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Short-term dietary restriction maintains synaptic plasticity whereas short-term overfeeding alters cellular dynamics in the aged brain: evidence from the zebrafish model organism

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ABSTRACT

Increased caloric intake (OF) impairs quality of life causing comorbidities with other diseases and cognitive deficits, whereas dietary restriction (DR) increases healthspan by preventing age-related deteriorations. To understand the effects of these opposing dietary regimens on the cellular and synaptic dynamics during brain aging, the zebrafish model, which shows gradual aging like mammals, was utilized. Global changes in cellular and synaptic markers with respect to age and a 12 week dietary regimen of OF and DR demonstrated that aging reduces the levels of the glutamate receptor subunits, GLUR2/3, inhibitory synaptic clustering protein, GEP, synaptic vesicle protein, SYP, and early-differentiated neuronal marker, HuC. DR significantly elevates levels of glutamate receptor subunits, GLUR2/3, and NMDA clustering protein, PSD95, levels, while OF subtly increases the level of the neuronal protein, DCAMKL1. These data suggest that decreased caloric intake within the context of aging has more robust effects on synapses than cellular proteins, whereas OF alters cellular dynamics. Thus, patterns like these should be taken into account for possible translation to human subjects.

1. Introduction

Obesity is an emerging problem worldwide, and its prevalence in the last four decades has almost tripled (World Health Organization, 2018). Moreover, obesity is linked to severe age-related diseases such as hypertension, diabetes, coronary heart disease and stroke (World Health Organization, 2018). Being overweight or obese not only leads to age-related metabolic impairments, it can also influence the central nervous system (CNS) and contribute to an accelerated aging brain profile that alters cognitive abilities sooner than anticipated. Studies including both human subjects and different model organisms have indicated that higher caloric intake leads to cognitive impairments even at younger ages and negatively impacts cognitive performance more severely at older ages (Hao et al., 2016; Kothari et al., 2017; Meguro et al., 2019; Sabia et al., 2009). Moreover, high caloric intake can alter the cellular and molecular mechanisms in the brain that underlie cognitive impairments. In rodent models, it has been shown that short-term high caloric intake in young animals causes significant reductions in the brain levels of pre- and post-synaptic integrity markers (Bocarsly et al., 2015; Kothari et al., 2017). Taken together, dietary regimens with high caloric intake are well-studied in terms of their impacts on body, but their effects on the brain are not as well-described. Moreover, it is also crucial to understand brain changes in response to diets in which the caloric intake is systematically varied by using same nutrient components among different dietary regimens.

Abbreviations: CNS, central nervous system; BMI, body-mass index; PSD95, Post-synaptic density 95; BDNF, Brain-derived neurotrophic factor; DR, dietary restriction; AL, ad libitum; DIO, diet-induced obesity; OF, overfeeding; DCAMKL1, doublecortin like kinase 1; SYP, Synaptophysin; GluR2/3, Glutamate receptor subunits 2 and 3; HuC, embryonic lethal, abnormal vision (ELAV; Drosophila) like 3; NR2B, N-methyl D-aspartate receptor subtype 2B; GEP, Gephyrin; GABA-A-alpha1, Gamma-Aminobutyric acid type A alpha 1 subunit; PCNA, proliferating cell nuclear antigen; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl D-aspartate; GABA, Gamma-aminobutyric acid; DCX, doublecortin

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As opposed to the detrimental effects of obesity and higher caloric intake, dietary restriction is an intervention that attenuates age-related deteriorations and extends lifespan across multiple organisms (Fontana et al., 2010; Martin et al., 2006; Mitchell et al., 2016). Dietary restriction (DR) corresponds to an overall reduction in total caloric intake without targeting a specific dietary component. Data suggest that this application will attenuate age-related cognitive decline in old rats (Adams et al., 2008; Markowska and Savonenko, 2002). To understand the cellular mechanisms underlying protective effects of DR on cognitive abilities, alterations in the expression of synaptic proteins have been investigated, and the results indicate that glutamate receptor protein levels tend to be preserved at older ages in animals that have been subjected to DR as opposed to *ad libitum* (AL)-fed subjects (Adams et al., 2008; Shi et al., 2007). DR has ameliorative impacts against aging-related molecular alterations on the brain, yet the literature is still limited in terms of investigating the differential effects of short-term DR initiated in both young and old subjects.

It is important to note that few studies have examined dietary interventions such as diet-induced obesity (DIO) and dietary restriction under the same experimental conditions, which would provide important insight into their complementary mechanisms related to successful versus unsuccessful brain aging. For this reason in the current study, the zebrafish model organism was used because it has become an important gerontological model to study molecular and cellular aging, as well as age-related cognitive decline (Adams and Kafaligonul, 2018; Gerhard and Cheng, 2002; Van houcke et al., 2015; Yu et al., 2006), due to its integrated nervous system and its gradual aging like humans (Kishi et al., 2003; Wullimann et al., 1996). There have been a few studies that have examined DIO or DR in this model organism. Studies have shown that short-term high-fat diet can impair cognitive function in young zebrafish and alter gene expression profile in the brain (Meguro et al., 2019; Montalbano et al., 2016). Data from our laboratory indicate that a 10 week short-term DR can alter cellular dynamics in the brain such as telomere length (Arslan-Ergul et al., 2016). In another study, 8 weeks of DR leads to an upregulation in the post-mitotic neuronal marker doublecortin like kinase 1 (DCAMKL1) protein levels (Celebi-Birand et al., 2020). Moreover, both of these DR treatments influenced whole body parameters and caused healthy weight loss (Arslan-Ergul et al., 2016; Celebi-Birand et al., 2020). These data establish this model organism for use in determining the neurobiological underpinnings of these complementary dietary interventions in promoting successful versus unsuccessful brain aging.

The purpose of current study was to examine the global levels of synaptic, neuronal and cellular proliferation markers with regards to age and diet in the context of overfeeding (OF), i.e., high caloric intake versus DR, low calorie diet. It was hypothesized that OF and DR dietary regimens will cause complementary global alterations in the levels of these markers. In the present study, a 12 week feeding intervention was initiated in young adult and old zebrafish. OF-fed fish received a diet that was high in calorie but with no changes in the nutritional components given to the AL and DR animals. Zebrafish that were in the DR group took in a lower caloric intake by being fed with an alternate day feeding regimen. Our results indicated that BMI values were reduced significantly in young and old DR animals as compared to AL and OF diets, while OF groups had increasing BMI values but it was not statistically significant as compared to AL. In the aged zebrafish brain, the protein levels of glutamate receptor subunits 2 and 3 (GluR2/3); Gephyrin (GEP), an inhibitory post-synaptic protein; Synaptophysin (SYP), a presynaptic protein; and embryonic lethal, abnormal vision (ELAV; Drosophila) like 3 (HuC), an early-differentiated neuronal marker declined significantly. Also, the levels of excitatory elements, Post-Synaptic Density 95 (PSD95) and GluR2/3, were upregulated in the brains of the DR animals which can reflect maintained synaptic plasticity. Furthermore, the post-mitotic neuronal marker, DCAMKL1, protein levels were increased but only in the old OF group, which suggests altered cellular dynamics in response to high calorie diet. To the best of our knowledge, the current study is one of the first to examine synaptic and cellular markers in the context of brain aging and diet including both OF and DR regimens.

Table 1
Feeding regimens of the diet groups.

Feeding Regimen	Dry flakes	*Artemia*
Ad libitum (AL)	180 mg/per day	3 times/week
Overfeeding (OF)	360 mg/per day	2 times/day
Dietary Restriction (DR)	90 mg/per 2 days	Once/week

2. Methods

2.1. Animals

A total of 74 wildtype zebrafish (AB), raised and maintained in a recirculating housing system (ZebTec, Techniplast, Italy) with a light:dark cycle of 14L:10D at a constant temperature of 27.5°C and pH of 7.5, were utilized in this study. Zebrafish were fed regularly with dry flakes and *Artemia* prior to the feeding interventions by the facility staff. Both male and female zebrafish were included in these experiments and randomly assigned to the different tanks designated for each feeding regimen; *ad libitum* (AL), overfeeding (OF) as well as dietary restriction (DR), and the fish were habituated in their new tank for one week prior to the initiation of the diets. This study was designed to determine the effects of aging and short-term dietary intervention. Therefore, AL, OF and DR groups at young (9 months old from birth) and old (20 months old from birth) ages were used, and feeding schedules were carried out starting at these ages. At the end of the feeding interventions, the young group was 12 months old, while the old group was 23 months old. These ages were included because it was previously shown that age-related cognitive decline in multiple behavioral measures was observed in 24 months old zebrafish as compared to 12 months old groups (Yu et al., 2006). Groups were fed with commercial dry flakes (TetraMin Flakes, Complete food for all tropical fish, Tetra Werke, Melle, Germany), having the following composition: 46% crude protein, 11% crude oils and fats, 3% crude fibre, and 6% moisture and freshly hatched *Artemia*. This live animal food source not only provides nutrients but also environmental enrichment for the fish, which is important for their well-being. The schedules for the feeding regimens for each diet group, which were determined from previous studies and (Arslan-Ergul et al., 2016; Celebi-Birand et al., 2020) and designed to be close to the facility dietary regimen for the AL-fed fish, are noted in Table 1. None of the dietary groups were deprived of a specific nutrient component but the total calorie intake varied among them. After a one week habituation period, the feeding regimens started and continued for 12 weeks. The duration of 12 weeks was chosen since short-term dietary interventions ranging between 8-11 weeks successfully affect both cognitive measures and molecular alterations in the brain (Arslan-Ergul et al., 2016; Celebi-Birand et al., 2020; Meguro et al., 2019), and a duration of 12 weeks would be past that required period. At the end of week 12, experimental animals were euthanized after cessation of opercular movements by submersion in cold system water with ice for 10 minutes. The dry weight and length of the zebrafish were recorded, and then the heads were separated from the body with a scalpel blade. The gills

