Caloric restriction reinforces the stem cell pool in the aged brain without affecting overall proliferation status

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ABSTRACT

Overfeeding (OF) and obesity increase the risk for brain aging and neurodegenerative diseases due to increased oxidative stress and neuroinflammation, which likely contribute to cellular dysfunction. In contrast, caloric restriction (CR) is an intervention known for its effects on extending both life- and health-span. In the current study, the effects on the aging brain of two short-term feeding regimens, OF and CR, were investigated. We applied these diets for 12 weeks to both young and aged zebrafish. We performed protein and mRNA level analysis to examine diet-mediated effects on any potential age-related alterations in the brain. Markers implicated in the regulation of brain aging, cell cycle, proliferation, inflammation, and cytoskeleton were analyzed. The most prominent result observed was a downregulation in the expression levels of the stem cell marker, Sox2, in CR-fed animals as compared to OF-fed fish. Furthermore, our data highlighted significant age-related downregulations in Tp53, Myca, and L-plastin levels. The multivariate analyses of all datasets suggested that as opposed to OF, the adaptive mechanisms increasing lifespan via CR are likely exerting their effects by reinforcing the stem cell pool and downregulating inflammation. The data reveal important therapeutic targets with respect to the state of nutrient uptake for the slowing down of the detrimental effects of aging, resulting in a healthy and extended lifespan, as well as lowering the risk for neurodegenerative disease.

1. Introduction

The risks for both cognitive impairment and neurodegenerative disorders increase as one ages. Aging-associated cognitive impairments include gradual declines in conceptual reasoning, memory, and processing speed (Harada et al., 2013). Moreover, aging is an inevitable biological process and a complex state that induces numerous cellular alterations in the organism such as dysregulation of nutrient sensing, stem cell exhaustion, epigenetic alterations, genomic instability, and loss of protein homeostasis, all of which could lead to the increased risk for neurodegeneration and its associated cognitive decline (López-Otín et al., 2013; Jones et al., 2014). For these reasons, it is important to find the most effective and easy-to-apply approaches to counteract any age-related changes to increase the health span of individuals. In this study, we focused on short-term dietary regimens in order to examine their effects on aging-related alterations in cell proliferation and inflammation in the brain.

Cell cycle regulation has been implicated as being susceptible to the effects of aging. For example, cellular senescence, the state in which cells enter a permanent cell cycle arrest and lose the ability to divide and grow, is known to be associated with phenotypes in conjunction with age-related pathologies (Campisi and Robert 2014). The consequences

of this will be affecting the downstream pathways related to cell renewal/repair. Although senescence may serve to prevent cancerous development in mammals (He and Sharpless 2017), it also restricts cell proliferation and cell renewal. Cell cycle progression can be promoted or inhibited by different targets affecting overall proliferation. Disruptions in cell cycle regulation were previously associated with neurodegenerative diseases (Copani et al., 2007; Ting et al., 2014; van Leeuwen and Hoozemans 2015), such that abnormal exits from cell cycle arrest cause differentiated neurons to raise tau phosphorylation, leading to apoptosis and progressing into Alzheimer's disease (Ueberham and Arendt 2005).

A positive regulator that may play a pivotal role in neural stem cell (NSC) proliferation during brain aging and neurodegenerative disease is the transcription factor Dp1 (Tfdp1). Dp1 is a cell cycle protein that controls G1 to S phase transition by forming a complex with E2f proteins (Erbaba et al., 2020a). A gain of function Tfdp1 mutation was previously reported to increase cell proliferation, migration, and invasion of colorectal cancer cells (Chen et al., 2014a). Together, Tfdp1 and E2f stimulate E2f dependent transcription of genes implicated in cell cycle progression (Erbaba et al., 2020a). While there is no study regarding the relationship between brain aging and/or neurodegenerative disease and Tfdp1, there is evidence to suggest that cell cycle progression events, collectively referred to as 'senescence,' are known to be associated with aging (Di Micco et al., 2021). Thus, it is important to understand through which mechanisms these alterations occur.

Neuroinflammation is a common underlying factor affecting the health of brain aging and the progression of aging-related neurodegenerative disease phenotypes. The main regulators of aging-related inflammation are microglial activation, increased levels of reactive oxygen species (ROS), accumulation of proinflammatory cytokines, and decreases in autophagy (Si and Liu 2014; Ward et al., 2015; Hickman et al., 2018; Guzman-Martinez et al., 2019). The nutritional status of the organism has a direct relationship with metabolic inflammation indicating that metabolic states influence brain health (Mattson and Arumugam 2018).

Accumulating evidence suggests that nutrient intake is a double-edged sword with excess nutrient uptake leading to exacerbated age-related symptoms and caloric restriction (CR) extending life and health span which guards against the detrimental effects of aging. Such possible detrimental effects of increased caloric intake on the brain include systemic inflammation, induction of synaptic stripping by microglia, gliosis that is the brain's injury response, impairments of hippocampal plasticity, disrupted connectivity, blood–brain barrier damage, and cognitive dysfunction (Miller and Spencer 2014; Tucsek et al., 2014; de Git and Adan 2015; Dorfman and Thaler 2015; Hao et al., 2016; Guillemot-Legris and Muccioli 2017). Furthermore, increased body weight and metabolic disorders were associated previously with the generation of excessive ROS and oxidative free radicals. These harmful byproducts then cause alterations in cell cycle regulation and cell signaling (Rani et al., 2016). On the other hand, caloric restriction (CR), a diet reached by 30–40% reduction in nutrient uptake, has previously been shown to decrease apoptosis, facilitate stem cell renewal, increase resistance to age-related neurodegenerative diseases, enhance neurogenesis, reduce DNA damage, and exert anti-oxidant and anti-inflammatory properties (Csiszar et al., 2009; Park and Lee 2011; Arslan-Ergul et al., 2016; Cavallucci et al., 2016; Mojaverrostami et al., 2020). However, exactly which key proteins in cell cycle regulation are responding to nutrient uptake status remains to be determined.

In the current study, we examined the effects of two opposing dietary interventions on markers of cell proliferation, and inflammation in the aging zebrafish brain. We used zebrafish model organism because it has become an important and appropriate gerontological model to study brain and aging, having similarities with humans (Celebi-Birand et al., 2018). They can live 3 years on average, age gradually and exhibit cognitive decline as in humans (Arslan-Ergul and Adams, 2014; Yu et al., 2006). They have an integrated nervous system and exhibit advanced behavior properties like memory and social behavior (Lieschke and Currie, 2007; Arslan-Ergul et al., 2013; Celebi-Birand et al., 2021). In order to quantify the differences between the effects of overfeeding (OF) and CR on the brains of young and old zebrafish, we applied a 12-week long diet of either *ad libitum* (AL)-feeding, OF, or CR similar to our previous studies (Arslan-Ergul et al., 2016a; Celebi-Birand et al., 2020; Karoglu-Eravsar et al., 2021). The differences in brain protein and/or mRNA levels were compared for selected markers related to cell proliferation (Pcna, Sox2), cell cycle regulation (Tfdp1, E2f5, Myca, Tp53), neuronal/glial identity (HuC, Gfap), neuronal migration/cytoskeletal regulation (Dcamkl1, L-plastin), and inflammation (Il1b, Tnfa). In addition, we added Alcamb in order to determine its relationship to stem cell engraftment potential (Jeannet et al., 2013) since we previously suggested it to be involved in brain aging (Erbaba et al., 2020b), and Rest for its strong correlations towards increased longevity and neuronal protection by repressing genes involved in oxidative stress, neuronal death and β-amyloid toxicity (Hwang and Zukin, 2018). In this study, Myca, Tp53, and Lcp1 expression levels were significantly decreased with advancing age and OF significantly elevated Sox2 expression levels in the brains of zebrafish. Finally, we showed with multivariate analyses that the OF diet groups clustered at higher values for proliferation and inflammation-related protein and gene expression levels, while CR diet groups clustered at lower values. These data suggest that OF is correlated with increases in cell proliferation and inflammation, while CR induces opposite effects. The results from this study identify alternative therapeutic approaches and targets for reversing neurobiological changes during brain aging.

