in zebrafish perception and perceptual acuity will be informative in this respect and are currently being performed in our laboratory. Preliminary data suggest there are subtle age-related differences that are gender-dependent [50].

Behavioral studies also support the notion that zebrafish provide a promising model of cognitive functioning [51, 52]. It has been found that zebrafish have both simple (e.g., habituation, dishabituation and sensitization) and relatively complex forms of learning (e.g., associative and spatial learning). They also displayed good performance on tasks requiring either short-term (e.g., object recognition) or long-term (e.g., avoidance) memory. More importantly, the age-related cognitive declines have been shown by behavioral studies on learning and memory. For instance, zebrafish exhibit decreased performance with age on tasks relevant to associative learning and also show defects in spatial learning and avoidance with a distinct onset throughout aging [53, 54, 14]. In general, research on zebrafish will provide insight into potential neurobiological changes that would allow for application of interventions that would alter their course and possible translation to human populations.

2.2. Neurobiological changes

Behavioral and cognitive alterations that occur during normal aging are one of the most explored areas in the aging research since these are the manifestations of the aging itself, not to be confused with those related to pathologies. Their biological underpinnings, on the other hand, help us understand not only the cellular and synaptic mechanisms that play a role in

aging, but also the very essence of the biology of behavior. To be able to understand the causes of cognitive decline that accompanies aging, for instance, we need to take all the biological components and their interaction with the environment into consideration. Previously, we described changes in cognitive processing that occur during aging and now we will focus on the neurobiological factors related to the hallmarks of aging [55] that likely underlie the changes in behavior and cognition (**Table 1**).

2.2.1. Epigenetic alterations and differential gene expression

Epigenetic mechanisms refer to structural or chemical modifications in RNA, DNA, and proteins without altering their primary sequence. These modifications play critical roles in major cellular processes such as regulation of gene expression, DNA replication, and cell cycle. Dynamic methylation/demethylation and acetylation/deacetylation events regulate structure of DNA and function of proteins, which consequently affect gene expression levels. DNA methylation at CpG dinucleotides, for example, is commonly associated with decreased DNA accessibility and turning genes off, although there are exceptions [56]. In contrast, histone acetylation generally results in an increase in gene expression [57]. The epigenetics of aging has been studied extensively, and researchers came up with a term, epigenetic drift, to define

Types of changes	Observations	Phenotypes	References
Epigenetic	Decrease in global DNA methylation	Disruption of gene expression and cellular differentiation	[59]
Gene expression	Decrease in expression of IGF signaling-related genes	Increase in lifespan	[16]
	Increase in *smurf2* expression	Replicative senescence	[16]
	Increase in *hsp1* expression	Impaired proteostasis	[11]
	Decrease in *tert* gene expression	Telomere shortening	[66]
Proteastasis	Decrease in Hsp70 levels	Impaired cellular stress response and proteostasis	[11]
	Increase in SOD2 activity		[64]
	Increased levels of lipofuscin		[65]
Genomic	DNA fragmentation	Senescence and/or cell loss	[60]
	Elevated apoptosis		[60]
Telomere attrition	Significant increase in the loss of telomere repeats	Telomere shortening	[66]
	Decrease in telomerase activity		[66]
Cellular and synaptic	Decrease in neurogenesis	Cognitive decline and altered behavior	[67, 68]
	Impaired oligodendrogenesis		[67]
	Altered balance in excitatory/inhibitory transmission		[75]

Table 1. Summary of age-related neurobiological changes.

age-related alterations in epigenetic patterns [58]. One well established epigenetic marker of aging is gradual decrease in global DNA methylation [59]. This decrease has been reported in humans, and other species including zebrafish. In zebrafish, while embryonic genome is highly methylated, gradual hypomethylation is observed throughout zebrafish lifespan [60]. These hypomethylation events are particularly observed at CpG islands, where several CpG dinucleotides cluster at regions typically involved with transcription regulation [60].

