Experimental Gerontology 149 (2021) 111346

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Environmental enrichment applied with sensory components prevents age-related decline in synaptic dynamics: Evidence from the zebrafish model organism

Elif Tugce Karoglu-Eravsar [a,b,c,d], Melek Umay Tuz-Sasik [a,b,c], Michelle M. Adams [a,b,c,e,*]

[a] Interdisciplinary Program in Neuroscience, Aysel Sabuncu Brain Research Center, Bilkent University, Ankara, Turkey
[b] National Nanotechnology Research Center (UNAM), Bilkent University, Ankara, Turkey
[c] Department of Molecular Biology and Genetics, Zebrafish Facility, Bilkent University, Ankara, Turkey
[d] Department of Psychology, Selcuk University, Konya, Turkey
[e] Department of Psychology, Bilkent University, Ankara, Turkey

ARTICLE INFO

Section editor: Tibor Hortobagyi

Keywords:
Environmental enrichment
Aging
Sexual dimorphism
Synapses

ABSTRACT

Progression of cognitive decline with or without neurodegeneration varies among elderly subjects. The main aim of the current study was to illuminate the molecular mechanisms that promote and retain successful aging in the context of factors such as environment and gender, both of which alter the resilience of the aging brain. Environmental enrichment (EE) is one intervention that may lead to the maintenance of cognitive processing at older ages in both humans and animal subjects. EE is easily applied to different model organisms, including zebrafish, which show similar age-related molecular and behavioral changes as humans. Global changes in cellular and synaptic markers with respect to age, gender and 4-weeks of EE applied with sensory stimulation were investigated using the zebrafish model organism. Results indicated that EE increases brain weight in an age-dependent manner without affecting general body parameters like body mass index (BMI). Age-related declines in the presynaptic protein synaptophysin, AMPA-type glutamate receptor subunits and a post-mitotic neuronal marker were observed and short-term EE prevents these changes in aged animals, as well as elevates levels of the inhibitory scaffolding protein, gephyrin. Gender-driven alterations were observed in the levels of the glutamate receptor subunits. Oxidative stress markers were significantly increased in the old animals, while exposure to EE did not alter this pattern. These data suggest that EE with sensory stimulation exerts its effects mainly on age-related changes in synaptic dynamics, which likely increase brain resilience through specific cellular mechanisms.

1. Introduction

Cognitive decline can be observed to varying degrees in individuals as they age even in the absence of neurodegenerative diseases. Previous studies suggest that age-related declines in cognitive processing are associated with subtle biological alterations regulating synaptic dynamics and plasticity in the brain and these likely underlie synaptic dysfunction in the aging brain (Adams et al., 2001; Mattson et al., 2001; VanGuilder et al., 2010). Additionally, there is a large heterogeneity in aged populations exhibiting susceptibilities to cognitive decline and associated alterations in biological markers of cellular aging (Gamberger et al., 2017). One element that influences brain aging is the environment in which the individual experiences. Evidence suggests that environmental manipulations can influence behavioral performance in

Abbreviations: EE, environmental enrichment; BMI, body mass index; PSD95, Post-synaptic density 95; 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-type receptor subunit 2B; GEP, Gephyrin; GABA-A-a1, Gamma-Aminobutyric acid type A alpha 1 subunit; PCNA, proliferating cell nuclear antigen; GFAP, glial fibrillary acidic protein; ROS, reactive oxygen species; AChE, acetylcholinesterase; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl D-aspartate; GABA, Gamma-aminobutyric acid; DCX, doublecortin; DCF, 2′,7′-Dichlorodihydrofluorescein diacetate; MDA, Malondialdehyde.

* Corresponding author at: Interdisciplinary Graduate Program in Neuroscience, Aysel Sabuncu Brain Research Center, Bilkent University, 06800, Bilkent, Ankara, Turkey.
E-mail address: michelle@bilkent.edu.tr (M.M. Adams).

https://doi.org/10.1016/j.exger.2021.111346
Received 24 January 2021; Received in revised form 31 March 2021; Accepted 3 April 2021
Available online 8 April 2021

conjunction with biochemical and structural changes in the brain, which determine the course of cognitive aging (Marcon et al., 2018; Mora et al., 2007). Another component that likely contributes to heterogeneity of the aging population is gender and sexually dimorphic patterns can be observed at many levels ranging from molecular changes in the brain to behavior (Grimm and Eckert, 2017; Gur and Gur, 2002; McCarrey et al., 2016). Therefore, it is crucial to determine the molecular mechanisms in the aged brain that will promote and retain successful cognitive abilities with respect to environment and gender, both of which can alter brain resilience throughout the aging process and likely underlie some of the heterogeneous profile of cognition in the elderly.