2. Material and methods

2.1. Animals

A total of 60 TgBAC(Gfap-GFP)zf167 zebrafish were included in this study, with 30 young (5-month) and 30 aged (25-month) animals. Both male and female animals were included in the current experiments. In order to obtain a gender balance, at the beginning of the feeding schedule, both male and female fish identified by eye were equally included in each tank. However, at the time of the dissections at the end of the feeding schedules there was a slight gender imbalance between the treatment and/or age groups. Since the gender balance was not perfect, no effects of gender were determined but this would be an important future study to do. All fish were kept in a controlled recirculating housing system designed by ZebTec (Tecniplast, Italy) with a 14-hour light and 10-hour dark cycle at 28 °C system water. For both ages, fish from an 8.5-liter tank were equally divided into three 3.5-liter tanks and assigned randomly to one of the following feeding groups: *ad libitum* (AL: normal feeding), OF and CR. Six animals per treatment and age group (overall n = 36) were assigned to Western blot experiments, and four animals per group (overall n = 24) to the RT-PCR experiments.

Fish were fed according to a feeding schedule based on Table 1. A 12-week diet intervention period was chosen considering the previous reports that it takes at least 8 weeks to affect both cognitive and molecular phenotypes (Arslan-Ergul et al., 2016; Meguro et al., 2019; Celebi-Birand et al., 2020; Karoglu-Eravsar et al., 2021). The nutrient composition of the commercial dry flakes consisted of the following: crude protein equal to 46%, crude oils and fats 11%, crude fiber 3%, and moisture content 6%. Freshly-hatched artemia were prepared and given to the fish as indicated in Table 1. The feeding protocol was applied for 12 weeks after a week of habituation to the tanks prior to beginning the dietary interventions. At the end of the 12 weeks, fish were euthanized in ice-cold tank system water as has been performed previously (Chen et al., 2014b), which was indicated by a cessation of movements that occurred for 10 min. The weights (g) and lengths (cm) of the young and aged fish were quantified at the end of the 12-week diet application, as well as the BMI (g/cm^2) values, which were calculated by dividing body weights by the square of the lengths. Following these measurements, the heads were decapitated from the body and then snap-frozen in liquid

Table 1
Weekly feeding schedule applied to fish for 12 weeks.

	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
AL	100 mg Dry Food 100 mg Dry Food 1x Artemia	100 mg Dry Food 100 mg Dry Food	100 mg Dry Food 100 mg Dry Food 1x Artemia	100 mg Dry Food 100 mg Dry Food	100 mg Dry Food 100 mg Dry Food 1x Artemia	100 mg Dry Food 100 mg Dry Food	100 mg Dry Food 100 mg Dry Food
CR	100 mg Dry Food		100 mg Dry Food 1x Artemia		100 mg Dry Food		100 mg Dry Food
OF	300 mg Dry Food 2x Artemia 300 mg Dry Food 2x Artemia	300 mg Dry Food 2x Artemia 300 mg Dry Food 2x Artemia	300 mg Dry Food 2x Artemia 300 mg Dry Food 2x Artemia	300 mg Dry Food 2x Artemia 300 mg Dry Food 2x Artemia	300 mg Dry Food 2x Artemia 300 mg Dry Food 2x Artemia	300 mg Dry Food 2x Artemia 300 mg Dry Food 2x Artemia	300 mg Dry Food 2x Artemia 300 mg Dry Food 2x Artemia

nitrogen in order to be stored at −80 °C for the molecular analyses. The animal protocol for this study was approved by the Bilkent University Local Animal Ethics Committee (HADYEK) with the approval date and number: Oct 10, 2019, no: 2019/37.

2.2. Protein and RNA isolation

For the Western blot experiments, individual whole brains were dissected from snap-frozen heads stored at −80 °C, and proteins were extracted according to a previously-described protocol from our group (Karoglu et al., 2017). Briefly, brain tissues were first homogenized with a 30-gauge syringe in 300 μl of RIPA lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, with protease inhibitor (Roche, Mannheim, Germany, 5892970001), and then homogenates were centrifuged after 30 min incubation on ice. Finally, total protein concentrations were quantified using the Bradford Assay (Bradford Reagent, B6916, Sigma, St. Louis, MO, USA) with respect to known concentrations of bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA).

For the real-time polymerase chain reaction (RT-PCR) experiments, whole brains were dissected in cold dPBS under the stereo microscope (Zeiss Stemi 1000, Germany) from the snap-frozen heads stored at −80 °C. Total RNAs were isolated by homogenizing the tissues with a 30-gauge sterile syringe using Thermo Fisher Scientific's TRIzol Reagent (Waltham, MA, USA) according to the manufacturer's instructions. The isolation protocols were followed by DNase treatment with an Ambion TURBO DNA-free Kit from Thermo Fisher Scientific following the manufacturer's instructions (Waltham, MA, USA). The concentrations of the isolated RNA samples were measured via a NanoDrop 2000 (ThermoScientific, Waltham, MA, USA), and the conversion of RNA to cDNA was performed using iScript cDNA Synthesis Kit (Biorad, Hercules, CA, USA).

2.3. Western blotting

For both young and old groups, 40 μg of protein was loaded into SDS-PAGE gels in order to have comparable bands. Following gel separation, proteins were transferred onto the PVDF membrane. TBS-T with 5% milk powder was used as the blocking agent in all the procedures. The primary antibody concentrations were decided in an assay-dependent manner based on the manufacturer's instructions (Table 2). Six brain protein samples were run as a cohort from each of the three feeding groups. Young and old animal group western blots were run separately. In order to minimize any potential systematic differences due to gel variation in the Western blotting experiments, the samples were loaded in alternating order. Lastly, chemiluminescent detection was performed using Thermo Scientific SuperSignal West Femto Maximum Sensitivity Substrate (Thermoscientific, Rockford, IL, USA: 34095) on ChemiDoc MP Imaging System (BioRad, Hercules, CA, USA). Band intensities were

Table 2
The primary and secondary antibodies used in the Western Blot experiments.

Antibody	Supplier	Use	Dilution
Anti-Dp1 (ab186831) (Supp. Fig. 1)	Abcam (UK)	Primary	1:1000
Anti-L-Plastin (ab210099) (Supp. Fig. 2)	Abcam (UK)	Primary	1:1000
Anti-GFP (ab6556) (Supp. Fig. 3)	Abcam (UK)	Primary	1:1000
Anti-Pcna (ab29) (Supp. Fig. 4)	Abcam (UK)	Primary	1:1000
Anti-HuC (ab78467) (Tuz Sasik 2020)	Abcam (UK)	Primary	1:1000
Anti-Dcamkl1 (ab109029) (Tuz Sasik 2020)	Abcam (UK)	Primary	1:5000
Anti-Tubulin (2146S) (Karoğlu 2017)	CST (USA)	Primary	1:5000
Anti-rabbit HRP linked antibody (7074S) (Tüz Şaşik 2020)	CST (USA)	Secondary	1:5000
Anti-mouse HRP linked antibody (ab97023) (Supp. Fig. 4)	Abcam (UK)	Secondary	1:5000

quantified by a blind analysis using ImageJ software (NIH, Bethesda, MD, USA). Following band intensity quantifications, within the gel and housekeeping protein (β-tubulin) normalizations were performed, as described previously (Karoglu et al., 2017).

2.4. Real-time polymerase chain reaction (RT-PCR)

RT-PCR was used to screen for gene expression differences in the brains of animals that were given a 12-week OF or CR diet as compared to AL-feeding. The primer sequences of genes of interest are provided in Table 3. For the RT-PCR experiments, LifeScience's LightCycler® 480 SYBR Green I Master kit (Roche, Germany) was utilized for use with the LightCycler® 480 machine (Roche, Basel, Switzerland). The protocol was completed with 2 μl of cDNA in a 20 μl reaction volume. Each sample had 3 technical replicates which were run in different plates. The measurements were performed by normalizing Ct values with regard to the geometric mean of two reference genes that included *beta-actin1* and the *60S ribosomal protein, L13 (rpl13a)* and log fold change values were calculated using the delta delta Ct method (de Oliveira et al., 2014).