In addition to the previously mentioned global methylation events, differential gene expression, that could result in changes in cellular and synaptic functioning have been documented in the aging brain. In our study characterizing gene expression changes in the brains of young and old, male and female zebrafish, it was show that there are over 200 differentially expressed genes that are involved in cell differentiation, growth, neurogenesis, and brain and nervous system development [16]. For example, detailed analysis showed that expression of insulin-like growth factor (IGF) signaling-related genes including, igf1, igf2bp3, and igfbp2a, which are related to cell growth, significantly decreases in the brains of old zebrafish. In contrast, SMAD specific E3 ubiquitin protein ligase 2 (smurf2) expression, which is implicated in replicative senescence, is higher in old zebrafish compared to young [16]. Taken together, these data indicate potential differences in cellular and synaptic functioning.

2.2.2. Impaired proteostasis

Maintenance of protein homeostasis (a.k.a. proteostasis) is crucial for a cell's response to stress. Aging cells are exposed to increasing levels of stress, and there is even more need for protein quality control in these cells to keep the proteostatic balance for survival. When the networks that regulates protein synthesis, folding, and clearance malfunction due to age-related accumulation of cellular damage, proteostasis becomes impaired, making the proteome more vulnerable to cellular stress. In most organisms, gradual loss of proteostasis is associated with aging, yet there are some long-lived organisms that have more stable proteomes, suggesting an evidence for the importance of a stable proteome for longer lifespan [61].

Proteostasis networks involve chaperone proteins and two proteolytic systems: ubiquitin-proteasome and lysosome-autophagy systems. Chaperones guide newly synthesized proteins through processes that fold, transport and target for degradation [62]. The fate of unfolded proteins is collectively decided by all the proteolytic components mentioned before, but which proteolytic pathway to be followed depends mainly on chaperones. For example, heat shock protein 70 (Hsp70) family is comprised of several chaperones that are involved in stabilization of the correctly folded proteins and targeting proteins for degradation. In zebrafish, Hsp70 protein levels decrease with age, while *hsp1* mRNA levels are increased in aged zebrafish, possibly to compensate for decreased ability of Hsp70 to function [11].

Activities of chaperone proteins are under the influence of age-related cellular changes such as dysregulation of cellular energetics. Reduced mitochondrial function, for example, impairs energy metabolism and limit the bioavailability of ATP. Aging cells respond to this reduction in ATP levels by switching to ATP-independent chaperones, as shown in aging human brain [63]. To overcome the effects of impaired energy metabolism and its harmful endproducts,

cells respond in various ways. For example, increase in the major antioxidant enzyme in mitochondria, superoxide dismutase 2 (SOD2) activity has been reported in the aged zebrafish, although not in the brain tissue. SOD2 activity, on the other hand, increases between 3 and 12 months of age, indicating high metabolism, but decreases after 18 months, and even further decrease was observed in older zebrafish [64].

Dysregulation in lysosomal degradation may be a contributing factor to age-related cognitive dysfunction. For example, increased levels of lipofuscin, a non-degradable end product of lysosomal digestion, and oxidized proteins have been observed in lateral and medial pallial areas of the zebrafish brain at the age of 2 years as compared to 12 month-old animals [65]. Cognitive abilities related to memory are impaired in these fish starting from the age of 18 months, which is after the levels of these cellular byproducts started to increase. This enhanced oxidative stress, as observed by increases in lipofuscin and oxidized proteins, may be causing cognitive impairments in the aging zebrafish after reaching a certain threshold level of accumulation.