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

Target Antigen	Supplier	Cat#	Use	Dilution	Blocking Solution	Total protein amount loaded into the gel
PSD95	Abcam	ab18258	Primary	1:5,000	5% NFDM in TBS-T	10 µg
SYP	Abcam	ab32594	Primary	1:10,000	5% NFDM in TBS-T	10 µg
GEP	SantaCruz	sc-6411	Primary	1:1,000	5% BSA in TBS-T	40 µg
GABA-A-alpha1	Abcam	ab211131	Primary	1:1,000	5% NFDM in TBS-T	40 µg
GluR2/3	LSBio	LS-C15368	Primary	1:1,000	5% NFDM in TBS-T	20 µg
NR2B	LSBio	LS-C25797	Primary	1:1,000	5% BSA in TBS-T	20 µg
DCAMKL1	Abcam	ab109029	Primary	1:1,000	5% NFDM in TBS-T	40 µg
HuC	Abcam	ab78467	Primary	1:2,000	5% NFDM in TBS-T	20 µg
PCNA	Abcam	ab18197	Primary	1:1,000	5% NFDM in TBS-T	40 µg
β-Tubulin	CST	#2146	Primary	1:5,000	5% NFDM in TBS-T	10-40 ug (housekeeping control)
Rabbit-HRP	CST	#7074	Secondary	1:5,000	5% NFDM in TBS-T	-
Goat-HRP	Abcam	ab97100	Secondary	1:10,000	5% BSA in TBS-T	-

Key: NFDM, nonfat dry milk; SYP, Synaptophysin; PSD95, Post-Synaptic Density95; GluR2/3, Glutamate Receptor 2 and 3; NR2B, N-methyl D-aspartate receptor subtype 2B; GEP, Gephyrin; GABA-A-alpha1, Gamma-Aminobutyric acid type A alpha 1 subunit; DCAMKL1, doublecortin like kinase 1; HuC, embryonic lethal; abnormal vision (ELAV; Drosophila) like 3; PCNA, proliferating cell nuclear antigen; TBS-T, tris-buffered saline with 0.3 Tween20; BSA, bovine serum albumin; HRP, horseradish peroxidase.

and eyes were removed, as well as the surrounding fatty tissue on the ventral surface of the skull. The skull was opened carefully and the brain was dissected and weighed. The gender of the animal was confirmed by the presence of ovaries or testis. Immediately after the dissections, the tissues and trunks were put in the tubes, then snap-frozen in liquid nitrogen and stored at -80°C. Cohorts of animals containing each of the age and diet groups were used for protein analyses. Thirty-six individual zebrafish (n = 6/group) were used for Western blot analyses. Moreover, the bodies of fifty-three individual zebrafish (n = 8-9/group) across these groups were used for the determination of trunk cortisol levels. The sex ratio of the groups was 1 male to 1 female. The animal protocol for this study was approved by the Bilkent University Local Animal Ethics Committee (HADYEK) with the approval date: Sept 6, 2017, no: 2017/12.

2.2. Protein Isolation

Proteins from the brain tissues were isolated with a protocol that has been used previously by our group (Celebi-Birand et al., 2020; Karoglu et al., 2017; Tuz-Sasik et al., 2020). Whole brain tissues were homogenized in 300 µl of lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP40, and protease inhibitor (2X stock, 05892970001, Roche, Mannheim, Germany) by passing the samples through syringe (1 ml, 26 gauge) multiple times. Homogenates were incubated on ice for 30 minutes and then centrifuged at 13,000 rpm for 20 minutes at +4°C. Supernatants were collected, aliquoted and stored at -80°C. Total protein amounts of the supernatants were determined by Bradford assay (Bradford Reagent, Sigma, St. Louis, MO, USA: B6916) using dilutions of bovine serum albumin as the standard.

2.3. Western Blot

For the detection of proteins of interest, the amount of total protein used for each antibody was indicated in Table 2. The Western blot protocol was followed as described previously (Celebi-Birand et al., 2020; Karoglu et al., 2017; Tuz-Sasik et al., 2020). Briefly, protein samples of the cohorts containing all 6 diet and age groups were loaded into 8-10% SDS-PAGE gels, run in reducing and denaturing conditions and transferred to PVDF membranes. Membranes were blocked with blocking solution at room temperature with gentle agitation and incubated with primary antibodies overnight with gentle shaking at +4°C. Both the blocking solutions and the primary and secondary antibodies utilized in the current study are listed in Table 2. Membranes were incubated with secondary antibodies (Table 2) for 1 hour at room temperature. After primary and secondary antibody incubations, membranes were washed with tris-buffered saline with 0.3% Tween20 (TBS-T). Protein bands were developed with Femto Supersignal (Thermoscientific, Rockford, IL, USA: 34095) and imaged with a ChemiDoc MP System (BioRad, Hercules, CA, USA). Band intensities were analyzed quantitatively with using ImageJ software (NIH, Bethesda, MD, USA) by authors ETK-E and MUT-S who were blind to the diet and age groups in the blots. Band intensities were first normalized with the average band intensity of the cohort, and then these values were divided by a corresponding value of the housekeeping protein β-tubulin (TUB). This method has been previously used by our laboratory in which we observed no statistical differences in the housekeeping protein with respect to age (Karoglu et al., 2017).

PSD95, GEP, SYP and β-tubulin (TUB) antibodies were previously optimized by our group with positive controls and zebrafish brain samples (Karoglu et al., 2017). Additionally, the commercially available GABA-A-alpha1 antibody has been shown to specifically react with zebrafish brain tissue and was utilized in this study. Its positive control is zebrafish brain lysate according to manufacturer's datasheet (ab211131, Abcam, Cambridge, UK). In the current study, antibodies directed against NR2B, GluR2/3, DCAMKL1, HuC and PCNA had not be optimized for use in zebrafish brains and were assayed with brain homogenates along with their positive control samples. All Western blot analyses using the untested antibodies above, yielded bands in the zebrafish brain samples and their positive controls at the expected molecular weight (Fig. 1).

2.4. Statistical Analysis

Statistical analyses were performed by using SPSS Statistics 19 (IBM, Istanbul, Turkey). Assumptions of normality were checked with Kolmogorov-Smirnov and Levene tests. When these assumptions were fulfilled, a two-way ANOVA with the factors of age with two levels (young and old) and diet with three levels (AL, OF and DR) was carried out. Since we did not observe any overall effects of sex, the gender groups were merged, however, an equal number of males and females were included in each group in order to maintain a balance. Univariate analyses were followed by post-hoc comparisons with a Bonferroni correction when the significant effects were observed. In the cases where the assumptions of normality were violated,