2.5. Statistical analysis

All statistical analyses were performed using IBM's SPSS statistical software (IBM, Armonk, NY, USA). Normality and homogeneity of variance assumptions were tested with Kolmogorov-Smirnov and Levene's tests, respectively. Whenever the assumptions were met, an analysis of variance (ANOVA) test with factors of age having two levels (young/aged), and feeding groups having three levels (AL/OF/CR)

Table 3
Primers used for RT-PCR experiments.

Purpose	Name	Forward Sequence (5′→3′)	Reverse Sequence (5′→3′)
Cell cycle regulation	Tfdp1	CAGGCTTTGACTGTTGGAAAA	GGCCTCTGAGGTGTGCTAAT
Cell cycle suppression	Tp53	CGCGGATTTGCTTTGTGGAT	CGTTTTGCGCCATTGCTTTG
Cell cycle activation	E2f5	AGCGGAGACACTCTTCTAGC	TTGACCATTGTGGCCCATTT
Cell adhesion behavior [previously associated with stem cell maintenance (Erbaba et al., 2020b)]	Alcamb	ACGGTGCAGTGGATTACCAA	TCACGTCCTCAACAAGCGAC
Cellular stemness	Sox2	GACCATTCATCGACGAAGCC	CCTCCGGGGTCTGTATTTGT
Neuroprotection	Rest	GCGTACACCACCATCAGTCA	GAATGGACGCTCACCAGTGT
Inflammation	Il1b	GATCCGCTTGCAATGAGCTAC	TCAGGGCGATGATGACGTTC
Inflammation [previously associated with overall inflammation in zebrafish (Kyritsis et al., 2012)]	Lcp1	GCCTTCACTAAAGTCGATGTGG	CGAAGGTGATCTTCCCGTCC
Cell growth/cell cycle regulation (Schmidt 1999)	Myca	AACGGCATTCGTTAAACACA	ATCCTCATCGTCGTTGTCG
Inflammation	TnfaF	AGGAGAGTTGCCTTTACCGC	GTGAGTCTCAGCACACTTCCA
Reference gene	Actb1	GCCTGACGGACAGGTCAT	ACCGCAAGATTCCATACCC
Reference gene	Rpl13a	ATGAACACCAACCCTTCCCG	ACCATGCGCTTTCTCTTGTC

followed by Bonferroni post-hoc tests in order to adjust for multiple comparisons. For the cases where the assumptions of normality and/or homogeneity of variance were violated, a non-parametric Kruskal Wallis test followed by Mann Whitney U tests for pairwise comparisons with Bonferroni corrected *p*-values were used. Where appropriate, pairwise comparisons were either done across treatment or age, as well as across individual animal groups. In order to investigate the relationships between the protein amounts and transcriptional differences among the selected markers, we formed correlation matrices separately for both protein and gene expression datasets using a Spearman correlation coefficient test (Supplementary Data). Further investigation of the protein and gene expression levels in the whole fish brains was performed using principal component analysis (PCA) to determine the changes in each protein/gene expression relative to each individual protein/gene. For component extraction, the eigenvalues were set above the 1.0 level. Significance levels in all analyses were set at $p < 0.05$ unless corrected as in the Mann Whitney U pairwise comparisons. Graphs of the data were made with Graphpad 8 software (San Diego, CA, USA). The PCA plots were generated using SPSS software.

3. Results

3.1. Fish weights, lengths, and body mass index (BMI) values were altered by age and dietary feeding regimens

The dietary interventions induced significant BMI differences among the groups (Fig. 1c). For all weight, length, and BMI data, there were both significant main effects of age (weight $U = 700$, $z = -5.899$, $p < 0.001$; length $U = 984$, $z = -4.115$, $p < 0.001$; BMI $U = 585.5$, $z = -6.128$, $p < 0.001$), and diet treatment (weight $H(2) = 33.772$, $p < 0.001$; length $H(2) = 35.592$, $p < 0.001$; BMI $H(2) = 29.382$, $p < 0.001$) (Fig. 1a-c). Overall, the CR animals had significantly lower weight, length, and BMI values than the AL ($p < 0.001$ for all) and OF groups (p

< 0.001 for all) at both young and old ages. There was also a significant difference between AL and OF groups with respect to the length data at young age ($p = 0.046$). However, there were no significant increases in the OF fish levels compared to the AL in weight ($p > 0.05$), and BMI data ($p > 0.05$) although there were numerical increases in OF group for all three data points compared to AL and CR groups.

3.2. Selected protein levels related to cellular proliferation, cell cycle regulation, cellular differentiation and migration, and inflammation were stable across the age and dietary groups
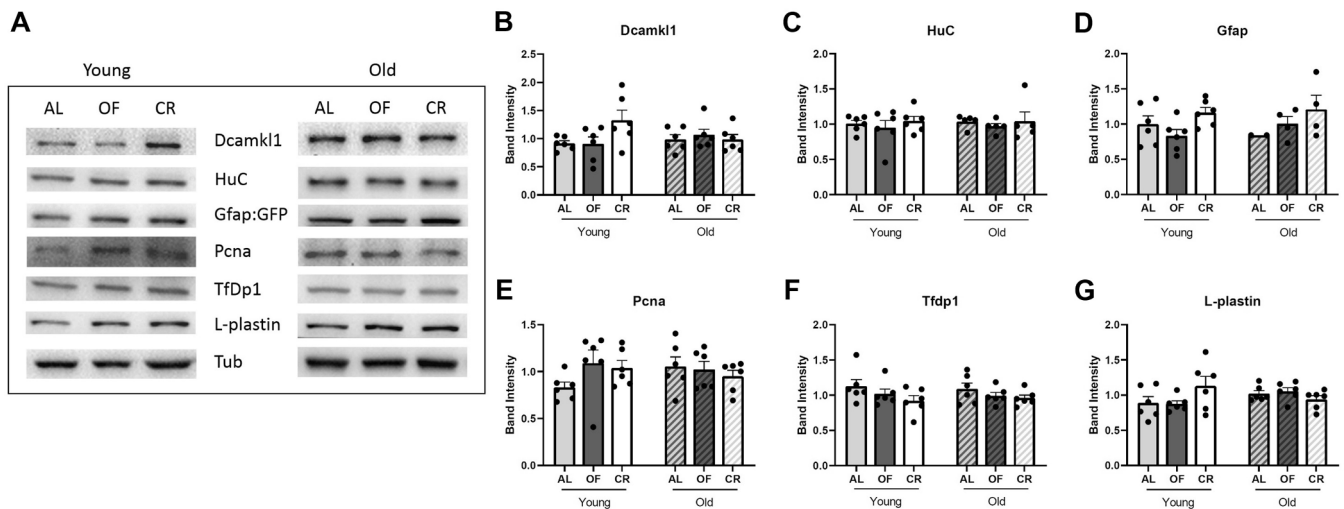
The brains from the different feeding groups were tested for possible age- and diet-related alterations in the amounts of target proteins related to cell proliferation, cell cycle regulation, neuronal/glial identity, neuronal migration/cytoskeletal regulation, and inflammation. Representative protein bands from the brains of young and old animals treated with the various dietary interventions are shown together in Fig. 2a.

Initially, Dcamkl1, a microtubule-polymerizing protein, was examined to determine if there were any effects of age or dietary manipulation related to microtubule organization or neuronal migration (Vreugdenhil et al., 2007). The results showed that there was no main effect of treatment ($F(2, 30) = 1.923$, $p = 0.164$) or age ($F(1, 30) = 0.179$, $p = 0.675$) on the protein levels (Fig. 2b). With respect to Dcamkl1 protein levels, there was only a marginally significant effect of the interaction between treatment and age ($F(2, 30) = 2.847$, $p = 0.074$). These data suggest that any effects of the diet might depend on the age of the animal to which the treatment is given.