2.2.3. Genomic instability and telomere attrition

In the aging brain, as well as the whole body, there is an increase in genomic instability and telomere attrition. Mild DNA fragmentation, which is a biomarker of genomic instability, has been shown in young fish but the levels of fragmentation significantly increase after 12 months in various zebrafish tissues including brain. These changes in genomic instability in the aging zebrafish result in elevated apoptosis, which could reflect an increased need for removal of senescent or damaged cells [60]. The shortening of telomeres due to loss of repeats with each replication event, is another biomarker of aging. The mean telomere length of zebrafish decreases from the young adult stage to the older adult stage, with significant decreases in telomere length occurring after 18 months. This contrasts with the period before young adulthood, in which telomere length has been shown to increase. The decrease in telomere length is occurring after changes in the telomerase enzyme activity and expression, which starts to decrease in the eye and brain at the age of 6–12 months [66]. Research from young zebrafish with mutations in the gene encoding a telomere repeat binding factor 2 (*terfa*$^{hi3678/hi3678}$) support the role of proper telomere functioning as it relates to cellular homeostasis. These *terfa*$^{hi3678/hi3678}$ mutants die at early stages of development due to severe telomere shortening, which leads to premature retinal neurodegeneration, increased cellular senescence in the brain and spinal cord, which is shown by high SA-b-gal activity, and smaller eyes and head compared with wild type [18]. Heterozygotes are viable but have a shorter lifespan than their wild type counterparts [18]. Taken together changes in genomic instability and telomere length will contribute to cellular homeostasis.

2.2.4. Changes in cellular and synaptic capabilities

Both the mammalian and zebrafish brain have the capacity for cellular proliferation, although it is more limited in mammals. Changes in cellular proliferation may affect cognitive processing in the aged individual, and thus, zebrafish provide a good model for understanding

mechanisms that regulate cell turnover in the brain. It has been observed by our research group and others that in both young and old adult zebrafish, neurogenesis is observed in the telencephalon, however, there is a significant decline in old adults [67, 68]. This decrease is attributed to the lengthening of cell cycle and decrease in the number of radial glia cells with self-renewal capability. In addition to this stem cell exhaustion, oligodendrogenesis has been shown to be impaired within the telencephalic parenchyma [67]. Therefore, similar to mammals, there is an age-related decrease in neural proliferation.

Loss of synaptic integrity may also play a major role in the age-related cognitive decline. Glutamate receptors, in particular, the N-methyl-D-aspartate (NMDA) receptor, have been implicated in age-related cognitive decline [69–71]. Decreases in the NMDA- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor levels have been shown to cause learning and memory impairments, whereas the increase leads to memory enhancement [72, 73]. While glutamate receptors remain a key molecular target that might be contributing to the age-related cognitive decline, it is important to understand changes in the excitatory/inhibitory balance and other neurotransmitter systems that might alter synaptic function with age. Compared to wild-types, mutants with impaired acetylcholinesterase function had better performance in spatial learning, entrainment and increased rate of learning [74, 14]. These findings suggest that cholinergic signaling may play a role in the age-related cognitive decline. Finally, we have begun to examine synaptic integrity in zebrafish brains. In a recent study, we examined changes in key synaptic proteins that reflect potential differences in both excitatory and inhibitory synapses across lifespan in young and old male and female zebrafish brains [75]. Our results show that the excitatory/inhibitory balance is altered differently in the brains of male and female zebrafish. These data indicate that there are age-related alterations in synaptic integrity that are gender-dependent and may contribute to cognitive decline.

3. Zebrafish models that delay or accelerate the aging process

Here, we will review several genetic and non-genetic interventions which are proposed to extend lifespan and healthspan, and discuss their use in zebrafish models in the context of aging.

3.1. Dietary restriction

Dietary restriction has been shown to have beneficial effects on both cognitive aging and the associated neurobiological changes in the aging brain. This has been shown in humans [76] and animal models [77–80]. Our research group proposed utilizing a dietary manipulation such as caloric restriction (CR) to alter the aging process in zebrafish. To date, only a few studies apart from our own have utilized a true CR in fish [68]. The previously published dietary restriction studies that have been performed in zebrafish did not reduce daily caloric intake but rather the fish were not given any food for an extended period, which would be considered as a starvation study [81, 82] and none had been done in aged animals or with regards to gender. Thus, designing the appropriate dietary intervention needed to be based on a wealth of literature that has accumulated from studies utilizing different animal models.