Environmental enrichment (EE) is considered an intervention that is non-invasive and easily translatable to elderly human subjects from studies utilizing animal aging models. EE, which is a method of exposure to a perceptually, socially, physically and cognitively-stimulating environment, has been shown to promote the maintenance of cognitive processing at older ages in both human and animal subjects (McDonald et al., 2018; van Praag et al., 2000). While the methods used for EE in animals and humans may be species-specific, there is considerable conservation among the plasticity-related processes and very likely the biological changes as well (Clemenson et al., 2015; McDonald et al., 2018). However, due to the constraints of studying biological changes at the cellular and synaptic level in the human brain, animal models are required in order to identify the neurobiological consequences of possible ameliorative effects of EE.

EE has been suggested as a promising intervention for attenuating age-related decrements and retain successful aging. There are important aspects of this intervention that need to be taken into consideration when deciding how to apply it, and one of these factors is the age at initiation. Studies have shown that the behavioral benefits and amelioration in the brain depend on the age of the animal. Firstly, EE has the possibility of being effective in old animals. For example, EE improves spatial memory acquisition and recall, as measured by performance on the radial arm maze and Morris water maze tasks (Frick and Fernandez, 2003; Bennett et al., 2006). These improvements in old enriched animals were accompanied by structural modifications in the brain, as well as the neurobiological changes (Frick and Fernandez, 2003; Mora et al., 2007; Pham et al., 2002). Additionally, differential effects of EE can be observed in young subjects as compared to old age groups. To illustrate, it was shown that radial maze performance of both young and mature rats were improved with the application of EE but neurochemical changes were prominently observed only in young groups (Mora-Gallegos et al., 2015). Moreover, similar age-dependent alterations were observed in zebrafish with results showing that neuroplasticity-related gene expression patterns were modulated by EE at younger ages more prominently compared to old animals (Manuel et al., 2015). Therefore, systematic investigation of the different age groups within the same EE paradigm will provide insights about possible age-dependent regulation of EE and the optimal time interval to observe the positive effects of this intervention.

Only a few studies of EE have included both male and female subjects within the same experimental design. This is important because those studies utilizing either male or female individuals have reported differential results regarding EE-dependent changes in the brain (Bennett et al., 2006; Frick and Fernandez, 2003), so it is unclear if both genders respond equivalently to EE. It is also evident that there are gender differences with respect to how individuals age (Grimm and Eckert, 2017; Gur and Gur, 2002; Karoglu et al., 2017; McCarrey et al., 2016) and this could be an important contributor to the heterogeneity with aging. Thus a critical aim of the current study was to include both males and females, which will provide crucial insights into the possible gender-dependent regulation of EE in the context of aging.

The zebrafish model organism was used as the gerontological model in the current study. This was done because it shows gradual age-related cognitive decline in spatial learning and memory, flexibility, generalization of the learned responses, and entrainment to spatial and temporal cues (Yu et al., 2006). Additionally, biomarkers of aging that include increased senescence-associated beta galactosidase activity, increased reactive oxygen species (ROS) activity, decreased neurogenesis, and altered synaptic proteome and transcriptome levels are similar across humans and zebrafish (Arslan-Ergul et al., 2016; Karoglu et al., 2017; Kishi et al., 2003). Moreover, gender-related patterns in brain aging have been described in zebrafish, in a manner similar to other aging mammals (Arslan-Ergul and Adams, 2014; Karoglu et al., 2017). Finally, studies have successfully applied enrichment methods to young adult zebrafish groups and indicated its ameliorating effects on both behavioral and biochemical measures (Marcon et al., 2018; Volgin et al., 2018; von Krogh et al., 2010), but EE paradigms in old zebrafish are still relatively limited in showing its possible effects on attenuating age-related alterations (Manuel et al., 2015). Therefore, systematic investigation of the interactions among age, gender and EE are still needed in order to understand the contribution of these factors in explaining the heterogeneity and nature of the susceptibility to age-related cognitive decline in humans, as well as establishing the zebrafish model for studying age-dependent effects of EE.

In the current study EE as a possible positive regulator in changing the course of the aging process with respect to global brain levels of neuronal, glial, proliferative, presynaptic and excitatory/inhibitory synaptic markers, as well as oxidative stress-related markers were investigated. A short-term EE that was sensory in nature and achieved through the addition of plants, swimming tubes, bottom gravel, and sea pictures was carried out in young adult and old male and female zebrafish for a duration of four weeks. Our results demonstrated that EE did not alter body mass index (BMI) values but a significant increase was seen in the brain weight of the old enriched animals as compared to old fish in the barren environmental condition. The results indicated EE prevented an age-related decrease in the levels of post-mitotic neuronal marker DCAMKL1 while other cellular protein markers were relatively stable. In terms of synaptic proteins, age-related declines were observed in the levels of the presynaptic marker, SYP, and excitatory synaptic marker NR2B in the old animals from the barren environment and this decrease was reversed by EE. Also, EE resulted in a significant increase in GEP, which is an inhibitory synaptic marker, in the old age groups. Moreover, in terms of oxidative stress markers including ROS activity and lipid peroxidation, aging-related significant increases were revealed that were independent of the environmental conditions. To the best of our knowledge, the current study is one of the first to investigate the effects of an EE application that is mostly sensory in nature on body parameters, synaptic and cellular markers, as well as oxidative stress-related indicators in the brains of both male and female zebrafish within the context of aging.