To infer the effects of diet on the number of cells of either neuronal or glial origin, HuC and Gfap protein levels were measured, respectively, across all of the feeding groups. For HuC protein levels, no observable change was detected in the brain with either any of the feeding interventions ($H(2) = 0.597$, $p = 0.742$) or with age ($U = 120$, $z = -0.542$, $p = 0.588$) (Fig. 2c). Gfap levels in the brains of the young and old
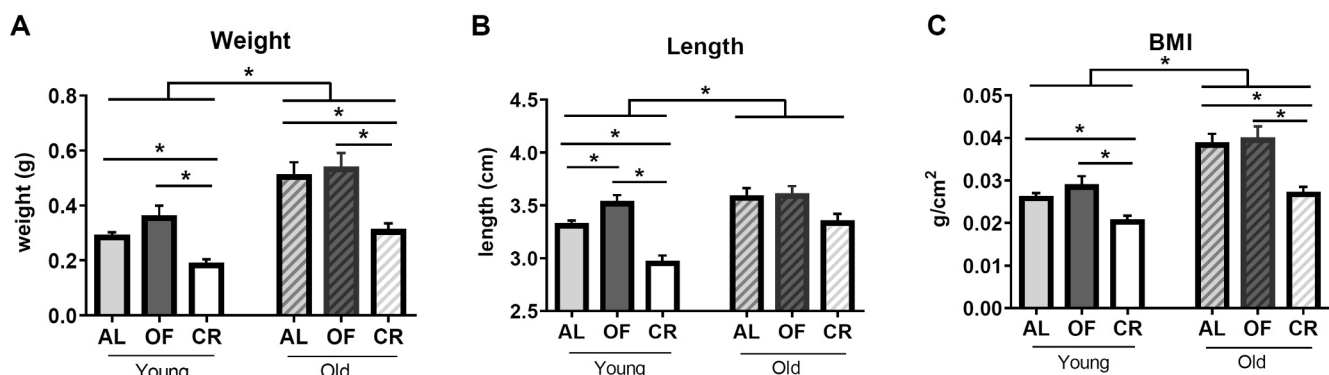
Fig. 1. Average (a) weight (N = 123), (b) length (N = 119), and (c) Body Mass Index (BMI) (N = 119) data across feeding groups are shown. All values are significantly increasing with age (p < 0.001) and there are significant main effects of treatment for all three data points (p < 0.001).

Fig. 2. (a) Representative Western blot images for Dcamkl1, HuC, Gfap:GFP, Pcna, Tfdp1, L-plastin, and β-Tubulin protein levels from the different feeding groups. Protein levels of (b) Dcamkl1 (N = 36), (c) HuC (N = 33), (d) Gfap (N = 28), (e) Pcna (N = 36), (f) Tfdp1 (N = 36), and (g) L-plastin (N = 36) across different feeding regimens and age. AL: Ad-libitum, OF: overfed, CR: Caloric restriction.

animals were measured to gain insight into the nature of the glial response. For this purpose, an anti-GFP antibody was used to measure GFAP levels (Lam et al., 2009). In the current study, our results demonstrated no main effect of age ($F(1, 30) = 0.038$, $p = 0.848$) nor showed any interaction ($F(2, 30) = 0.740$, $p = 0.489$) on Gfap levels. A marginally significant main effect of treatment was observed for Gfap levels ($F(2, 30) = 3.221$, $p = 0.059$). Overall, CR feeding appeared to increase Gfap protein levels in the brains compared to AL and OF treatments (Fig. 2d).

In order to examine whether age or diet affects total cell proliferation the protein levels of Pcna were measured. This antibody is used to mark the actively dividing cells (Zhang and Jiao 2015). In our experiments, the Pcna protein levels were not found to be statistically different with respect to diet or age (treatment: $F(2, 30) = 0.758$, $p = 0.477$; age: $F(1, 30) = 0.076$, $p = 0.785$; interaction: $F(2, 30) = 1.759$, $p = 0.190$)) (Fig. 2e).

In terms of the cell cycle regulatory components, a comparison in the brain protein level differences of Tfdp1 showed that there was no effect of age or interaction (age: $F(1, 30) = 0.027$, $p = 0.871$; interaction: $F(2, 30) = 0.163$, $p = 0.850$). There was a marginally significant main effect of treatment on Tfdp1 protein levels ($F(2, 30) = 2.807$, $p = 0.076$) (Fig. 2f). This may suggest that CR diet is likely decreasing the Tfdp1 protein levels compared to the AL-fed subjects and could alter cell cycle progression.

The last protein marker of interest measured in the brains was L-plastin, which is involved in regulating the cytoskeleton. The results demonstrated no significant change in L-plastin protein levels in the brain with any of the short-term feeding interventions ($H(2) = 0.263$, $p = 0.877$) or with age ($U = 125$, $z = -1.171$, $p = 0.242$) (Fig. 2g). Stability of L-plastin levels suggests that neither diet nor age causes a drastic and direct influence on cytoskeletal regulations.

3.3. Multivariate analysis of proteins demonstrates an upregulation in cell proliferation with an OF regimen and downregulation in proliferation and potential induction in neuronal plasticity with CR

Principal component analysis (PCA) was performed to explore the pattern of alterations in the levels of each protein as compared to each of the others. Analysis was performed for the complete dataset of proteins of interest regarding the Western blot band intensities. Three main components were extracted by the analysis, accounting for 70.1% of the variance in the protein expression data (Table 4). Visualization of the data in two dimensions is shown in Fig. 3.

Table 4

Each component of the PCA analysis of the protein datasets and the percentage of the explained variance is shown, as well as the factors having highest loading scores for each component. The results demonstrated that 28.82% of the variance was explained by PC1, 22.77% by PC2, and 18.52% by PC3. Only the factors with the loading scores of 0.5 and above are presented.

		% of Variance Explained	Component Loading Scores (>0.5)		
Principal Component (Protein data)	PC1	28.82	L-Plastin (0.713)	HuC (0.685)	Gfap (0.554)
	PC2	22.77	Gfap (-0.654)	Pcna (0.519)	HuC (-0.517)
	PC3	18.52	Tfdp1 (-0.774)	Dcamkl1 (0.671)	
	Total	70.1			

The first principal component (PC1) of the protein data was driven positively by L-plastin, a cytoskeletal regulator; HuC, a neuronal marker; and Gfap, a marker for astroglial reactivity (Anderson et al., 2014) (Table 4). For the PC1, the young CR samples partially clustered at higher values than the young OF group (Fig. 3a). The second principal component (PC2) of the data was mainly driven positively by Pcna, a marker for the actively dividing cells; and negatively by Gfap and HuC. For the PC2, the young OF group scored higher values than the young AL animals (Fig. 3c). Finally, the third principal component (PC3) was influenced negatively by Tfdp1 and positively by Dcamkl1. Regarding the clusters of the young group, CR treated young samples had higher PC3 values than young AL and OF treated animal brains. Simple correlational analyses were also performed on the selected protein levels, which showed significant positive correlations between L-plastin and Dcamkl1 ($p = 0.033$), as well as between L-plastin and Tfdp1 ($p = 0.016$) (Supplemental Table 1).

3.4. CR significantly lowers Sox2 gene expression levels compared to OF, while Lcp1, Myca and Tp53 were significantly downregulated by age