Initially, our research group established a protocol designed as a daily reduction in caloric intake in zebrafish. For this task, cohorts of both young and old fish raised in the zebrafish facility were moved to round glass aquaria (**Figure 1a** and **b**). Fish were fed individually in 600 mL beakers during the weekdays (**Figure 1c** and **Table 2**). On the weekend, fish were fed similar amounts of food in the housing aquaria. It should be noted that the animals were not housed continually in the beakers since zebrafish are highly social [83, 84], and continuous social isolation would increase their stress levels [85]. Since the effects of CR are thought to be modulated through the target of rapamycin (TOR) pathway [86], we aimed to test whether we could mimic the effects of CR with rapamycin treatment, a TOR inhibitor. Rapamycin is a macrocyclic compound produced by bacterium *Streptomyces hygroscopicus* and approved for patient use by the Food and Drug Administration (FDA, USA) [87]. The rapamycin group was treated daily with 100 nM rapamycin dissolved in DMSO. The fish in all treatment groups

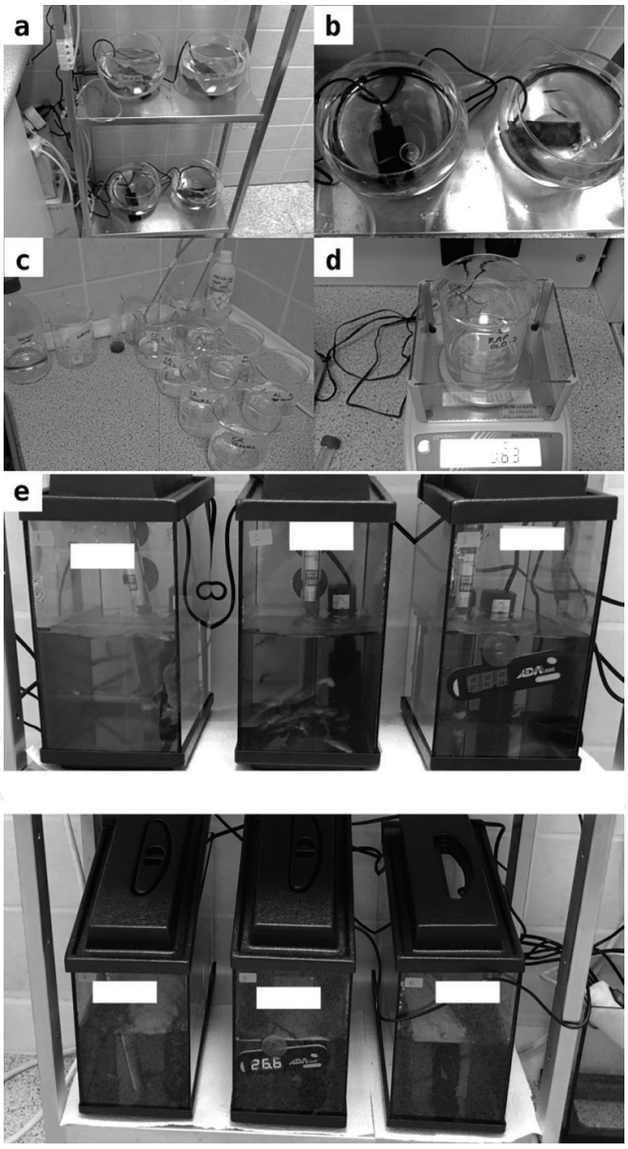


Figure 1. Aquaria set-up for CR and rapamycin treatment (a–d), and IF and rapamycin treatment (e) experiments.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Ad libitum (control for CR and rapamycin-treated groups)	20 mg*	20 mg*	20 mg*	20 mg*	20 mg*	20 mg*	20 mg*
Rapamycin treatment	20 mg*	20 mg*	20 mg*	20 mg*	20 mg*	20 mg*	20 mg*
Caloric restriction	1 mg*	1 mg*	1 mg*	1 mg*	1 mg*	1 mg*	1 mg*
Ad libitum (control for IF and rapamycin-treated groups)	90 mg food** and artemia†	90 mg food**	90 mg food** and artemia†	90 mg food**	90 mg food** and artemia†	90 mg food**	90 mg food**
Rapamycin treatment	90 mg food** and artemia†	90 mg food**	90 mg food** and artemia†	90 mg food**	90 mg food** and artemia†	90 mg food**	90 mg food**
Intermittent feeding	45 mg food**		45 mg food** and artemia†		45 mg food**		45 mg food**
Overfed	180 mg food** and artemia**	180 mg food** and artemia**	180 mg food** and artemia**	180 mg food** and artemia**	180 mg food** and artemia**	180 mg food** and artemia**	180 mg food** and artemia**

*Food per fish per day.