2. Methods

2.1. Animals

A total of 93 wild-type zebrafish (AB strain) were used in the current study including both young (6 month-old), and old (27 month-old) male and female animals. These ages were chosen due to the fact that age-related cognitive decline starts at 24 months of age in zebrafish (Yu et al., 2006), and this would permit the determination of whether or not environmental enrichment (EE) might ameliorate the cellular effects of aging-related deteriorations related to cognitive alterations. Additionally, zebrafish are considered as adult-like after passing 3–6 months of age (Kimmel et al., 1995), therefore, using 6 month-old animals as the young group ensures that the observed effects were not confounded by any latent brain maturation processes. All zebrafish prior to the enrichment intervention were maintained in a controlled and recirculating housing system, ZebTec (Techniplast, Italy), in the Bilkent University Molecular Biology and Genetics Fish Facility. This system enables the maintenance of a constant temperature of 27.5 °C and pH of 7.5, as well as stable water quality parameters for the well-being of the

fish. All fish were subjected to a light:dark cycle of 14L:10D. In the standard system conditions, zebrafish were fed twice with dry flakes and once with *Artemia* per day.

2.2. Experimental tanks

Individual glass aquaria with the dimensions of 50 × 15 × 30 cm were used and enrichment was achieved via introduction of bottom gravel, swimming tubes and artificial plants. The biggest challenge for performing EE in zebrafish is maintaining similar water quality parameters in both the enriched and barren-control environments. In the literature, generally enrichment paradigms were conducted using separate tanks from the control groups (Manuel et al., 2015; Marcon et al., 2018). However this procedure has limitations because artificial plants and bottom-gravel can release some materials into the water, as well as increase the overall surface area which can lead to an accumulation of biological waste. Thus, any differences due to the EE paradigm might be associated with a confounding factor of the variance in the water quality parameters across the enriched and control tanks. Our experimental tanks illustrated in Fig. 1 were designed by us taking into consideration these issues.

The length of the aquarium (50 × 15 × 30 cm) was divided into two equal compartments with dimensions of 23 × 15 × 30 cm. Between the compartments, two plexiglas, opaque black separators (15 × 30 cm with a 3 mm thickness) were placed into glass notches, which were attached to the walls of aquarium (Fig. 1A). In order to permit water flow between the compartments, holes with a radius of approximately 1.5 mm were drilled into the plexiglas plates. Since the holes in the separator might lead to visual contact and sensory stimulation between the compartments, two separators with holes in non-overlapping locations were used. There was a small compartment, which was 3 cm in length, between the separators that was used to empty and fill water in the tank, which permitted us to not disturb the fish. Both compartments in one tank were equipped with an air pump and a heater (Fig. 1B). To eliminate visual interference from irrelevant stimuli, the three walls of tanks were covered, and for the EE compartment, the paper had pictures of the sea and plants with the barren environment having dark blue paper (Fig. 1C). The color of the paper surrounding the tank was important due to the fact that the fish were raised in system tanks that were blue in nature and they were habituated to this basic type of environment. Moreover, previous studies indicated that this background color did not elicit any significant stress response in zebrafish as compared to bright background colors (Pavlidis et al., 2013).

2.3. Experimental procedure

When the experimental zebrafish had reached the required age of maturity, they were randomly assigned to one type of environmental condition, either enriched or barren. Although shorter durations of EE, such as one week, can have effects on zebrafish brains (von Krogh et al., 2010), other studies have indicated that longer periods of an enrichment, such as four weeks, will alter molecular signatures that include reactive oxygen species (ROS) activity and gene expression patterns, as well as reduce the vulnerability against extrinsic factors like stress (Manuel et al., 2015; Marcon et al., 2018). Thus, a duration of four weeks was chosen for the current study. Initially the different zebrafish