In the current study, 10 genes were examined with RT-PCR and those were related to promoting cell cycle (Tfdp1, E2f5, Myca), cellular senescence (Tp53), progenitor state (Sox2), cell adhesion which is implicated for roles of stem cell engraftment (Alcamb), cytoskeletal plasticity/immunity (Lcp1), inflammation (Tnfa, Il1b), and neuroprotection (Rest).
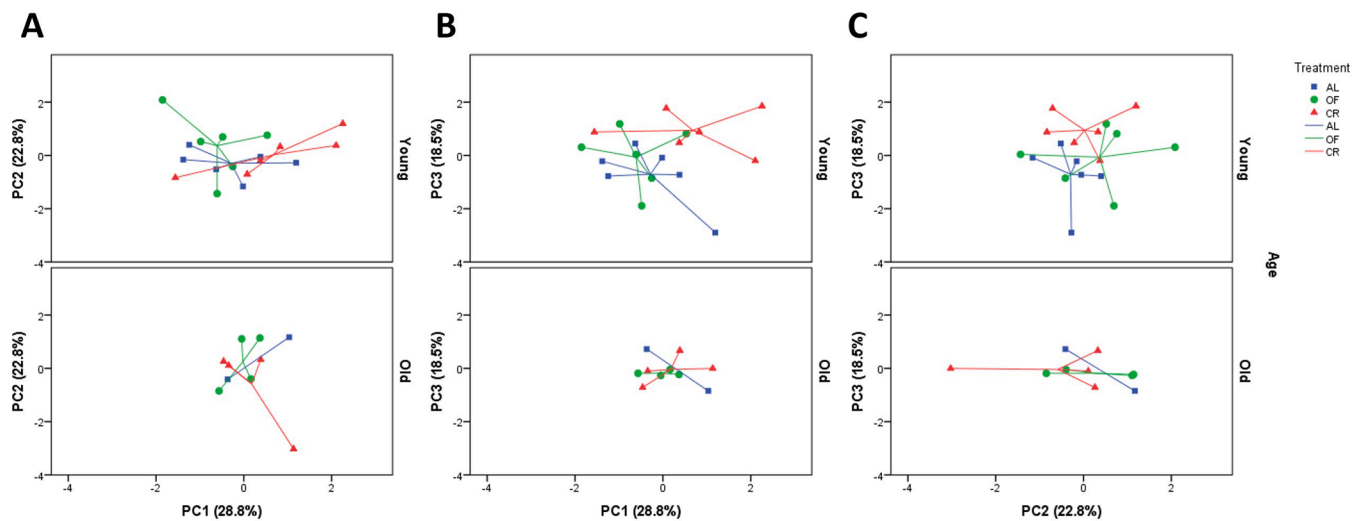
Fig. 3. Expression levels of proteins of interest clustering in an age- and treatment-specific manner in the principal component analysis. Data is visualized in 2-dimensions using the PC1, PC2, and PC3. Data shown in scatterplots on A, B, and C are arranged by the factor of age. Groups are denoted by different colors as shown from the legends. AL: *Ad libitum*-feeding, OF: Overfeeding, CR: Caloric restriction feeding.

The mRNA levels of Tfdp1 did not show statistically significant change neither with age ($F(1, 24) = 0.506, p = 0.486$) nor with diet ($F(2, 24) = 0.774, p = 0.476$) (Fig. 4a). In the case of E2f5, the binding partner of Tfdp1, again no main effects of dietary treatment ($H(2) = 2.855, p = 0.24$) or age ($U = 61, z = -0.635, p = 0.551$) were found to be statistically significant (Fig. 4b).

In terms of cell proliferation markers, we examined Sox2, which is a marker for neural stem/progenitor cells, across multiple organisms (Mercurio et al., 2019). In our data, we found no effect of age ($F(1,24) = 1.225, p = 0.283$). However, there was a main effect of treatment on Sox2 expression levels in the zebrafish brain ($F(2, 24) = 4.866, p = 0.02$) (Fig. 4c). Bonferroni post-hoc tests revealed that in the CR group Sox2 mRNA levels were significantly lower compared to OF treatment groups ($p = 0.018$). Yet, there was no further significant differences in the pairwise comparisons among separate age groups, ie. between young CR vs young OF ($p = 0.089$), and old CR vs old OF ($p = 0.171$). We also measured Myc protein, Myca in zebrafish, which is a proto-oncogene known to stimulate proliferation (Uribesalgo et al., 2012). Myca brain mRNA levels were significantly decreased with age ($F(1, 24) = 6.456, p = 0.02$), although there was no significant alteration in Myca expression levels by diet ($F(2,24) = 0.974, p = 0.397$). This effect of age was driven by the CR group such that young CR fish Myca expression levels were significantly higher than those observed in aged CR animals ($p = 0.006$) (Fig. 4d). No statistically significant differences were found between young and old AL and OF groups.

Next, the mRNA levels of the negative proliferation regulator/tumor suppressor, Tp53, were examined in the context of diet and aging (Perry and Levine 1993). There was an overall effect of age on Tp53 gene expression levels. They were found to be significantly lower in the old animals compared to the young fish ($U = 16, z = -3.233, p = 0.001$) (Fig. 4e). In contrast, there was no main effect of treatment on the Tp53 mRNA levels ($H(2) = 1.235, p = 0.539$). Further pairwise comparisons demonstrated that this change with age was significant between young CR and old CR ($p = 0.001$), as well as between young OF and old OF groups ($p = 0.021$). In case of the AL diet, there were no difference between young and old groups ($p = 0.666$).

Alcam is a cell adhesion molecule with various roles, but in the brain, it mostly regulates leukocyte transmigration through the BBB to the CNS, and therefore it is related to neuroinflammation (Swart 2002; Cayrol et al., 2008; Thelen et al., 2012; Jeannet et al., 2013; Shaha-duzzaman et al., 2015; Kim et al., 2017; Lécuyer et al., 2017; Erbaba et al., 2020b). Our results showed no effect of age in terms of the Alcamb

expression levels ($U = 68, z = -0.231, p = 0.843$). Results demonstrated only a marginally significant main effect of dietary treatment on Alcamb transcription levels ($H(2) = 4.82, p = 0.09$) (Fig. 4f).

The RE1-silencing transcription factor (Rest) has been implicated in promoting longevity and neuronal survival during healthy brain aging, and therefore, is considered neuroprotective (McGann et al., 2021). In the current study, no significant changes in Rest mRNA levels were found for the main effect of age ($F(1,24) = 1.406, p = 0.251$) or diet treatment ($F(2, 24) = 0.021, p = 0.979$) (Fig. 4g). This suggests that our dietary interventions did not promote or reduce any neuroprotective potential of this marker.

In order to make diet-regulated connections to specific inflammatory pathways, we measured Il1b, Tnfa and Lcp1 expression levels (Lopez-Castejon and Brough 2011; Kyritsis et al., 2012; Hayashi et al., 2013). No main effects of dietary treatment or age were observed for Il1b (age: $U = 70, z = -0.115, p = 0.932$; treatment: $H(2) = 1.625, p = 0.444$) (Fig. 4h) and Tnfa (age: $U = 53, z = -1.097, p = 0.291$; treatment: $H(2) = 3.155, p = 0.206$) (Fig. 4i). Our data did not show significant alterations in Lcp1 expression levels following the short-term diets ($F(2, 24) = 0.587, p = 0.566$). However, a significant main effect of age was found on Lcp1 expression levels showing a significant decline in older subjects ($F(1, 24) = 8.269, p = 0.01$) (Fig. 4j). Following pairwise comparisons, the effect of age was found to be significant between young CR and old CR ($p = 0.021$), and young OF and old OF groups ($p = 0.026$). There was no significant alteration between young and old groups of AL diet ($p = 0.976$).

3.5. Multivariate analysis of expression levels of genes demonstrates increases in proliferation and inflammation with an OF diet, and decreases in proliferation and inflammation with CR

In order to explore the variation of expression levels of each gene with respect to one another, PCA was applied to the entire gene expression dataset. An analysis of the Ct values was run for the complete dataset of genes. Three main components were extracted from the analysis, accounting for 65.26% of the total variance in the gene expression data (Table 5). Visualization of the data in two-dimensions is shown in Fig. 5. As was observed in the protein data, the PCA for gene expression data did not explain much of the variance in the old diet groups.