**Twice a day.

†Once a day.

Table 2. Different feeding paradigms applied by our research group to study the effects of dietary restriction (i.e. CR and IF) or overfeeding compared to drug treatment (i.e. rapamycin-treated) on zebrafish aging.

were weighed individually in beakers throughout the experiment (**Figure 1d**). The results demonstrated small losses in body weight in all groups (**Figure 2a**). While these data indicated that CR caused a significant weight loss, a better and more efficient CR protocol was needed since all the fish lost weight, and this likely indicates that all animals were under some stress.

CR can be performed as a daily reduction in caloric intake or as an every-other-day feeding regimen, also known as an intermittent fasting (IF) paradigm. Research has shown that the effects on body weight and markers related to cellular and synaptic plasticity in the brain are not different for these two paradigms [88]. In our recent study [68], we utilized an IF regimen that included dry flakes and artemia (**Table 2**). An IF paradigm would not disrupt any social hierarchy of the fish or cause any unnecessary social isolation or netting stress in the fish since the animals would be kept in their home tanks. Artemia is not only an extra source of protein but also provides environmental enrichment to the fish [89]. The diet continued for 10 weeks and the results indicated that while there was no prevention of an age-related decline in newly born neurons, IF treatment stabilized an age-related decline in telomere shortening [68]. Thus, some of the beneficial effects of dietary restriction maybe done through subtly altering the cell cycle dynamics.