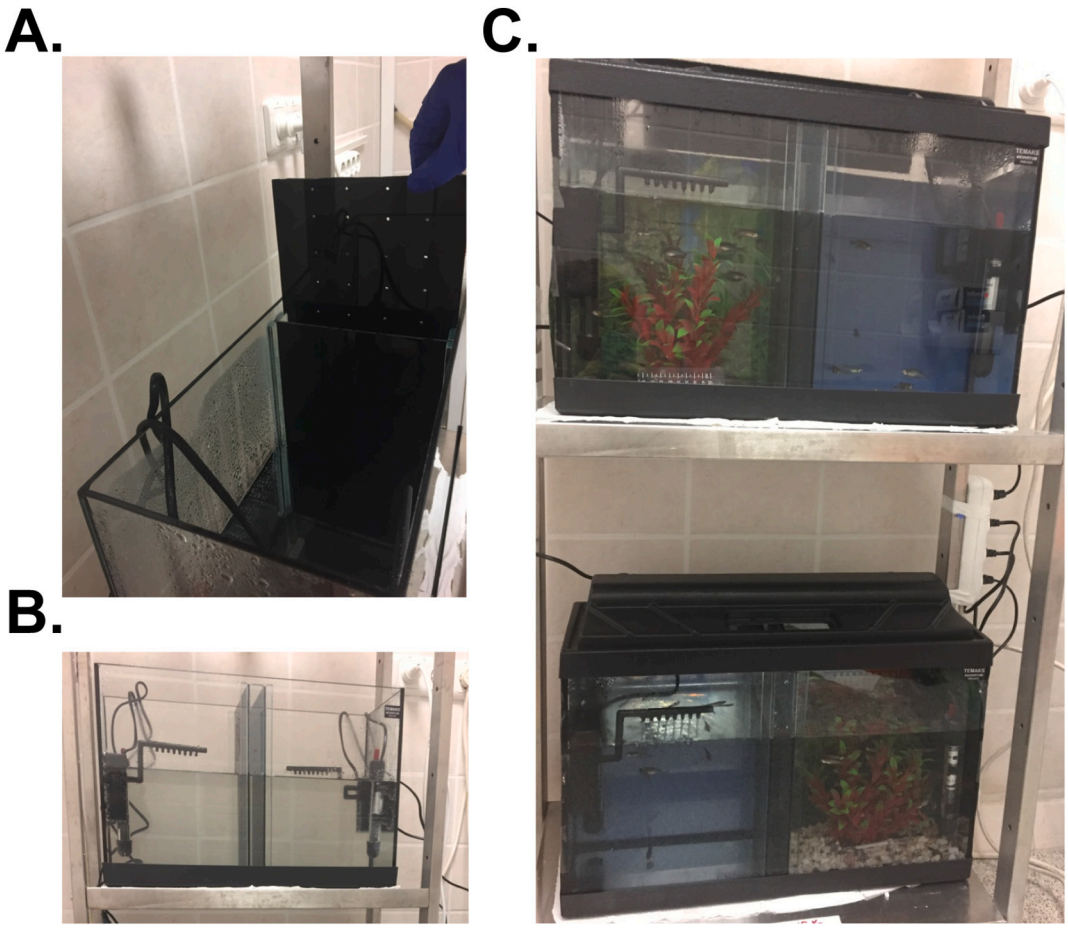

Fig. 1. Representative photographs of the tank system used for environmental enrichment. (A) Two plexiglas separators with holes in non-overlapping locations. (B) The separate compartments equipped with an air pump and a heater. (C) The experimental tanks with both the enriched and barren environmental conditions. The enriched side contains bottom gravel, an artificial plant and tubes, which were used as sensory enrichment materials, and the barren side contains no accessories.

groups were habituated to the tank for one week, and following that period exposed to the experimental protocol for 4 weeks. Since our setup was not a recirculating system, one-third of water was emptied and fresh system water was added in intervals of every 2 days. Additionally, the air pumps were cleaned thoroughly throughout the entire duration of the experiment. Prior to each tank cleaning, water samples from tanks were collected, and these samples were subjected to pH and nitrate tests for monitoring the water quality. The temperatures of tanks were recorded daily, and the fish were fed with dry flakes two times a day and *Artemia* once a day. The feeding regimen was the same standard protocol as that given to the animals in the main recirculating system in the zebrafish facility (Bilkent University, Ankara, Turkey).

After the experiments were terminated, zebrafish were anesthetized by submersion in ice-cold system water for 10 min, and when full movement ceased they were decapitated with a scalpel blade. The weight and length of the animals were recorded. The head was opened from the ventral surface. Following detachment of the gills, the eyes were dissected from the optic nerves, then the skull was opened carefully and the brain was removed and weighed. The trunk was examined to determine the gender of each animal. Fish that contained testes were designated as males and those with ovaries were identified as female. All tissues, including the brain, gills, eyes and trunk were snap frozen in liquid nitrogen, and then stored at −80 °C for further experimental analysis. The experimental protocol for this study was approved by the Bilkent University Local Animal Ethics Committee (HADYEK) with the approval date: Sep 12, 2018 and no: 2018/28.

2.4. Protein isolation

Brains were homogenized in 250 μl of lysis buffer (150-mM NaCl, 1.0% NP-40, 0.1% SDS, 50-mM Tris-HCl pH 8.0) containing protease inhibitor (Roche, Mannheim, Germany: 05892970001) by passing the tissue through a syringe (1 ml, 26 gauge). Lysates were incubated on ice for 30 min and centrifuged (Himac CT15E, VWR Hitachi, Darmstadt, Germany) at 13,000 rpm for 20 min at +4 °C. Supernatants were collected and aliquoted. Total protein amounts of the supernatants were determined using a Bradford reagent (Sigma, St. Louis, MO, USA: B6916) and bovine serum albumin (BSA) as the standard.