PC1 of the gene expression data was driven by Rest, Tfdp1, Lcp1, Sox2, Tp53, Myca, Tnfa, and Il1b (Table 5). Therefore, PC1 is reflecting
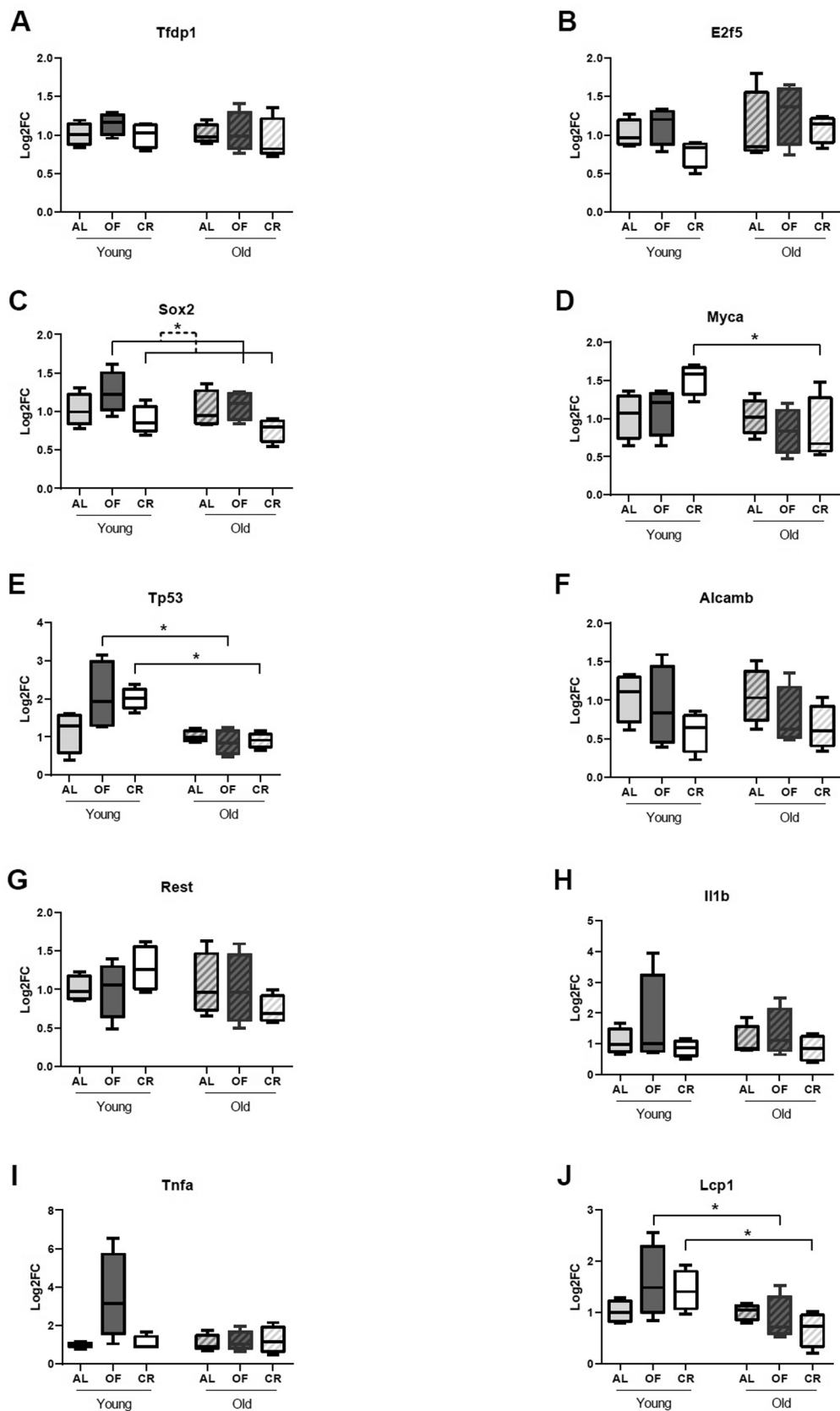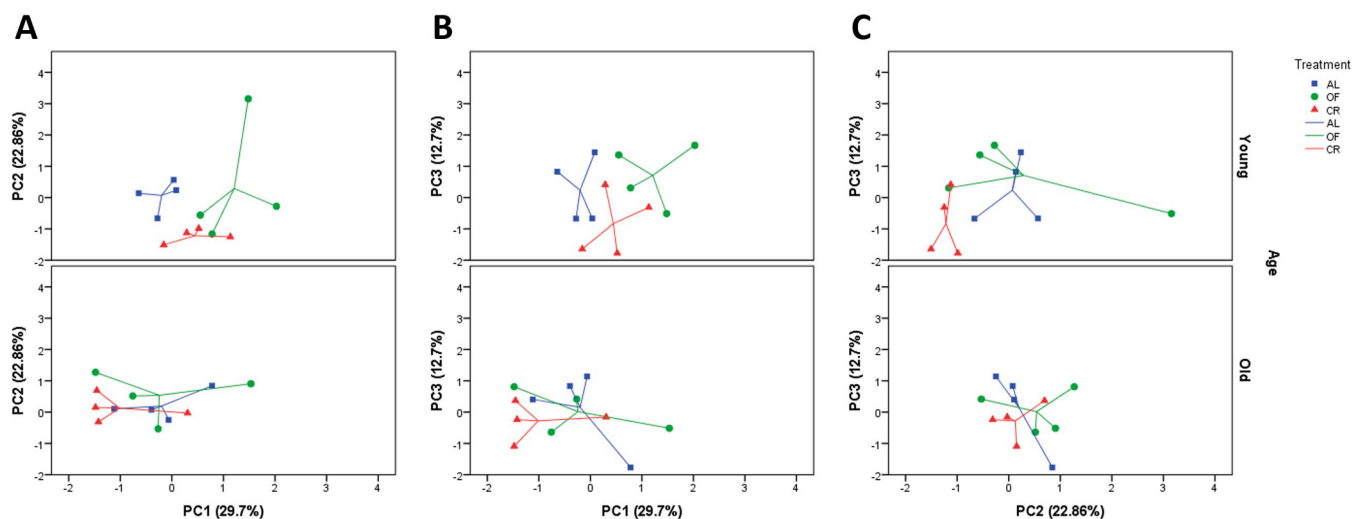
Fig. 4. mRNA level comparisons of selected genes analyzed by RT-PCR; (a) Tfdp1 (N = 24), (b) E2f5 (N = 24), (c) Sox2 (N = 24), (d) Myca (N = 24), (e) Tp53 (N = 24), (f) Alcamb (N = 24), (g) Rest (N = 24), (h) Il1b (N = 24), (I) Tnfa (N = 24), (j) Lcp1 (N = 24).

Table 5

Percentage of explained variances, and factors having highest loading scores for each component in the PCA of gene expression level dataset. The results demonstrated that 29.7% of the variance was explained by principal component 1 (PC1), 22.86% by PC2, and 12.7% by PC3. AL: *Ad libitum*-feeding, OF: overfeeding, CR: Caloric restriction feeding.

		% of Variance Explained	Component Loading Scores (>0.5)							
Principal Component (Expression data)	PC1	29.70	Rest (0.687)	Tfdp1 (0.674)	Lcp1 (0.611)	Sox2 (0.601)	Tp53 (0.579)	Myca (0.572)	Tnfa (0.565)	Il1b (0.545)
	PC2	22.86	Il1b (0.718)	Myca (−0.658)	E2f5 (0.651)	Alcamb (0.583)	Tnfa (0.537)			
	PC3	12.70	Sox2 (0.608)	Rest (−0.560)						
	Total	65.26								

Fig. 5. Expression levels of genes of interest cluster in an age- and treatment-specific manner in the principal component analysis. Data is visualized in 2-dimensions using the PC1, PC2, and PC3. Data shown in scatterplots on a, b, and c are arranged by the factor of age. Groups are denoted by different colors as shown from the legends. AL: *Ad libitum*-feeding, OF: overfeeding, CR: Caloric restriction feeding.

a large-scale pattern composed of suppression of neuronal excitation with Rest, proliferation stimulation with Tfdp1, Sox2, and Myca, increase in inflammatory status with Tnfa, and Il1b, induction of growth arrest/repair mechanisms with Tp53, and cytoskeletal rearrangements with Lcp1. The young OF animals scored higher for PC1 component compared to young AL group (Fig. 5a). An age-dependent decrease in the PC1 scores of OF animals was observed in Fig. 5a. The PC2 was mainly influenced positively by Il1b, E2f5, Alcamb, and Tnfa, as well as negatively by Myca. Among those, Il1b, Alcamb and Tnfa alter

inflammatory outcomes, while E2f and Myca are proliferation-related genes. The young CR group samples clustered at lower PC2 values than the young AL and OF groups. PC3 was impacted positively by Sox2 and negatively by Rest. Although there doesn't seem to be a distinct grouping of samples for PC3, the CR samples cluster at lower values than OF animals (Fig. 5b). The data from the multivariate analyses were supported by correlational analyses (Supplemental Table 2).