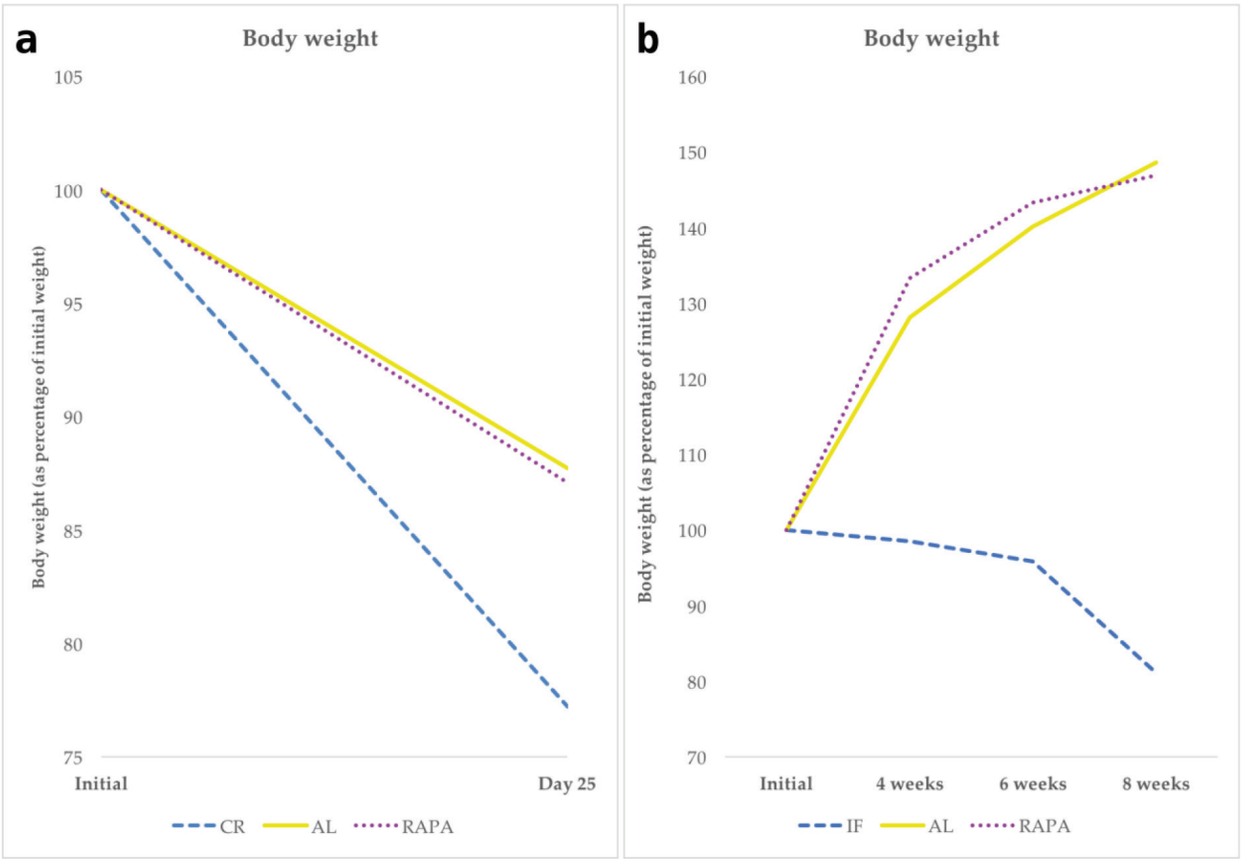


Figure 2. (a) Final body weight on day 25 as percentage of the initial body weight. AL and RAPA animals lost 12 and 13% of their initial weight, respectively, whereas CR animals lost 23%. (b) The effect of IF and rapamycin treatment on body weight of animals. Body weights at 4, 6 and 8 weeks were compared to the average weight at the beginning of the experiment. While AL and RAPA animals showed a very similar pattern of weight gain throughout 8-week experiment, average weight of IF animals was first stabilized, then after 4 weeks they continuously lost weight, with the largest decline occurring between 6 and 8 weeks.

Our group has extended this paradigm to include a rapamycin-treated group to test whether rapamycin could mimic CR's effects [90], and careful design was necessary since the fish remained continuously in the tanks (**Figure 1e**) while receiving the drug treatment and the half-life of the drug needed to be considered. According to the literature, half-life of rapamycin is 3 days [87] so at least half and at most three-quarters of the tank water was replaced with fresh water supplemented with 100 nM rapamycin every 3 days to keep the active drug levels consistent (**Table 2**). We applied the same protocol for water replacement for *ad libitum* (AL) and IF groups without adding rapamycin, since the change of aquaria water also permitted us to keep nitrate levels low and pH levels stable. Our preliminary data demonstrated that body weight decreased significantly after 6–8 weeks of IF treatment but was not different in the AL or rapamycin-treated animals [91] (**Figure 2b**). These data suggest that an IF paradigm in zebrafish can significantly reduce body weight in young and old animals, and the effects of IF can be studied in conjunction with a drug-treated group. Current analysis is being performed

as to whether these treatments alter the course of some of the neurobiological alterations that were referred to in Section 2.2. For example, CR may regulate DNA methylation at individual loci by increasing the activities and/or expression levels of certain DNA methyltransferase (DNMT) enzymes in different animal models [92, 93]. In addition to the regulation of DNMTs, histone acetylation and deacetylation are affected by CR [94]. Induction of neuroprotection-related gene expression patterns by CR, such as upregulation of *mir-98-3p* in the cerebral cortex of rats have also been shown [95]. Also, CR regulates synaptic proteins in the aging brain and delays cognitive declines in memory [96]. Therefore, the durations of CR or IF alongside potential mimetics of dietary restriction are important to examine in zebrafish for potential translation to human studies.

3.2. Overfeeding

Contrary to CR and IF paradigms that decelerate the aging process, overfeeding has been proposed to accelerate aging. For example, the effects of obesity on behavioral and neurobiological changes have been investigated in humans [76] and animal models [97]. Zebrafish, like humans, will overeat if exposed to large amounts of food [98]. Although studies have examined the effects of overfeeding in zebrafish [99–101], few studies have utilized these animals as a model to study obesity, and to a lesser extent to study the effects of brain aging. A recent study demonstrated that a life-long high caloric diet in adult zebrafish caused significant increases in body weight, and eventually obesity, along with high cortisol levels and decreased rate of neurogenesis which were similar to the observations on older fish [101].