2.5. Western blot

The protocol for the Western blotting experiments was followed as described previously (Celebi-Birand et al., 2020; Karoglu et al., 2017; Tuz-Sasik et al., 2020). For protein detection, 15–30 μg of total protein from each protein sample was loaded into 10% SDS-PAGE gels in cohorts that included one representative animal tissue sample from each experimental group, and the places of cohorts in the gel were changed for the technical replicates to avoid any introduction of systematic gel-to-gel variability. The protein samples of the cohorts from each group were separated under reducing and denaturing conditions and transferred for 90 min at 100 V to PVDF membrane. Following the transfer, the membranes were blocked using a blocking buffer that contained 5% non-fat dried milk powder in Tris-buffered saline with Tween 20 (137 mM NaCl, 20 mM Tris-HCl, 0.3% Tween 20, pH 7.6) (TBS-T) at room temperature for 1 h. Following this blocking procedure, the membranes were then incubated with the primary antibodies of interest at +4 °C overnight with gentle agitation. Primary antibody incubation was followed by multiple washing steps with TBS-T for 30 min, and then secondary antibodies were incubated for 1 h at room temperature, which was followed by washing with TBS-T. Primary and secondary antibodies utilized in the current study are listed in Table 1. All of the listed antibodies, except those directed against NR2B and GluR2/3, have been optimized by our group previously for use with zebrafish brain samples (Celebi-Birand et al., 2020; Karoglu et al., 2017). For the antibodies directed against NR2B and GluR2/3 were tested with zebrafish brain lysates and positive control protein samples to determine if they were producing the expected molecular weight band. The visualized bands for these two antibodies were observed at the expected molecular weights in both zebrafish brain lysates and positive controls (Supplementary Fig. 1).

Protein bands were detected and visualized with Femto Supersignal (Thermoscientific, Rockford, IL, USA: 34095) and a ChemiDoc MP System (BioRad, Hercules, CA, USA). Quantitative analyses of band intensities were conducted using ImageJ software (NIH, Bethesda, MD, USA) by author MUT-S, who was blind to the age, gender and environmental condition of each of the groups of cohorts. Band intensities were normalized as described previously (Karoglu et al., 2017; Tuz-Sasik et al., 2020). First, band intensities were normalized to their averaged cohort intensity within the same membrane and then these intensities to their corresponding β-tubulin intensity, which was used as a housekeeping control.

2.6. Brain acetylcholinesterase (AChE) activity measurements

For measurement of AChE activity, the brains were homogenized in 150 μl of ice-cold phosphate buffered saline (PBS; Biowest, Nuaille, France: L0615) by passing the brain tissue through a 1 ml, 26 gauge syringe, and then centrifuged (Himac CT15E, VWR Hitachi, Darmstadt, Germany) at 10,000 ×g for 10 min at +4 °C. Supernatants were collected and total protein amounts were determined by using a Bradford reagent (Sigma, St. Louis, MO, USA: B6916) and bovine serum albumin (BSA) as the standard. Samples were subjected to an AChE Assay (Abcam, Cambridge, UK: ab138871) using the manufacturer's instructions. The range of the standard concentrations was between 1000 mU/ml – 1 mU/ml. From the brain samples, 40 μg of total protein was used for each reaction and each test sample and standards were loaded in duplicates. The absorbance of the samples and the standards were measured with a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The optical densities (ODs) were measured at 410 nm and means of the plate blanks were subtracted from each value. Linear fitting was used between the ODs of the standards and their known concentrations to calculate the concentrations of the test samples.

Table 1
Primary and secondary antibodies utilized in the Western blot experiments.

Antigen	Supplier	Cat#	Use	Dilution	Total protein loading amount into the gel
PSD95	Abcam	ab18258	Primary	1:5000	15 μg
SYP	Abcam	ab32594	Primary	1:10,000	15 μg
GEP	Abcam	ab185930	Primary	1:2500	30 μg
GABA-A-a1	Abcam	ab211131	Primary	1:1000	30 μg
GluR2/3	LSBio	LS-C15368	Primary	1:1000	15 μg
NR2B	LSBio	LS-C25797	Primary	1:1000	30 μg
GFAP	Abcam	ab53554	Primary	1:2000	30 μg
DCAMKL1	Abcam	ab109029	Primary	1:1000	30 μg
HuC	Abcam	ab78467	Primary	1:2000	30 μg
PCNA	Abcam	ab18197	Primary	1:1000	30 μg
β-Tubulin	CST	#2146	Primary-Housekeeping	1:5000	15–30 μg
Rabbit-HRP	CST	#7074	Secondary	1:5000	–
Goat-HRP	Abcam	ab97100	Secondary	1:10,000	–

PSD95: Post-Synaptic Density 95; SYP: Synaptophysin; GEP: Gephyrin; GABA-A-a1: Gamma-Aminobutyric acid type A alpha 1 subunit; GluR2/3: Glutamate Receptor 2 and 3; NR2B: *N*-methyl D-aspartate receptor-type subunit 2B; GFAP: Glial fibrillary acidic protein; DCAMKL1: Doublecortin like kinase 1; HuC: Embryonic lethal abnormal vision (ELAV; Drosophila) like 3; PCNA: Proliferating cell nuclear antigen; HRP: horseradish peroxidase.