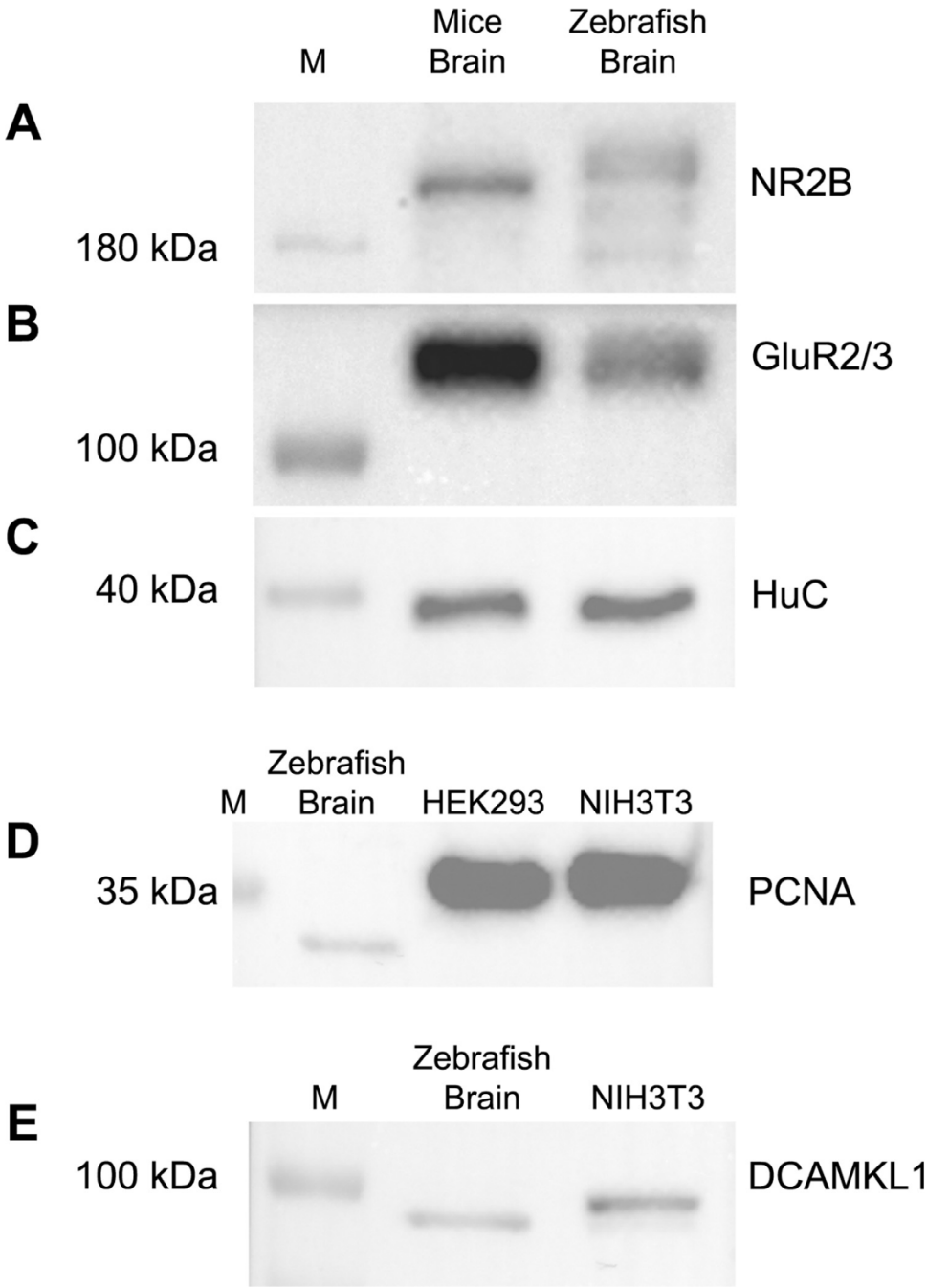

Fig. 1. The validation of NR2B, GluR2/3, HuC, PCNA, and DCAMKL1 antibodies in the zebrafish brain. Commercially available (A) NR2B (LS-Bio, LS-C25797), (B) GluR2/3 (LS-Bio, LS-C15368), and (C) HuC (Abcam, ab78467) antibodies were validated with mouse brain lysate as the positive control and both mouse brain lysate and zebrafish brain lysate gave the bands at the expected molecular weight, approximately 180 kDa, 110 kDa and 40 kDa, respectively. (D) HEK293 and NIH3T3 cell lines were utilized as the positive controls to validate commercially available PCNA antibody (Abcam, ab18197) and the zebrafish brain lysate had the expected molecular weight of 29 kDa. (E) Also, NIH3T3 cell line was the positive control of DCAMKL1 antibody (Abcam, ab109029) and both zebrafish brain lysate and NIH3T3 positive control gave the bands at the expected molecular weight, approximately 82 kDa. M: PageRuler Prestained Protein Ladder, 10 to 180 kDa.

a non-parametric Kruskal-Wallis test was applied and a Mann-Whitney-U test was used for pairwise comparisons with a correction depending on the number of comparisons. Significance levels in all analyses were set at $p < 0.05$ unless they were corrected and more stringent for the non-parametric Mann-Whitney-U pairwise comparisons. The graphs were drawn using Graphpad 8 (San Diego, CA, USA) and SPSS Statistics 19 (IBM, Istanbul, Turkey).

3. Results

3.1. Body parameters were differentially altered by age and dietary regimens

All three dietary regimens were applied for 12 weeks to young and old age groups. At the end of the dietary interventions, dry weights and lengths of each animal were recorded to calculate

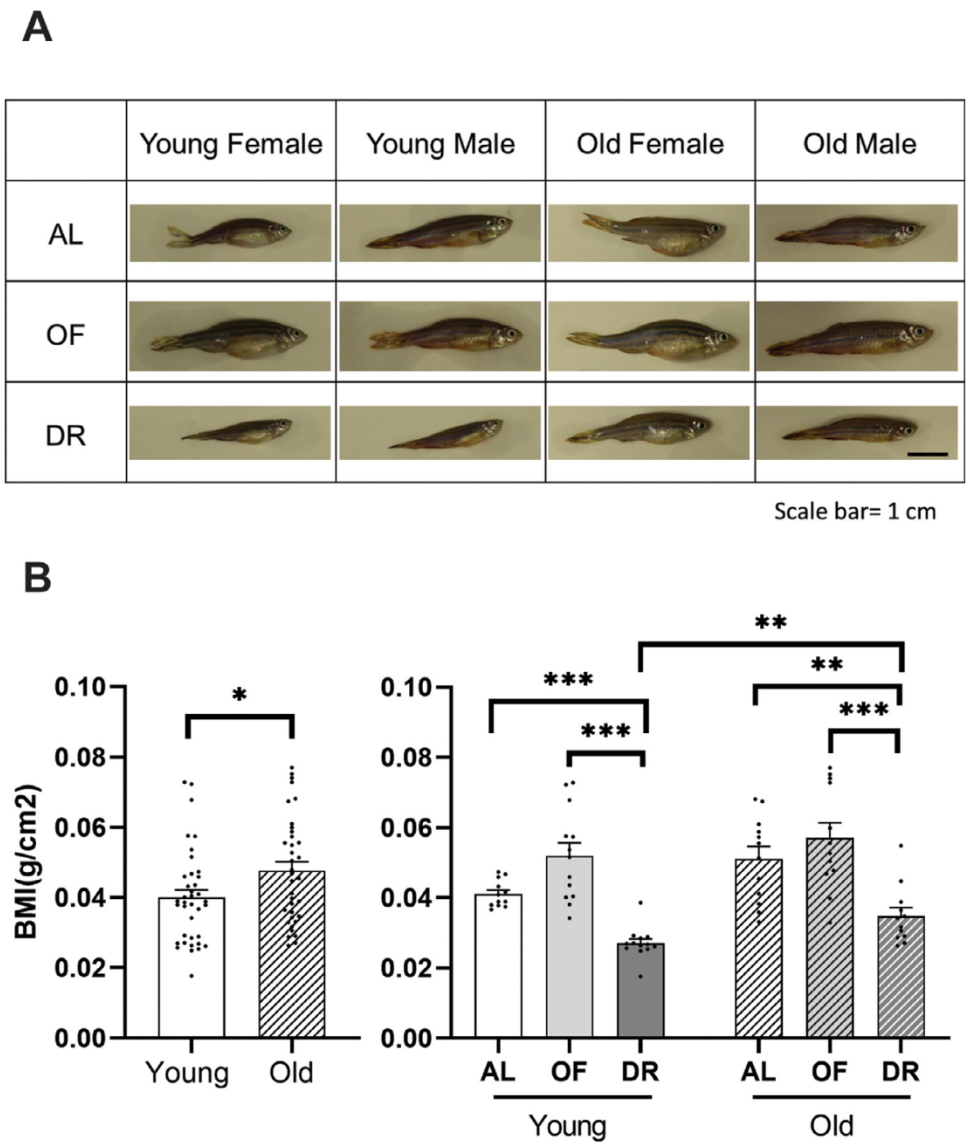

Fig. 2. (A) Representative images of animals from the different cohorts at the end of each of the 12-weeks dietary regimens. Scale bar = 1 cm. (B) BMI values significantly increased with age, old group had significantly higher BMI as compared to young and this effect was prominent in DR fish. Diet significantly altered BMI, DR subjects at both young and old ages had significantly lower BMI as compared to other dietary regimens. The group means + standard error (SE) are represented. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. AL: *Ad libitum*; OF: Overfeeding; DR: Dietary Restriction.