In order to compare our dietary restriction and drug manipulations with overfeeding, our research group decided to test whether short-term overfeeding in fish will lead to premature neurobiological changes in the aging brain in young adults and even further accelerated brain aging in older fish. For this paradigm, we increased the amount of dry food per feeding to two times the normal quantity and supplied artemia every day instead of three times a week. These overfed fish will be compared back to our regular AL- and IF-treated groups (**Table 2**). Our initial observations indicate that the animals exposed to overfeeding have an increase in body weight in comparison to AL-fed and IF animals. This type of feeding paradigm, along with examining the effects of short-term dietary restriction, has important implications for possible interventions and translational studies for humans.

3.3. Mutant models

Accelerated or decelerated brain aging may be affected by environmental manipulations such as overfeeding or dietary restriction. However, it is difficult to establish the link between direct cellular and molecular mechanisms affecting the behavioral or neurobiological changes upon such dietary manipulations. As was mentioned earlier, one of the advantages of the zebrafish animal model is the ease of manipulating genes. This can be done with forward genetics screening where one can identify mutated genes responsible for a particular disease phenotype, or it is possible to introduce a mutation in a specific gene of interest by using reverse genetic screens such as the transposon-mediated insertional mutagenesis (Tol2) or CRISPR-Cas9 system. Using inducible tissue- or cell-specific promotors a gene could be

expressed at anatomically distinct areas in the brain, and thus a more refined understanding of the gene function. Taken together, all of these available genetic tools and approaches allow zebrafish researchers to perform large-scale genetic screens in order to understand the function of corresponding genes during development and adulthood.

Identifying genes which play role in decelerated and accelerated aging models are critically important. One such example, which was obtained from a chemical screen, is a fish line with a specific mutation in the acetylcholinesterase gene (*ache*^{sb55}) that results in a loss-of-function of this enzyme and an increase in the levels of acetylcholine [102]. In the normal aging brain, there is a loss of acetylcholine that is thought to contribute to age-related cognitive decline [103], so the fish with increased acetylcholine levels should have delayed brain aging and cognitive deficits. While homozygous mutants die in very early developmental stages, interestingly, mutants with a heterozygous mutation will develop and live until late adulthood. Furthermore, Yu et al. demonstrated that age-related spatial learning deficits are delayed in these animals, which is consistent with the hypothesis that acetylcholine levels affect age-related cognitive function [14]. Thus, these data suggest that preventing age-related declines in acetylcholine can alter the pattern of age-related phenotypes.

In order to understand the direct cellular mechanism of dietary restriction, we are currently examining the effects of altering the TOR signaling pathway in zebrafish. We have two models with which we are currently working. The first is a zebrafish line with a knockout of the *tor* gene using the Tol2 system that resulted in a mutated TOR [104]. In the second we are creating transgenic models of zebrafish using the Tol2 system that will express one of the following types of TOR, (1) an overactivated mouse TOR complex, (2) an overactivated mouse TOR complex that is rapamycin resistant, (3) a reduced or an inactive mouse TOR complex, or (4) a reduced or an inactive mouse TOR complex that that is rapamycin resistant. This will allow us to examine the effects of dietary restriction and rapamycin separately. While these data regarding *ache*^{sb55} mutants and the data from potential TOR transgenics are interesting, a confound still exists that needs to be addressed, which is that in both cases the mutations are active throughout the entire life of the animal, including early developmental time periods. Thus, future studies need to be directed at creating tissue- or cell-specific lines that are inducible, which can be easily be done in zebrafish.