2.7. Brain reactive oxygen species (ROS) levels measurements

Homogenates in PBS used for the AChE assay were also tested to determine ROS levels. The 2′,7′-Dichlorodihydrofluorescein diacetate (DCF), a fluorescent probe, was utilized to detect the content of the free radicals. The protocol for this assay was followed as described previously (Marcon et al., 2018). Briefly, 25 μl of supernatants were mixed with 5 μl of 1 mM DCF (Sigma, St. Louis, MO, USA: D6883) in a 96-well plate with a final reaction volume of 200 μl. Plates were incubated at 37 °C for 30 min, then the fluorescent wavelength of the plates was read with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) with a 488 nm excitation and 525 nm emission. The results are given as relative fluorescence units.

2.8. Brain lipid peroxidation levels assessments

To investigate brain lipid peroxidation levels across the comparison groups, a commercially available kit detecting the malondialdehyde (MDA) content, which is a reactive molecule and produced when there is a lipid peroxidation, was utilized (Abcam, Cambridge, UK: ab118970) following manufacturer's instructions. Briefly, snap frozen brains were homogenized in 303 μl of MDA lysis buffer by passing the brain tissue through a 1 ml, 26 gauge syringe. Homogenates were centrifuged at 13,000 ×*g* for 10 min at 4 °C, and then supernatants were collected and total protein amounts were assessed with a Bradford reagent (Sigma, St. Louis, MO, USA: B6916). From the supernatants and standards a volume of 200 μl was mixed with 600 μl of TBA solution and incubated at 95 °C for 60 min, following that period samples were placed on ice for 10 min. From these reactions, 200 μl of the samples and standards were loaded into a 96 well plate in duplicates and optical densities (ODs) were measured at 532 nm with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.9. Statistical analysis

Statistical analyses were conducted using SPSS 19 (IBM, Istanbul, Turkey). Assumptions of normality including normal distribution and homogeneity of variance were checked with both Shapiro-Wilk and Levene's tests, respectively. In the cases where the assumptions of normality were fulfilled, a three-way ANOVA was carried out with the factors of environment with two levels (enriched and barren), age with two levels (young and old), and gender with two levels (male and female) to determine their effects on body parameters, protein expression levels, ROS and AChE activity and lipid peroxidation measurements. Significance levels in all cases were set to $p < 0.05$. In the cases of significant main effects or interactions, Bonferroni post-hoc comparison and simple effects analyses followed by ANOVA were performed. In the situations where the assumptions of normality were violated, the non-parametric Kruskal-Wallis and Mann Whitney *U* tests were utilized with *p* values adjusted for the number of comparisons, which make them more stringent. These are the non-parametric equivalents of the parametric tests. GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used for the generations of the graphs illustrated in the following figures.

3. Results

3.1. Environmental enrichment increases brain weight in an age-dependent manner without inducing changes in body weight parameters

Body mass index (BMI) measurements were not significantly affected by age ($\chi^2(1) = 0.003, p = 0.954$, Fig. 2A) due to the fact that both length and weight measurements, which are used to calculate the BMI, were increasing with the age of the animal. On the other hand, effect of gender was statistically significant ($\chi^2(1) = 63.253, p < 0.0005$, Fig. 2A) with females having higher BMIs as compared to males and this difference was significant across all ages and environmental treatment groups (barren young: $p < 0.0005$; barren old: $p < 0.0005$; enriched young: $p = 0.003$; enriched old: $p = 0.001$). Environmental enrichment (EE) did not alter the BMI significantly ($\chi^2(1) = 0.901$, $p = 0.343$, Fig. 2A), which implies that this intervention does not lead to changes in feeding behavior or overall locomotor activity, both of which could influence the BMI. Moreover, in a pilot study we have analyzed overall locomotor activity including the swimming speed and distance moved of animals from both the barren environmental condition as compared to enriched environment. From these video analyses no significant locomotor activity change between the environmental conditions was observed. This suggests that there is no altered physical activity depending on the environmental condition (Supp. Table 1 and Video 1).

Brain weight measurements showed a significant main effect of age ($F(1,85) = 129.579$, $p < 0.0005$, Fig. 2B), with old zebrafish from both the barren and enriched environments having significantly higher brain weight as compared to their young counterparts (barren: $p < 0.0005$, enriched: $p < 0.0005$). Additionally, a main effect of gender was also found to be significant ($F(1,85) = 42.622$, $p < 0.0005$, Fig. 2B), with males having higher brain weights compared to females and pairwise comparisons indicated that this gender difference is significant in the young barren ($p = 0.004$), young enriched ($p = 0.003$), old barren ($p = 0.002$) and old enriched ($p < 0.0005$) zebrafish. The effect of environment was marginally significant ($F(1,85) = 3.782$, $p = 0.055$) with enriched animals tending to show increased brain weight as compared to animals in the barren group. Interestingly, a significant age by environment interaction was observed in the brain weight measurements ($F(1,85) = 6.594$, $p = 0.012$, Fig. 2B). While there was no change in the young groups, old animals in the enriched condition have significantly increased brain weight in relation to old subjects in the barren environment condition ($p = 0.002$). Interactions between the other factors were not found to be significant (age by gender: $F(1,85) = 0.422$, $p = 0.518$; gender by environment: $F(1,85) = 0.135$, $p = 0.714$; age by gender by environment: $F(1,85) = 0.116$, $p = 0.734$). These results indicate that EE is likely exerting its effect on the brain in an age-dependent manner without altering the overall body parameters.