body-mass index (BMI) and Fulton K-factor value (Fig. 2A, Supp. Table 1). It is well-established that both the weight and length of the zebrafish increase as the animals age (Arslan-Ergul et al., 2016; Celebi-Birand et al., 2020). Therefore, the weight measurement alone is not enough to define diet-induced higher or lower body weight differences especially as it relates to aged animals. Previous reports utilized both the BMI and Fulton K-factor as an indicator of overfeeding (OF) or dietary restriction (DR) (Celebi-Birand et al., 2020; Landgraf et al., 2017; Oka et al., 2010; Ran et al., 2017; Vargas and Vásquez, 2017). In the current study the same calculations were used to demonstrate the effects of the different dietary regimens. For the BMI (g/cm^2) values, a significant overall main effect of age ($\chi^2(1) = 4.725$, $p = 0.030$) was observed which indicated that older animals have a higher BMI than young (Fig. 2B). This pattern is consistent with previously published studies (Arslan-Ergul et al., 2016; Celebi-Birand et al., 2020). Furthermore, the age effect was very apparent in the DR group, as the aged animals had higher BMI values than young zebrafish had ($p = 0.002$). Additionally there was a significant overall effect of diet on the BMI values ($\chi^2(2) = 39.377$, $p < 0.0005$; Fig. 2B). Consistent with our previous study (Celebi-Birand et al., 2020), *ad libitum* (AL) animals had a significantly higher BMI than the DR group at both young and old ages ($p < 0.0005$ and $p = 0.001$, respectively). Moreover, OF-fed zebrafish weighed more than the DR animals in both the young and old groups as measured by the BMI ($p < 0.0005$ and $p < 0.0005$, respectively). This difference between DR and OF dietary regimens at young age was accompanied by a significant decrease of trunk cortisol levels in DR animals as compared to young OF zebrafish which may indicate that the robust increases in calorie intake can lead to endocrine alterations (Supp. Figure 1). On the other hand, no significant difference was seen between the OF and AL animals in both young and old groups ($p = 0.060$ and $p = 0.356$, respectively). For all of the dietary interventions, the results of the Fulton K-factor, which is an indicator of the well-being of the fish (Siccardi et al., 2009; Williams, 2000), showed similar patterns (Supp. Table 1). The weight and length

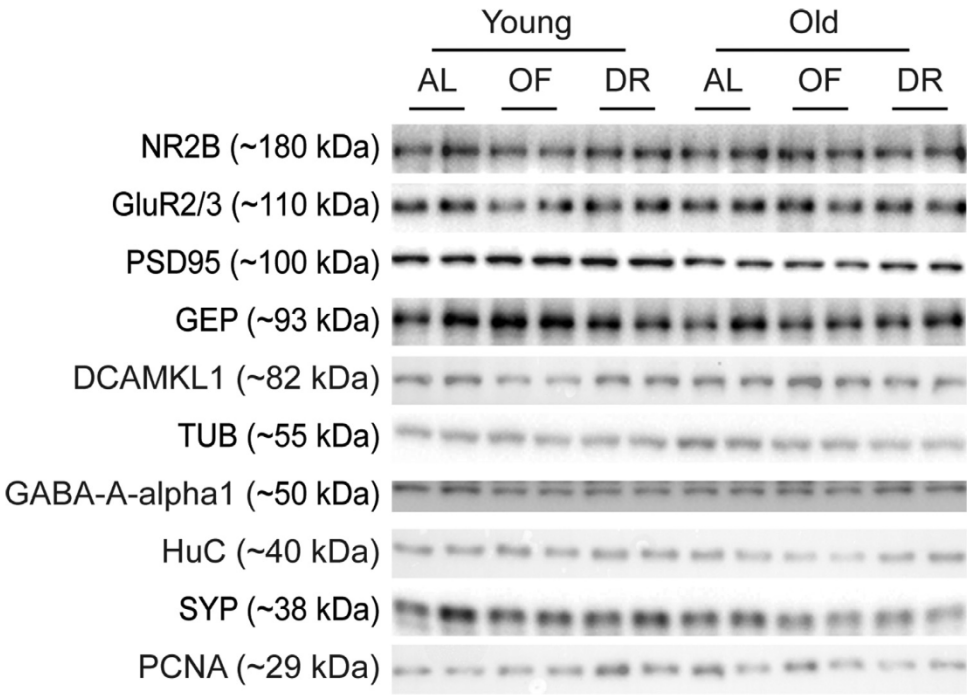

Fig. 3. Images are from one cohort and representative Western blot for synaptic and neuronal proteins analyzed in the current study for the effects of age and diet. All bands ran at the expected molecular weight. AL: *Ad libitum*; OF: Overfeeding; DR: Dietary Restriction.

data used for the calculation of BMI along with the Fulton K-factor are shown in Supp. Table 1. Our results demonstrated that the applied dietary regimens had an overall effect on the BMI measurements of the zebrafish.

3.2. Age and dietary regimens affected the protein levels of synaptic and neuronal markers

In order to investigate alterations in synaptic protein levels with respect to diet and age, elements of excitatory neurotransmission: post-synaptic density 95 (PSD95), glutamate receptor subunits 2 and 3 (GluR2/3) and *N*-methyl D-aspartate (NMDA) receptor subunit 2B (NR2B); a marker of presynaptic integrity synaptophysin (SYP) and elements of inhibitory neurotransmission, gephyrin (GEP), gamma-aminobutyric acid (GABA) receptor subunit alpha-1 (GABA-A-alpha1) were assessed (Fig. 3). To understand whether changes in the synaptic markers were happening in parallel with global changes in neurons and cells, protein expression levels of neuronal and cell proliferation markers, embryonic lethal, abnormal vision (ELAV; Drosophila) like 3 (HuC), doublecortin like kinase 1 (DCAMKL1), and proliferating cell nuclear antigen (PCNA) were examined (Fig. 3). For the quantitative analyses of the brain protein levels, lysates from young and old brains from each diet group were run in cohorts in the gel as depicted in Fig. 3.

3.2.1. Synaptic proteins that comprise excitatory and inhibitory synapses were differentially altered by age and diet

In the case of PSD95 protein levels, which is the main clustering protein for NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, a significant main effect of diet was observed ($\chi^2(2) = 10.613$, $p = 0.005$; Fig. 4A). The DR animals tended to have higher levels of PSD95 compared to the other diet groups. Moreover post-hoc analyses indicated that in the old animals this difference was statistically significant between AL and DR groups ($p = 0.010$), while there was no significant change between old OF and DR animals ($p = 0.074$). Additionally at young age, no significant difference in PSD95 levels was revealed among diet groups. In the context of overall age effects, no significant alteration was observed in PSD95 levels ($\chi^2(1) = 2.919$, $p = 0.088$). With respect to GluR2/3 protein levels, which are the subunits comprising the primary for the adult-like AMPA receptor that alter calcium permeability (Hollmann and Heinemann, 1994), a significant main effect of age was observed, ($F(1, 30) = 4.716$, $p = 0.038$; Fig. 4B) with its levels declining in old groups compared to young. Moreover, a significant main effect of diet was revealed in the levels of GluR2/3, ($F(2, 30) = 9.913$, $p < 0.0005$). The young DR animals had significantly elevated levels of this protein compared to young AL ($p = 0.006$) and OF ($p = 0.034$) fish. This trend was also observed in the old fish but increased levels of GluR2/3 in the DR subjects were not significantly different from AL ($p = 0.080$) and OF ($p = 0.062$) animals. In contrast the protein levels of NR2B, which is an NMDA receptor subunit that when overexpressed promotes increased synaptic plasticity (Philpot et al., 2001), showed no significant main effect of age, ($\chi^2(1) = 1.445$, $p = 0.229$) or diet ($\chi^2(2) = 3.047$, $p = 0.218$; Fig. 4C). These results suggest that the elements of excitatory neurotransmission and synaptic integrity, including PSD95 and GluR2/3, tend to be elevated in the DR animals, and this diet-driven trend seems to be differentially affecting the age groups.

SYP, which is a presynaptic vesicle protein used to give an overall picture about the total presynaptic population, and this was affected by age but not diet. There was an overall significant main effect of age ($\chi^2(1) = 7.929$, $p = 0.005$; Fig. 4D) with SYP decreasing in the brain of older animals. In contrast, the effect of diet was not statistically significant in SYP levels ($\chi^2(2) = 0.038$, $p = 0.981$). These results suggest that alterations in this presynaptic element are mainly driven by age rather than the diet.