	Aging		Diet				
			Young			**Old**	
			CR	**OF**	**CR**	**OF**	
Body Parameters	BMI	⬆	BMI ⬇	BMI ⬆	BMI ⬇	BMI ⬆	
Expression Level Alterations	Tp53	⬇	Sox2 ⬇	Sox2 ⬆	Sox2 ⬇	Sox2 ⬆	
	Myca	⬇					
	Lcp1	⬇					

Fig. 6. Summary figure illustrating the significant alterations with respect to age and diet. Created with Microsoft Powerpoint.

4. Discussion

In the current study we applied both a short-term OF and CR regimen to understand the neurobiological underpinnings of how these different diets contribute to brain aging. The results demonstrated that the 12-week CR regimen significantly decreased the BMI compared to AL- and OF-fed animals at both young and old ages (Fig. 6). Moreover, aged animals had higher body-mass index (BMI) values than the young groups, which was consistent with previous studies (Arslan-Ergul et al., 2016a; Celebi-Birand et al., 2020) (Fig. 6). The effects of age and diet were tested on proteins and genes of interest that have previously been implicated in aging process. These include makers of cell proliferation, cell-cycle regulation, inflammation, and cytoskeletal regulation. While many were stable, our results showed that the OF regimen significantly upregulated the expression levels of Sox2, a proliferation marker, compared to the CR diet. Moreover, we observed age-related declines in the levels of the proliferation marker, Myca, the negative proliferation marker, Tp53, and the inflammatory marker, Lcp1, expression levels (Fig. 6).

In the current study, cell proliferation was tested by measuring the protein levels of Proliferating cell nuclear antigen (Pcna) and the mRNA levels of Sex determining region Y box2 (Sox2). Pcna is a marker of the actively dividing cells (Zhang and Jiao, 2015). In our study, no significant changes were observed in Pcna protein levels with respect to age or diet. Another proliferation marker, Sox2, a well-known stem cell marker that is also active in neural stem cells NSCs) was examined (Mercurio et al., 2019). Its gene encodes a transcription factor known to promote cell proliferation, regulate various physiological processes (Zhang et al., 2020). Its dysregulation/overexpression was implicated in cancer research, including brain cancer, due to gene amplification and protein overexpression, and stated to be positively correlated with the malignancy grade (Mansouri et al., 2016; Capatina et al., 2019; Zhang et al., 2020). In our study, consistent with Pcna measurements, no significant differences were observed in Sox2 expression with age. However, Sox2 expression levels were upregulated following OF-feeding as compared to a CR diet. Sox2 is widely regarded as an NSC marker in both embryo and adult tissues (Mercurio et al., 2019). Actually, Sox2 is better known as an NSC maintenance factor promoting the self-renewal or pluripotency of the cells, than being a proliferation marker like Pcna (Sato et al., 2019). With no effect of our dietary application on Pcna, here we suggest that Sox2 upregulation with OF, may not imply a direct increase in overall proliferation but may infer an increase in stem cell proliferation, leading to a fast depletion of the stem cell reserve. Thus, the significant Sox2 upregulation with OF treatment as compared to CR could be interpreted as an increase in the proliferative status of stem cells with an OF diet that could have detrimental effects as compared to CR. Therefore, this may be one mechanism as to how CR promotes health-span in individuals.

Indeed, CR was previously found to inhibit cell proliferation in various tissues including the brain. This is likely due to the mechanisms being energetically expensive, and conversely, excess nutrients were stated to exhaust NSCs accelerating brain aging (Lu et al., 1993; Cavallucci et al., 2016; Seim et al., 2016; Erbaba et al., 2020a). CR has been shown to push the cells to spend more time regulating genomic stability and suppress cell cycle progression, which eventually helps decrease the cancer risk (Seim et al., 2016). Mercken et al. (2013) also found a metabolic shift in skeletal muscle cells subjected to CR from proliferation to repair and maintenance. Therefore, our data suggests that the decrease in cell proliferation via CR increases the time that cells spend in the G0/G1 phase of cell cycle. This may form the basis of how CR-mediated protective mechanisms are thought to be exerted at the cellular level. Thus, the damaged cells would have more time for repairing their DNAs before entering S phase (Charnley and Tannenbaum 1985; Lu et al., 1993). On the other hand, increasing cell proliferation, was determined to be associated with decreased time spent for DNA repair adding to the risk for unrepaired DNA synthesis (Charnley and Tannenbaum 1985), eventually increasing DNA damage and driving

towards the potential for cancer (Tudek et al., 2010). This suggests an excellent protective mechanism achieved by CR against both cancer and aging compared to OF diet (Gensler and Bernstein 1981; Warner and Price 1989; Tudek et al., 2010; Nicolai et al., 2015). To our knowledge, it is the first time that Sox2 is suggested as one of the regulators of diet-mediated improvement of neuroprotection mechanisms in the brain.

After we examined how changes in cell proliferation were regulated by CR and OF, positive regulators of cell cycle were measured. Among many of the cell cycle regulatory genes, we were specifically interested in the effects of dietary interventions and age on Tfdp1. The reason for this comes from a previous result from a preliminary microarray study from our group, in which Tfdp1 was significantly downregulated in aged animal brains following a short-term CR (Erbaba et al., 2022). Tfdp1 and its binding partner E2f5 are known for their roles in promoting the cell cycle progression (Vaishnav and Pant 1999; Chan et al. 2002). Previously no known association has been made between Tfdp1 and CR or age in the brain. For both Tfdp1 and E2f5 gene expression levels, there were no significant changes with age or diet in the brains, although, Tfdp1 protein levels showed a marginally significant main effect of treatment with numerically decreased values in the CR group compared to AL and OF. We also observed a significant downregulation following short-term CR in aged animal brains in a previous pilot study using microarray analysis (Erbaba et al., 2022).

With aging there is a gradual deterioration in the immune system, also referred to as immunosenescence. This concept is viewed as a possible evolutionary adaptation or a remodeling rather than solely detrimental as such changes are also attributed to the extended lifespan/longevity (Fulop et al., 2018). Myc protein, Myca in zebrafish, is a proto-oncogene, which is known to stimulate proliferation, and Tp53 functions as a tumor suppressor by arresting the cell cycle, or inducing apoptosis. Our data demonstrated statistically significant decreases in Myca and Tp53 mRNA levels with advanced age.

The downregulation of Myca and Tp53 with age could be attributed to immunosenescence and its association with healthy brain aging. This hypothesis is supported by the roles of the genes that were examined. Myc is related to proliferation stimulation. It stimulates the cell cycle progression by facilitating transition from G_1 to S phase. To promote cell growth, c-Myc activates cell cycle promotion genes such as Cdks and E2f, and suppresses transcription of cell cycle arrest genes, such as p21 (Vermeulen et al., 2003). There is an inverse correlation of Myc with longevity (Hofmann et al., 2015). c-Myc was previously reported as having dual functions depending on a phosphorylation switch deciding its path to proliferation and increased malignancy, or differentiation and reduced tumorigenesis (Uribesalgo et al., 2012). In this study, the age-dependent decrease in Myca expression was driven by the CR group such that in aged CR fish Myca expression levels were significantly lower than those observed in young CR animals ($p = 0.006$) (Fig. 4d). This pattern of diet changing within the context of age reflects the complexity of brain aging as an independent contributor to neurobiological alterations in the brain. This age-dependent decrease in Myca expression that we encountered might be caused by a phosphorylation switch induced by aging as Uribesalgo et al. (2012) suggested.

With respect to the downregulation of p53 expression with age, this could be a result of the aging-accumulated byproducts in senescent cells. These cells have been shown to accumulate factors that are known to repress Tp53 functions (Gudkov and Komarova 2016). In this way, the repression of Tp53 in senescent cells of aged brains might further be increasing their survival rates by preventing the Tp53 induced apoptotic events. Our conclusion related to immunosenescence with a concomitant decrease in proliferation induced by the downregulation of Myca and Tp53 with age, is supported by the gene expression multivariate analysis.