3.2. Quantitative Western blot analyses shows that post-mitotic neuronal, presynaptic, excitatory and inhibitory proteins are differentially altered by environmental enrichment in an age-dependent manner with no robust changes in glial and proliferation markers

In order to reveal the effects of EE and its possible interactions with age and gender on specific cellular populations and proliferation dynamics, the levels of two neuronal markers of embryonic lethal, abnormal vision (ELAV; Drosophila) like 3 (HuC) and doublecortin like kinase 1 (DCAMKL1), a glial marker of glial fibrillary acidic protein (GFAP), and a global proliferation marker, proliferating cell nuclear antigen (PCNA), were analyzed (Fig. 3). Moreover, these interventions can also change the synaptic dynamics in the absence of cellular or proliferative alterations. For this reason, synaptic protein levels were also investigated (Fig. 3). Synaptophysin (SYP) was assessed as an indicator of presynaptic integrity. Post-synaptic density 95 (PSD95), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor subunits 2 and 3 (GluR2/3), and *N*-methyl D-aspartate (NMDA)-type receptor subunit 2B (NR2B) were used to determine any effects on elements of excitatory neurotransmission. Finally, the components of inhibitory neurotransmission, gephyrin (GEP) and gamma-aminobutyric acid (GABA) receptor subunit alpha-1 (GABA-A-a1) were examined. As a housekeeping control protein, β-tubulin (TUB) was utilized for further normalization. TUB has been previously used for normalization by our laboratory in which we observed no statistical differences with respect to age, gender and environmental interventions such as diet (Celebi-Birand et al., 2020; Karoglu et al., 2017; Tuz-Sasik et al., 2020). To examine the quantitative differences of the brain protein levels, protein lysates from young and old, male and female brains

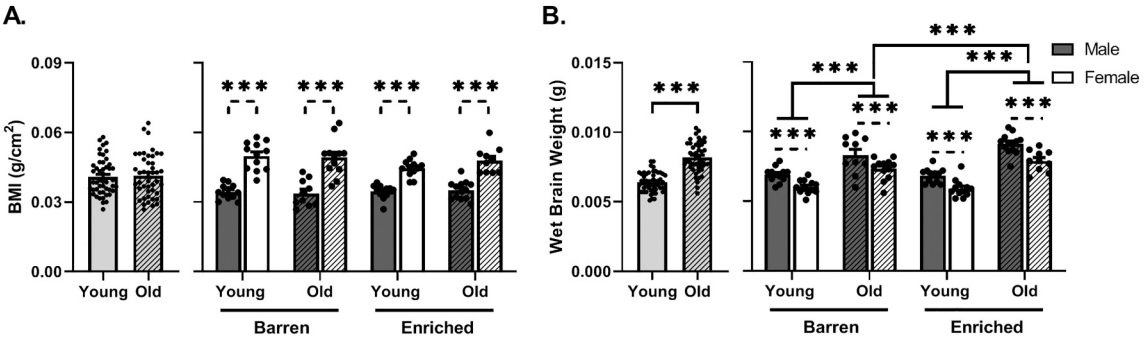

Fig. 2. Effects of environmental enrichment on BMI and brain weight. (A) BMI values significantly increased in female subjects as compared to males but there was no effect of age or environment. (B) Brain weight of zebrafish increased significantly with aging, and males had significantly higher brain weights as compared to females. A significant age by environment interaction indicates that the effect of EE is significant in old animals, which have significantly increased brain weight compared to old subjects in the barren environment. The continuous lines indicate significant differences between age and environment groups, dashed lines represent significant changes between gender groups, ***: $p < 0.005$, Error bars = +SE.