GEP is a clustering protein at inhibitory synapses that interacts with $GABA_A$ receptors at the post-synaptic membrane (Tyagarajan and Fritschy, 2014). A significant main effect of age in GEP protein levels was revealed ($\chi^2(1) = 5.481$, $p = 0.019$; Fig. 4E),

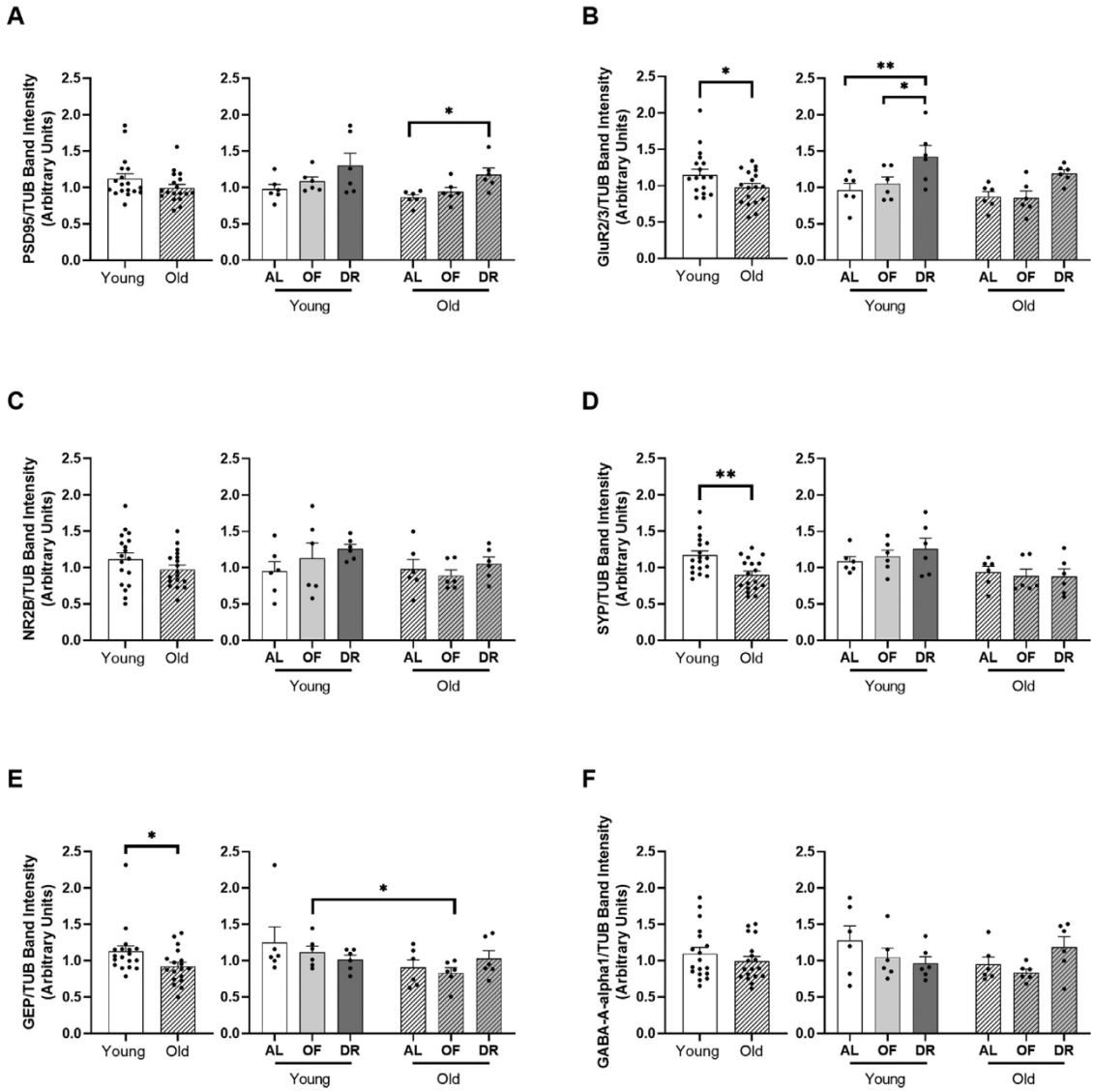

Fig. 4. Analysis of synaptic protein levels in zebrafish whole brain. (A) Protein levels of the excitatory element, PSD95, significantly changed among diet groups with increases in the DR animals, old DR subjects had elevated levels of PSD95 as compared to the old AL group. (B) GluR2/3 levels declined significantly with age and the effect of diet was significantly affecting the GluR2/3 levels, with significant increases being observed in young DR subjects as compared to fish given other dietary regimens. (C) NR2B protein levels were not altered significantly. (D) The protein levels of presynaptic marker SYP decreased significantly with age. (E) An age-related decline in GEP levels was observed with a prominent decrease in the OF group. (F) GABA-A-alpha1 protein levels did not change significantly. The group means + standard error (SE) are represented. *: $p < 0.05$, **: $p < 0.01$. AL: *Ad libitum*; OF: Overfeeding; DR: Dietary Restriction.

with declines being observed in the old animals. Specifically, a significant age-related decrease in GEP levels was demonstrated in the OF group ($p = 0.025$). By comparison, no significant main effect of diet was found on GEP levels ($\chi^2(2) = 0.416$, $p = 0.812$). The clustering partner of GEP, GABA-A-alpha1, is a one of the major subunits of the ionotropic $GABA_A$ receptors (Tyagarajan and Fritschy, 2014). In the case of the levels of GABA-A-alpha1, no significant main effects of age, ($\chi^2(1) = 0.842$, $p = 0.359$) and diet ($\chi^2(2) = 1.272$, $p = 0.529$; Fig. 4F) were found. These results suggest that any effects of age and diet could be altering inhibitory function through GEP but not $GABA_A$ receptors.

3.2.2. Early-differentiated and post-mitotic neuronal markers were altered with diet and age with no evident change in the global proliferation

Since age and diet both affected the protein levels of key synaptic markers, alterations in the protein levels of neuronal markers were further analyzed. An early-differentiated neuronal marker, HuC (Kim et al., 1996) and a post-mitotic neuronal marker, DCAMKL1 (Shin et al., 2013) were investigated. HuC protein levels were differentially expressed in young brains compared to old brains in a significant manner ($F(1, 30) = 9.706$, $p = 0.004$; Fig. 5A), whereas there was no main effect of diet ($F(2, 30) = 0.020$, $p = 0.980$). Furthermore, pairwise comparisons indicated that the HuC protein levels were significantly decreased in the aged brain of both the OF ($p = 0.023$) and DR groups ($p = 0.007$). As HuC is a marker of early-differentiated neurons, we also investigated DCAMKL1 as an adult neuronal marker, since it has continuous expression in post-mitotic neurons (Shin et al., 2013). There were neither main effects of age ($F(1, 30) = 0.005$, $p = 0.945$) nor diet ($F(2, 30) = 2.337$, $p = 0.114$; Fig. 5B) in the case of DCAMKL1 levels. Nevertheless, a significant interaction between age and diet ($F(2, 30) = 3.903$, $p = 0.031$) was observed in DCAMKL1 protein levels with the OF groups showing significant increases in these levels