3.4. Transient gene knockdowns and overexpression in adults and embryos

One of the useful genetic tools available for use in zebrafish is injections of both morpholino antisense oligonucleotides and *in vitro* transcribed capped mRNA. The morpholinos create a transient knockdown of a specific gene of interest and are an essential tool for understanding the specific role of that gene. Microinjecting morpholino antisense oligonucleotides targets and blocks access of the cellular components to specific RNAs and by doing so, can prevent translation, splicing, or ribozyme activity depending on their design, and results in a loss of a functional protein [105, 106]. Microinjection of *in vitro* transcribed capped mRNA, on the other hand, serves as a powerful tool for overexpressing a gene of interest to understand its function *in vivo*, which is in contrast to the knockdown approach with morpholinos [107–109]. From a mechanistic point of view, morpholino antisense oligonucleotides

can be designed in several ways, either translation-blocking or splice-blocking [105, 106]. As its name implies, a translation-blocking morpholino oligonucleotide binds to its target RNA and blocks the sites where the translation initiation machinery accesses the target RNA molecules. The splice-blocking morpholino oligonucleotide inhibits the splicing of pre-mRNA. Besides these traditional types of morpholino, there is also the vivo-morpholino, which is a promising tool for transient gene silencing in adult animals and cell culture [110–115]. These vivo-morpholino experiments can also be compared to injections of *in vitro* transcribed capped mRNA into the adult brain to overexpress a gene of interest. Taken together these techniques allow for silencing or overexpressing a gene of interest in both embryos and adults.

Injections of both morpholino antisense oligonucleotides and *in vitro* transcribed capped mRNA can be easily done in embryos and adults. The injections into embryos are done in the 1–4 cell stage. A method utilized for both vivo-morpholino and *in vitro* capped mRNA injections in the adult brain is performed by cerebroventricular microinjection (CVMI). The CVMI technique was used by Kizil et al. [116, 117] to investigate the effects of overproduction of amyloid protein on cellular, molecular and functional aspects of the adult zebrafish brain, and has been shown to be a useful method for inactivation studies in zebrafish and other vertebrates [118–123]. These techniques in the adults hold promise to provide an understanding of the role of a gene of interest without the possible confounding effects of early developmental changes on the future adult animal. Moreover, the embryo injections not only give insight into the developmental role of a gene of interest but might also provide a platform for an *in vivo* cell culture model for examining the cellular mechanisms of aging and aging-related interventions.

4. Conclusions

Based on the similarities to the aging process in humans, the zebrafish is emerging as a promising model to understand the mechanisms that lead to age-related behavioral and neurobiological alterations. Additionally, there are many genetic tools such as mutagenesis protocols and transient knockdown/overexpression approaches that can easily be applied to the zebrafish to intervene in this aging process, in addition to the many potential environmental manipulations. Moreover, it is possible to study different behavioral paradigms such as visual motion processing and spatial learning and memory abilities that change with increasing age. In these behaviorally-characterized animals it will be feasible to measure the expression levels of neurobiological markers to understand their functional role in cognition, and determine the success of potential interventions. The zebrafish is also a powerful model for the use of drug screening and provides information about promising therapeutics that can be eventually translated to human populations. All in all, this gerontological model will be very useful for unlocking the secrets of the aging brain and potentially helping to uncover anti-aging therapies in order to restore brains back to their youthful capacity.

Acknowledgements

The current studies by our group mentioned in this chapter are being supported by grants (214S236 and 215S701) under the 1001 project scheme from the Scientific and Technological Research Council of Turkey (TÜBİTAK). The initial caloric restriction/rapamycin study was supported by an Installation Grant from the European Molecular Biology Organization (EMBO).

Conflict of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

The authors would like to thank Tülay Arayıcı for providing excellent technical assistance for all the experiments mentioned in this chapter that were conducted in the Department of Molecular Biology and Genetics Zebrafish Facility in Bilkent University. Additionally, the authors thank Dr. Ayça Arslan-Ergül, Göksemin Fatma Şengül, and Narin Ilgım Ardıç for help with feeding and tissue harvesting in the experiments. Finally, the authors thank Elif Karoğlu for helpful discussion with regards to the information for the design of the overfeeding experiments.

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