In order to check neuronal identity, Dcamkl1 and HuC protein levels were investigated. Dcamkl1 regulates microtubule polymerization and is involved in the dynamic rearrangement of the cytoskeletal machinery with roles in neuronal migration, neuronal development, and formation

of axonal projections related to axonal wiring (Koizumi et al., 2006; Shimomura et al., 2007; Vreugdenhil et al., 2007). Regarding the Dcamkl1 protein levels in our study, there were no significant alterations with respect to age or diet. For the early-differentiated neuronal marker HuC protein levels, in the current study, we did not detect any clear alterations with respect to age or diet. Thus, neuronal levels were not affected drastically with our dietary manipulations.

Glial cells are responsible mainly for supporting and nurturing neurons for proper functioning. Age-dependent alterations in glial cells have been characterized by a gradual loss of function, which facilitates neurodegenerative processes, in addition to shifting regional gene expression patterns (Soreq et al., 2017; Verkhratsky et al., 2019). Gfap is an intermediate filament protein that has roles in cytoskeletal function, cell communication and functioning of the blood–brain barrier, as well as being a widely accepted marker of CNS astrocytes. We detected Gfap protein levels with a GFP signal in a transgenic line, which was utilized in the current study to compare Gfap protein levels among the groups. In this transgenic line, the fusion of the Gfap-GFP protein very closely mimics endogenous Gfap (Lam et al., 2009). The use of this line aided us for the Western blotting experiments in the zebrafish brain, as most of the commercial antibodies against Gfap are developed mostly for mice or human and result in many extra nonspecific bands that would jeopardize the reliability of the analysis. Thus, the signals using the GFP antibody (ab6556, Abcam, UK) worked well and gave a clear specific band with zebrafish brain tissues, which was previously used by Lam et al. (2009). In our results, there was no main effect of age with respect to Gfap protein levels, which is consistent with another study published from our group (Celebi-Birand et al., 2020). However, our findings revealed a marginally significant main effect of diet treatment, in which CR upregulated Gfap protein levels in the fish brains compared to AL and OF-fed animals. The increase that was observed with CR compared to AL and OF diets, might be due to an astroglial response.

Since there is heterogeneity in astrocyte reactivity, the observed upregulation needs to be considered in a context-dependent manner as the response can be either beneficial or detrimental. For example, expression level changes in astrocytes after the induction of reactivity were found to be shifting the cells either towards repair and protective functions, which is common after stroke and/or ischemia, or driving the transcriptome towards increasing the detrimental inflammatory immune responses, such as after LPS induction, a potent injury model (Zamanian et al., 2012). In our study, the inflammatory markers were not triggered with a CR diet as demonstrated by the gene expression data. Therefore, the astroglial response is unlikely to result from detrimental inflammatory responses. From this perspective, compared to the AL and OF regimens, the CR diet might presumably be inducing a low amount of stress resulting from a process called 'hormesis', the term of which is defined as the beneficial effects of a low dose of stress. The occurring hormesis state in the cells then might trigger the astroglial responses in the case of CR, shifting the cells towards repair and protective functions.

Activated leukocyte cell adhesion molecule (Alcamb) is a cell adhesion protein that is associated with leukocyte cell adhesion, engraftment of stem cells, immune cell migration into the CNS, neural development, synaptic transmission modulation, axonal outgrowth, and maintaining blood–brain barrier (BBB) integrity, in addition to being associated with neuroinflammation (Swart 2002; Cayrol et al., 2008; Thelen et al., 2012; Jeannet et al., 2013; Shahaduzzaman et al., 2015; Kim et al., 2017; Lécuyer et al., 2017; Erbaba et al., 2020b). For Alcamb expression levels, though the effect of age was not profound, we observed a marginally decreasing trend especially in the CR groups as compared to AL groups. It has been suggested by Swart et al. (2002) that Alcam functions in tumor growth and induction of cell migration, as well as invasiveness contributing to the growth of tumor cells. Thus, from many aspects, Alcam seems to be highly correlated with the inflammatory and cancerous states of the brain by attracting and recruiting leukocytes to the inflammation site. Its expression is found among many cells including neurons, fibroblasts, epithelial cells, lymphoid and myeloid cells, so Alcam is thought to be upregulated in response to neuro-inflammation to attract and recruit leukocytes to the inflammation site. Therefore, the decreasing trend in its expression observed within the CR group as compared to the AL and OF groups may suggest a role for CR exerting anti-inflammatory properties in the brain. Therefore, it is very likely to be a good target for further investigation of its roles in the anti-inflammatory responses of specific cell types exposed to nutrient restriction. To our knowledge, this is the first time that a role has been proposed for Alcamb with respect to nutrient intake.

Multivariate statistical testing revealed significant relationships that were age- and diet-dependent for both the protein and gene expression datasets. Overall, the PCA results for both datasets demonstrated that the CR group data scored higher values for the components with factors related to cytoskeletal/migratory/plasticity regulation (L-plastin, Dcamkl1, HuC), while having lower scores or being negatively associated with factors related to proliferation/cell cycle promotion (Pcna, Sox2, Tfdp1, E2f5), inflammation (Il1b, Tnfa) and tumor suppression (Tp53). In contrast, the data from the OF group had trends toward higher values in the components with factors related to proliferation/cell cycle promotion (Pcna, Sox2, Tfdp1, Myca, E2f5), and inflammation (Il1b, Tnfa), tumor suppression (Tp53). Such nutrient-driven regulations in brain cells might be an indication of sophisticated molecular adjustments underlying the status of the cell survival mechanisms. The regulation mechanisms mediated via CR, likely show their effects on lifespan by decreasing cell proliferation, inducing neural stem cell quiescence, protecting the stem cell reserve, decreasing overall inflammation, and protecting the brain by decreasing cancer incidence, while the OF diet, in contrast, is increasing the proliferation and inflammation status of the cells, which supports the hypothesis of Cavallucci et al. (2016). In fact, CR could be exerting an outcome resembling senescence in the case of cell proliferation, thereby inhibiting cell cycle progression and providing a protective barrier against cancerous development. Decreasing proliferation, therefore, can be viewed preferably as an adaptive response rather than detrimental one (Erbaba et al., 2020a). Unlike CR, OF might consume the stem cell reserve by forcing it to proliferation, thereby leading to excessive production of metabolic by-products and accumulation of DNA damage, which are the drivers of inflammation. This hypothesis is also supported by the correlation analysis, in which we observed strong positive correlations of Il1b with E2f5; also, of Tnfa with Tfdp1 and Sox2 (Supplementary Table 2). Considering that Tfdp1, E2f5 and Sox2 are markers associated with cell proliferation, and Tnfa and Il1b are inflammatory-related markers, any correlations detected imply a positive relationship between cell proliferation and inflammation. As a result, increases in proliferation might lead to an elevation in inflammatory mechanisms. The outcome of a positive correlation between proliferation and inflammation is in agreement with that OF related obesity is known to increase inflammatory processes (Guillemot-Legris and Muccioli 2017). There is likely a perfectly-tuned balance that emerges by the level of nutrient intake deciding between two fates: a) excess proliferation regardless of a proper DNA damage control, that increases the tumor development and probably decreasing the chance of survival due to mutation accumulations or b) cell cycle arrest or accurately controlled slower cell cycle progression leading to a senescence-like quiescent state, however providing a healthy and longer lifespan.

In summary, our data demonstrate that the negative effect of CR on cellular proliferation likely reinforces the stem cell pool in the brain to maintain quiescence, which is important for the conservation of the limited energy produced due to the low amount of nutrient uptake. Moreover, CR also helps promote DNA repair and protects the cells in the brain against the accumulation of DNA damage. On the other hand, OF induces a higher metabolism demand by promoting cell proliferation, as well as decreasing the time spent on DNA damage control, and the consequence is the exhaustion of the stem cell pool. While our data suggest important cellular mechanisms related to the effects of aging,

there are limitations such that further studies examining the protein levels of these markers need to be performed. In conclusion, our data point to new drug targets focusing on cell cycle mechanisms with respect to the state of nutrient uptake for slowing down the detrimental effects of aging, resulting in a healthy and extended lifespan, as well as lowering the risk for neurodegenerative disease.

CRediT authorship contribution statement

Begun Erbaba: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Duygu Maca-roglu:** Methodology. **N. Ilgim Ardic-Avci:** Methodology. **Ayca Arslan-Ergul:** Conceptualization. **Michelle M. Adams:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.147026.

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