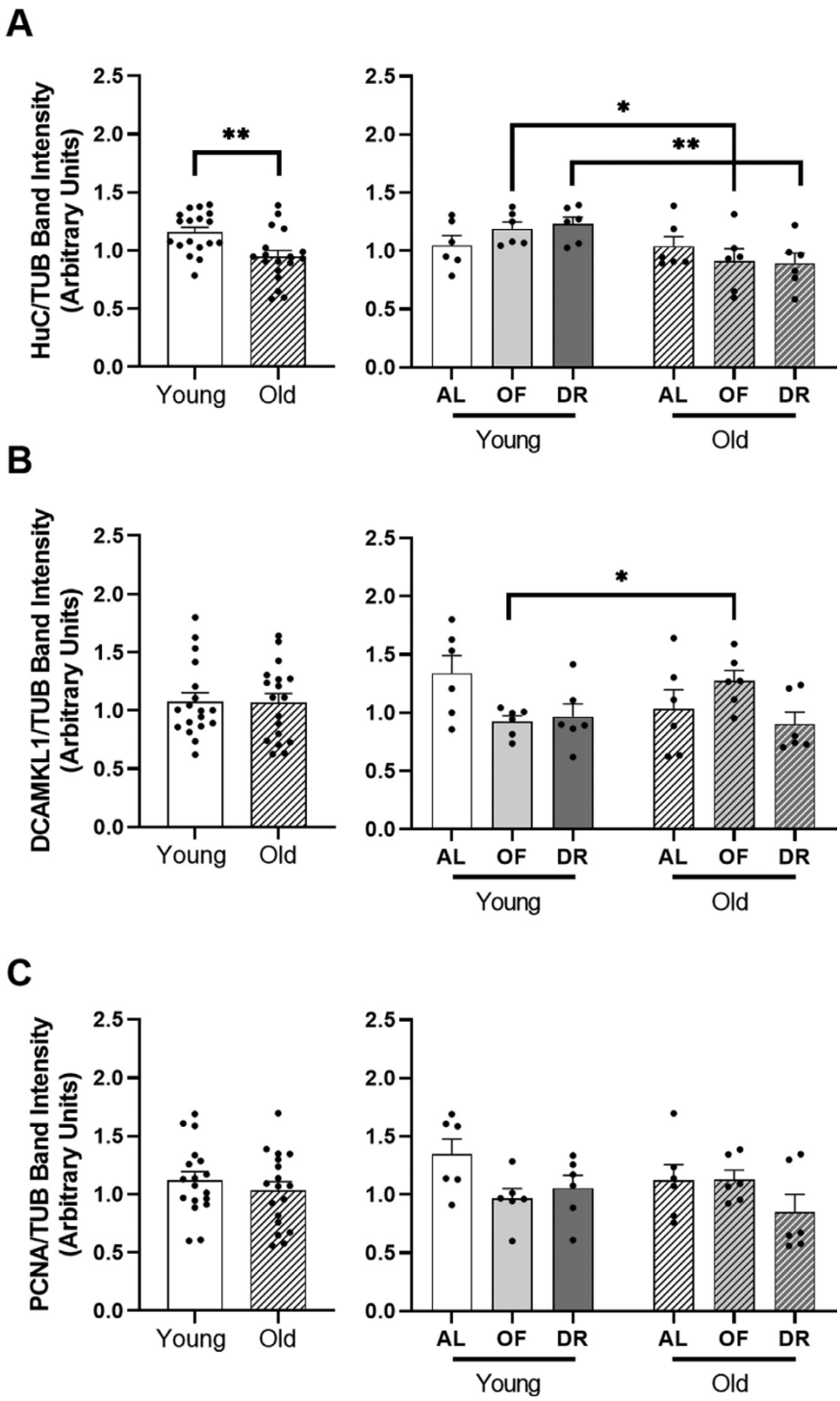

Fig. 5. Analysis of neuronal and proliferation markers in zebrafish whole brain (A) HuC protein levels significantly decreased with age; an age-related decline was prominent in both the OF and DR groups. (B) An interaction between age and diet was observed on DCAMKL1 protein levels with elevated levels of DCAMKL1 in the OF group during aging. (C) PCNA, a global proliferation marker, was not changed significantly by age and diet. The group means + standard error (SE) are represented. *: $p < 0.05$, **: $p < 0.01$. AL: *Ad libitum*; OF: Overfeeding; DR: Dietary Restriction.

in the aged brain ($p = 0.046$; Fig. 5B). The important finding was that the OF diet increased the levels of DCAMKL1 significantly in the aged brain, while the levels of an early-differentiated neuronal marker, HuC, decreased in the OF group during aging. This data may be interpreted such that age- and diet-dependent expression levels of neuronal markers, HuC and DCAMKL1, can reflect altered cellular dynamics.

Previous research demonstrated that the number of PCNA positive cells is less in the aged zebrafish brain compared to young brain (Edelmann et al., 2013). Hence, PCNA protein level, which is the indicator of global proliferation, was analyzed in the current study to determine potential cellular mechanisms that may have contributing roles to the altered dynamics. There were no main effects of age ($F(1, 30) = 0.808$, $p = 0.376$; Fig. 5C) and diet ($F(2,$

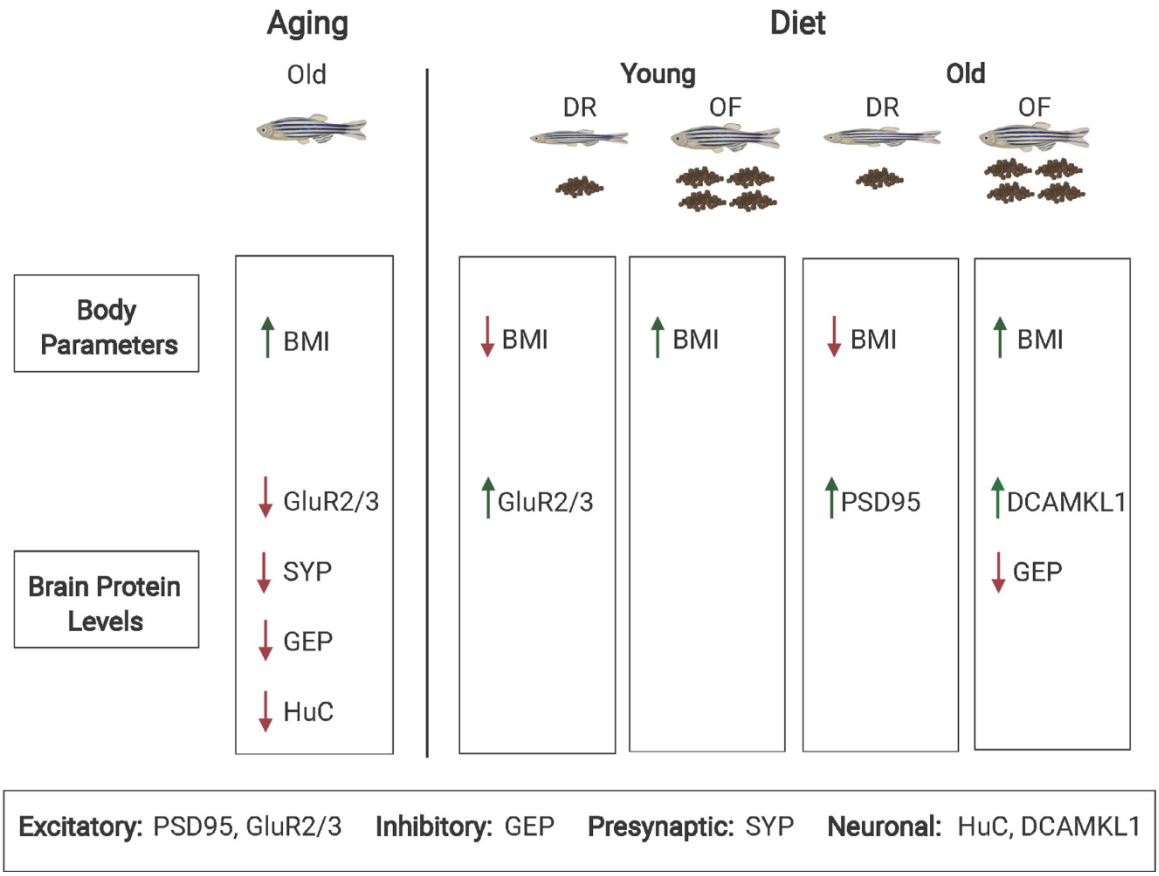

Fig. 6. Summary figure depicting age- and diet-related changes in young and old animals. The positive effects of DR may be increased plasticity by refining calcium signaling through GluR2/3 and PSD95, and the effects of OF are altering cellular dynamics. OF: Overfeeding; DR: Dietary Restriction. The figure was created with BioRender.com.

30) = 2.836, $p = 0.074$). Furthermore, the interaction between age and diet was not significant ($F(2, 30) = 1.699$, $p = 0.200$). Taken together, these data suggest that changes in neuronal markers were affected by age and diet in the absence of global changes in proliferation.

4. Discussion

The current study showed that short-term overfeeding (OF) and dietary restriction (DR) dietary interventions altered body parameters. Specifically, we have demonstrated that a 12 week dietary regimen of DR significantly reduced the body-mass index (BMI) measurements in both young and old animals, whereas OF only lead to increases that were significantly greater than DR subjects but not *ad libitum* (AL) fish (Fig. 6). The main aim of this study was to examine global changes in the brain depending on a high calorie versus low calorie diet and to reveal possible interactions with aging. Age-related declines were found in the levels of glutamate receptor subunits 2 and 3 (GluR2/3), an excitatory post-synaptic protein, synaptophysin (SYP), a presynaptic protein, gephyrin (GEP), an inhibitory post-synaptic protein, and embryonic lethal, abnormal vision (ELAV; Drosophila) like 3 (HuC), an early-differentiated neuronal marker (Fig. 6). Moreover, diet-driven effects, which were age-dependent, were observed in the levels of GluR2/3, post-synaptic density 95 (PSD95), and doublecortin like kinase 1 (DCAMKL1) (Fig. 6). These synaptic and neuronal changes occurred in the absence of alterations in the levels of a global marker of proliferation. Taken together, these data indicate the positive effects of DR may be increased plasticity by refining calcium signaling through GluR2/3 and PSD95, and the effects of OF alter cellular dynamics along with changes in inhibitory neurotransmission.

In the current study at the end of 12 weeks of the OF and DR dietary regimens, the animals given the OF diet had a significantly higher BMI and Fulton K-factor than the DR-fed subjects. Additionally, the subjects fed with a DR regimen had a significantly lower BMI than their AL counterparts which was consistent with previous studies (Arslan-Ergul et al., 2016; Celebi-Birand et al., 2020). Our results demonstrated that although there was an increasing pattern in OF-fed fish in terms of BMI values, the statistical difference between OF and AL groups was not significant in both young and old animals. However, it does not imply that the OF diet was not altering body parameters since there can be changes in the body fat volume which cannot be assessed by BMI. Previous studies have shown that a short-term OF regimen in zebrafish leads to significant increases in the volume of fat tissue without evident changes in body weight (Meguro et al., 2019, 2015). Additionally, in young age groups, a significant reduction in trunk cortisol levels was evident in the DR group compared to OF young animals. This indicates that increases in total calorie intake can elevate trunk cortisol levels, as shown in a previous work utilizing the zebrafish model (Wang et al., 2017). Previous analyses in humans indicated that application of DR can elevate the plasma cortisol levels at initial periods, but after the several weeks of the diet, cortisol levels were decreased to the baseline levels (Nakamura et al., 2016). It was also shown that applications of low-calorie diets in obese individuals were associated with alterations in cortisol response and metabolism without evident fluctuations in plasma cortisol levels

(Johnstone et al., 2004; Yanovski et al., 1997). However, in the current study, cortisol was extracted from the whole trunk of the zebrafish in which blood was present as well as other tissues such as fat which can regulate the local cortisol levels differentially. Future studies will be aimed at investigating the cortisol regulation in different samples such as plasma and fat tissue to understand sample-specific regulations and obtain more similar end-points to the human data. Taken together, these changes in body parameters induced by varying calorie intake will indirectly affect the brain in terms of synaptic and cellular architecture either positively or negatively (Hao et al., 2016; McLean et al., 2018; Meguro et al., 2019; Wang et al., 2017; Zhou et al., 2018).

The main purpose of the current study was to investigate the alterations in the levels of synaptic proteins having roles in the regulation of excitatory/inhibitory neurotransmission and maintenance of synaptic integrity with respect to diet and age. Three synaptic proteins, PSD95, GluR2/3 and N-methyl D-aspartate receptor subtype 2B (NR2B) were used in order to estimate properties of excitatory neurotransmission. PSD95 is a clustering protein found at post-synaptic sites of the glutamatergic neurons and scaffolds alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl D-aspartate (NMDA) receptors (Prange et al., 2004). PSD95 alters synaptic activity and plasticity by regulating the abundance and expression of the both NMDA and AMPA receptors which then mediate calcium influx that modulate synaptic maturation and strength (Colledge et al., 2003; Lin et al., 2004). Our results indicate no significant effect of age on PSD95 levels, while a significant effect of diet was observed with the old DR groups having significantly higher levels of PSD95 compared to AL-fed old animals. Consistent with our results, it has been shown that applications of short- and long-term DR increase the levels of PSD95 in models of pathological aging and after diet there is a reversal in response to lower calorie diets (Hao et al., 2016; Rühlmann et al., 2016). More importantly our results indicated that short-term DR-dependent modulations of PSD95 levels can occur at old age even during in normal non-pathological aging and this would likely lead to an overall maintenance of synaptic plasticity with DR at older ages.

GluR2 and GluR3 are two subunits of the AMPA receptor involved in plasticity. Both the GluR2 and GluR3 subunits contain short cytoplasmic carboxyl tails, which are their unique and common features compared to the other AMPA receptor subunits (Malinow et al., 2000). Both the GluR2 and GluR3 subunits have regulatory roles in trafficking, stabilization, and maintenance of AMPA receptors associated with their short carboxyl termini enabling interactions with cytosolic proteins (Shi et al., 2001). More specifically, the GluR2 subunit is mainly involved in regulating calcium permeability via RNA-editing with the adult form of the receptor rendered relatively impermeable to calcium (Isaac et al., 2007). On the other hand, the functions of GluR3 are not as well-addressed, but it is also subjected to RNA editing processes and regulates the activity of neural networks (Steenland et al., 2008). AMPA receptors are recycled rapidly in the synapses, and these dynamic alterations in the expression levels of AMPA receptors regulate synaptic plasticity and efficacy through changing local calcium concentrations (Chetkovich et al., 2002). Previous studies in rat brains indicated that GluR2 levels in the CA1 and CA3 regions of hippocampus are significantly reduced between young and old ages, moreover, these studies showed that GluR2 levels tend to be stable in the DR group throughout the aging process (Adams et al., 2008; Shi et al., 2007). Our results also indicated a significant decrease in GluR2/3 levels with advancing age in zebrafish. Additionally, we have observed a significant effect of diet with the young DR group having increased GluR2/3 levels compared to their AL and OF counterparts, while no prominent changes were observed at old age. This diet effect is pointing to potential changes in synaptic plasticity with a DR regimen, as well as potentially neuroprotective mechanisms because the presence of GluR2 renders the AMPA receptor relatively impermeable to calcium (Hollmann and Heinemann, 1994). Additionally, the elevations of GluR2/3 in the DR group may indicate the maintenance of continuous trafficking of AMPA receptors regulated by both subunits (Shi et al., 2001).

In the DR groups, we have observed a coordinated increase in the levels of PSD95 and AMPA receptor subunits, GluR2/3. Published data has shown that through protein-protein interactions, GluR2/3 can regulate stabilization and trafficking of the AMPA receptors, and through GluR2- and PSD95-dependent mechanisms, calcium signaling can be refined (Chetkovich et al., 2002; Colledge et al., 2003; Gómez-Varela et al., 2012). In the current study, the antibody used for the detection of GluR2/3 recognized the carboxyl terminus peptide found in both the GluR2 and GluR3 subunits. Therefore, our results likely reflect an overall promotion of AMPA receptor trafficking dynamics and their maintenance with short-term DR application driven by changes in both GluR2 and GluR3 levels. However, expression levels of these subunits are not equally abundant in the brain, and it was demonstrated that subunits of AMPA receptors are mainly composed of GluR1/GluR2 and GluR2/GluR3 heteromers (Malinow et al., 2000; Shi et al., 2001). This may indicate that our results mostly reflect GluR2 alterations and elevations with a DR regimen might show increased synaptic plasticity through refining calcium signaling. Further investigations will use antibodies targeting individual specific subunits of AMPA receptors working with zebrafish samples to understand subunit-specific changes. On the other hand, no overall effects of age or diet were observed in NR2B, which is one of the subunits of the NMDA receptors that regulates synaptic plasticity (Clayton and Browning, 2001; Philpot et al., 2001). Taken together, diet-driven changes in excitatory markers PSD95 and GluR2/3 imply that elevated PSD95 levels will increase the capacity for AMPA receptors at the synapses and maintain synaptic plasticity and resilience in DR subjects during aging.

GEP and gamma-aminobutyric acid type A alpha 1 subunit (GABA-A-alpha-1) were used to estimate the regulation of inhibitory neurotransmission in the context of the factors of age and diet. GEP is a clustering protein that is found at postsynaptic sites of inhibitory GABAergic neurons and scaffolds ionotropic $GABA_A$ and glycine receptors (Tyagarajan and Fritschy, 2014). In our data, we have observed a significant effect of age as GEP levels decreased in old animals. In the literature, studies have shown that GEP levels decrease in the visual cortex of humans (Pinto et al., 2015). Additionally, an age-dependent significant decline was only observed in OF regimen among the diet groups, with OF-fed old animals experienced a steeper decrease in GEP levels with aging. Decrements in GEP levels were previously observed in neurodegenerative conditions and have made animals more vulnerable to neurotoxicity (Agarwal et al., 2008). Therefore, evident fluctuations in OF diet can manifest age-specific susceptibilities in this group to excitotoxic insults.

Furthermore, inhibitory and excitatory components can also be thought of as acting together within the context of the excitatory/inhibitory balance. Overall, the markers including PSD95, GluR2/3 and GEP have a shared decreasing pattern with aging, so there is no specific shift in this balance that would favor excitation or inhibition since all components tend to be reduced slightly. This is similar to data that was recently published by our group (Celebi-Birand et al., 2020). However, our group has previously shown that this balance with aging might be shifted towards excitation but this is solely in old females (Karoglu et al., 2017). In the current data the effect of diet was likely stronger than any gender effects and probably blunted any changes in the protein levels

driven by sex. As far as diet is concerned, there were no direct effects on GEP levels, and this is similar to our recently published work (Celebi-Birand et al., 2020). GABA-A-alpha1 is the predominant subunit of the $GABA_A$ receptors, which are clustered by GEP (Tyagarajan and Fritschy, 2014). In the literature, it has been shown that with aging this subunit tends to be stable in the hippocampus (Palpagama et al., 2019). Likewise, in the current study we did not observe a significant main effect of age or diet on GABA-A-alpha1 levels. However, more studies should focus on inhibitory markers within the context of dietary interventions since GEP is directly interacting with nutrient-sensing signaling pathways including the mammalian target of rapamycin (mTOR), which is affected by increasing and decreasing total caloric intake (Sabatini et al., 1999).

SYP is a transmembrane glycoprotein that can be found in synaptic vesicles located in presynaptic terminals (Kwon and Chapman, 2011) and used as an overall indicator of presynaptic integrity. Previously, in different model organisms it has been shown that SYP levels decreased in the aging brain (Adams et al., 2008; Karoglu et al., 2017; Pinto et al., 2015; VanGuilder et al., 2010). Our data followed similar age-related significant declines in SYP levels but we did not observe any effect of diet on SYP. In a previous study conducted using male rats in which the SYP levels were measured in the CA3 region of the hippocampus, it was demonstrated that while age-related declines in SYP were observed in AL groups, DR groups tend to have stable levels of SYP, so at older ages, the DR group has higher levels of SYP compared to AL subjects (Adams et al., 2008). In another study conducted with mice in which whole brain extracts were utilized for the analyses, it has been shown that while high-fat diet was decreasing the elements of excitatory transmission including PSD95, no change was observed in SYP levels (Kothari et al., 2017). One reason that we did not observe any diet effect on SYP levels is probably due to using whole brain extracts rather than focusing on a specific region. Additionally, it could be due to the fact that these studies used male mice and also did different durations of dietary regimens. For future studies, possible region-specific alterations in SYP levels are being investigated with respect to age and diet. Moreover, to dissect more specific mechanisms, rather than using a marker giving information about an overall synaptic integrity, more specific presynaptic markers such as vesicular transporters of glutamate and GABA can be further investigated within this context.

Subtle synaptic alterations affected by age and dietary interventions could lead to neuronal changes at the cellular level. To answer this question, we examined the protein expression of two neuronal markers, HuC and DCAMKL1. The HuC protein is recognized as an early-differentiated neuronal marker and DCAMKL1 is a post-mitotic neuronal marker (Kim et al., 1996; Shin et al., 2013). Previously, Edelmann et al., (2013) indicated that newly generated neurons labeled with Bromodeoxyuridine (BrdU) and HuC/D declined in the aging zebrafish brain. Furthermore, it has been shown that the number of HuC/D positive neurons decreases with advanced age in the case of the human subthalamic nucleus (Zwirner et al., 2017). Consistent with these studies, our data analysis also demonstrated an age-related decline in HuC protein levels that was significant and this age effect was prominent in both the OF and DR groups. In terms of this early-differentiated neuronal marker, our data is consistent with previous reports (Edelmann et al., 2013; Zwirner et al., 2017).

DCAMKL1 is a post-mitotic marker with continuous expression in adult neurons and its precise level is required to achieve a balance in adult neurogenesis (Shu et al., 2006) similar to doublecortin (DCX) (Boekhoorn et al., 2008; Friocourt et al., 2007; Saaltink et al., 2012; Shu et al., 2006; Tanaka et al., 2006; Vreugdenhil et al., 2007). DCAMKL1 is also homologous to the calcium/calmodulin-dependent protein kinase (CAMK) at the C-terminal region and through its kinase domain, DCAMKL1 negatively regulates the post-synaptic protein content including PSD95 (Shimomura et al., 2007; Shin et al., 2013). Our data demonstrated that its levels were affected by an age by diet interaction, which means any age-related changes are dependent on the dietary status of the fish. The results indicated that aged OF-fed zebrafish have significantly higher DCAMKL1 expression levels than young OF animals. However, increases in DCAMKL1 protein levels does not always mean more neurogenesis since the upregulation of DCAMKL1 causes mitotic arrest and if the arrested cells exit the cell cycle, then they adopt their neuronal fate (Shu et al., 2006). Additionally, the levels of an early-differentiated neuronal marker, HuC, decreased in the OF group during aging. The pattern was different between these two neuronal markers pointing out the potential regulatory roles of DCAMKL1 besides the structural changes. Moreover, a recent study indicated that DCAMKL1 regulates α-Synuclein protein levels post-transcriptionally via its kinase domain and its knockdown rescues α-Synuclein toxicity in mouse models of synucleinopathy (Vázquez-Vélez et al., 2020). It has been also demonstrated that overexpression of DCAMKL1 in the mouse brain leads to reductions in the AMPA/NMDA ratio along with a decreased synaptic content of AMPA (Shin et al., 2013). Taken together, these results imply that there is a shift in the precise balance controlled by DCAMKL1 in a response to higher caloric intake, i.e. OF, during aging that will alter the synaptic and cellular dynamics in the brain.

To gain comprehensive insight into the effects of aging and diet on the brain, alterations in the synaptic and neuronal proteins were studied in the context of global cellular proliferation. The levels of the global proliferation marker, proliferating cell nuclear antigen (PCNA) were examined and this marker is an indicator of proliferating neural stem cells (NSCs), which can be utilized to label the cycling population of progenitors, neuroblasts (Edelmann et al., 2013; Zhang and Jiao, 2015). In the present study, age and/or diet did not alter the protein levels of PCNA. In the literature, it has been shown that the number of PCNA positive cells is less in the aged zebrafish compared to young (Edelmann et al., 2013). Furthermore, it has been shown that in the mouse brain PCNA positive cells in dentate gyrus are less in number during aging (Jinno, 2011; Oh et al., 2020). The differences between the published work (Edelmann et al., 2013; Jinno, 2011; Oh et al., 2020) and our current data is that their studies utilized anatomical methods to estimate the number of PCNA positive cells and the current study uses Western blot techniques to examine protein levels.

Taken together our results indicate that synaptic and cellular markers robustly declined in an age-dependent manner, but short-term dietary interventions were more prominently affecting synaptic components with respect to brain aging. This likely suggests that short-term DR is having effects on synaptic proteins without inducing gross alterations in cellular markers. These observations are consistent with the literature because it was shown that life-long DR modified the synaptic protein levels in the hippocampus while structural measures were not altered significantly by diet (Adams et al., 2008; Shi et al., 2007). This evidence demonstrates that short-term and life-long DR can have comparable effects and both have the potential to alter synaptic elements with no evident changes in the cellular markers. On the other hand, the levels of cellular elements were affected by an age by OF diet interaction, which means any age-related changes may be dependent on high calorie intake. However, the effects of high calorie diets on the brain are not well-described within the context of aging. Further studies are still required to gain more insights into the link between cellular changes and OF-related interventions.

5. Conclusion

Previous studies, as well as the current work, have shown that the zebrafish is a suitable model organism to study overfeeding/diet-induced obesity (OF/DIO) and dietary restriction (DR) since it shares the common physiological outcomes in response to these dietary interventions (Celebi-Birand et al., 2020; Oka et al., 2010). The current study was aimed at unveiling the neurobiological consequences of the dietary interventions that included OF and DR in both young and old animals. The current short-term dietary regimens were found to affect the levels of neuronal and synaptic proteins in the aged zebrafish brain, as well as the body parameters. According to the present results, the effects of OF maybe driven by both unregulated increases in the neuronal protein, DCAMKL1 and decrements in the levels of inhibitory synaptic protein GEP with aging. On the other hand, DR has positive impacts by increasing synaptic plasticity and promoting the maintenance of receptor trafficking dynamics through the excitatory elements, GluR2/3 and PSD95. The present findings will help to increase the limited knowledge about the effects of short-term high and low caloric dietary interventions on the brain that have implications for maintaining cognitive performance. Moreover, these data provide insight into the mechanisms that may be occurring in human populations for use in translational approaches.

Disclosure statement

The authors declare that they have no conflict of interest.

Authors contribution

Elif Tugce Karoglu-Eravsar: Conceptualization, Methodology, Formal Analysis, Investigation, Writing-Original Draft, Visualization. **Melek Umay Tuz-Sasik:** Conceptualization, Methodology, Investigation, Writing - Original Draft, Visualization. **Michelle M. Adams:** Conceptualization, Methodology, Resources, Writing - Original draft, Supervision, Project administration, Funding acquisition.

Verification statement

1. The authors declare that there are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) our work.
2. Our institution does not have any contracts relating to this research through which it or any other organization may stand to gain financially now or in the future.
3. There are no other agreements of ours or our institution that could be seen as involving a financial interest in this work.
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5. The data contained in the manuscript being submitted have not been previously published, have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging.
6. The animal protocol for this study was approved by the Bilkent University Local Animal Ethics Committee (HADYEK) with the approval date: Sept 6, 2017, no: 2017/12.
7. All authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data.

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Supplementary materials

Supp. Table 1. Total body weight (g), body length (cm) and Fulton K-factor (100 g/cm^3) values.

Supp. Fig. 1. Diet significantly altered cortisol levels (ng/g trunk weight). Cortisol was extracted from snap frozen trunks and its levels were determined with commercially available cortisol kit (R&D systems, Minneapolis, MN, USA: KGE008B). A significant main effect of diet was revealed, ($F(2,47) = 3.856$, $p = 0.028$). Animals in the DR groups demonstrated the lowest levels of trunk cortisol compared to the animals treated with other dietary regimens. Post-hoc analyses revealed that in the young group DR animals had significantly lower cortisol levels than the OF-fed fish ($p = 0.040$). Neither a significant main effect of age nor an age by diet interaction were observed on the trunk cortisol levels ($F(1,47) = 1.326$, $p = 0.255$ and $F(2,47) = 0.729$, $p = 0.488$, respectively). The group means + standard error (SE) are represented. *: $p < 0.05$. AL: *Ad libitum*; OF: Overfeeding; DR: Dietary Restriction.

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