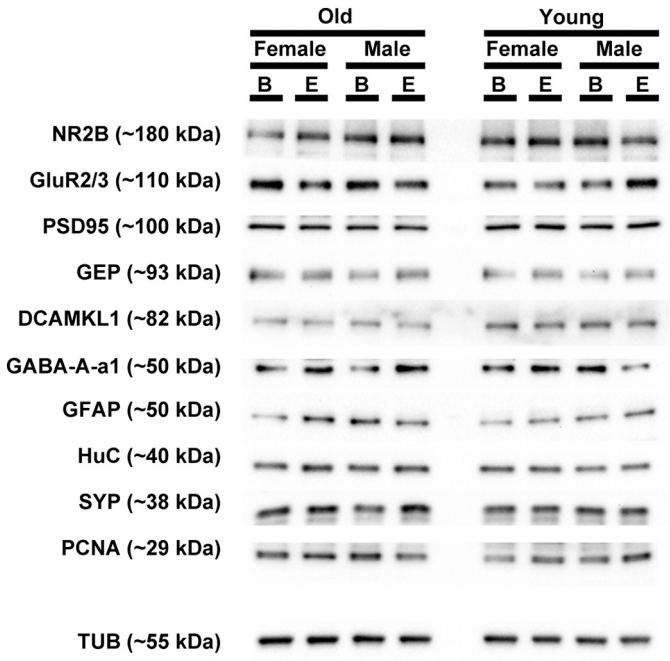

Fig. 3. Representative images from one cohort for Western blot analysis of synaptic, neuronal, glial and proliferative protein levels investigated in the present study for effects of age, environment and gender. All antibodies yielded bands at the expected molecular weights. B: Barren Environment; E: Enriched Environment.

from each environmental condition were run in cohorts in the same gel as shown in Fig. 3.

3.2.1. *Environmental enrichment prevents age-related declines in post-mitotic neuronal protein levels, while early-differentiated neuronal, glial and proliferative markers are not prominently affected*

Alterations in the protein levels of neuronal markers were investigated with respect to EE. An early-differentiated neuronal marker, HuC (Kim et al., 1996) was not significantly altered by the factors of environment, age and gender or their interactions (environment: $F(1,24) = 0.041$, $p = 0.841$; age: $F(1,24) = 0.015$, $p = 0.904$; gender: $F(1,24) = 0.135$, $p = 0.716$; age by gender: $F(1,24) = 0.029$, $p = 0.866$; age by environment: $F(1,24) = 1.119$, $p = 0.301$; gender by environment: $F(1,24) = 0.008$, $p = 0.930$; Age by gender by environment: $F(1,24) = 0.0001$, $p = 0.988$, Fig. 4A). DCAMKL1 was analyzed as a post-mitotic neuronal marker (Shin et al., 2013). A significant main effect of age was revealed on the DCAMKL1 levels, older zebrafish had significantly declined levels of DCAMKL1 ($F(1,24) = 6.206$, $p = 0.020$, Fig. 4B). Pairwise comparisons indicated a significant age-dependent reduction in barren environmental condition ($p = 0.038$), while no significant decline was observed in the subjects exposed to the enriched environment ($p = 0.199$). The main effects of environment and gender or any interaction among the factors were not statistically significant on DCAMKL1 levels (environment: $F(1,24) = 3.589$, $p = 0.070$; gender: $F(1,24) = 0.052$, $p = 0.822$; age by gender: $F(1,24) = 0.004$, $p = 0.950$; age by environment: $F(1,24) = 0.388$, $p = 0.539$; gender by environment: $F(1,24) = 0.638$, $p = 0.432$; age by gender by environment: $F(1,24) = 0.002$, $p = 0.962$, Fig. 4B). These data suggest that EE does not significantly alter an early-differentiated neuronal marker but has implications on preventing age-related declines in the levels of a post-mitotic neuronal marker.

A glia-specific marker, GFAP, was also assessed in order to gain insights about glial populations that might be affected by EE, age and gender (Middeldorp and Hol, 2011). Apart from the glial population, GFAP expression can be found in progenitor cells, which give rise to adult neurogenesis in the brain (Garcia et al., 2004). The effects of age, environment and gender were not statistically significant on GFAP protein levels (age: $\chi^2(1) = 0.688$, $p = 0.407$; environment: $\chi^2(1) = 1.945$, $p = 0.163$; gender: $\chi^2(1) = 0.818$, $p = 0.366$, Fig. 4C). Additionally, PCNA was examined as a marker of global cellular proliferation (Edelmann et al., 2013) with respect to environment, age and gender. The main effects of age, environment and gender, as well as their interactions was not statistically significant (age: $F(1,24) = 0.533$, $p = 0.472$; environment: $F(1,24) = 3.740$, $p = 0.065$; gender: $F(1,24) = 0.120$, $p = 0.732$; age by gender: $F(1,24) = 0.053$, $p = 0.820$; age by environment: $F(1,24) = 0.974$, $p = 0.334$; gender by environment: $F(1,24) = 0.246$, $p = 0.625$; age by gender by environment: $F(1,24) = 0.793$, $p = 0.382$, Fig. 4D). Overall, no significant differences were observed in the levels of glial and proliferative markers with EE.

3.2.2. *Declining levels of a presynaptic marker and an NMDA receptor subunit are prevented and levels of an inhibitory scaffolding protein are elevated with environmental enrichment in old animals*

Although no robust changes were observed on the cellular markers, subtle synaptic alterations may still be observed in the brain, which could explain potential ameliorative effects of EE during brain aging. Synaptophysin (SYP) was investigated as an indicator of overall presynaptic integrity (Smith et al., 2000). A significant main effect of environment was shown on SYP protein levels ($F(1,24) = 6.706$, $p = 0.016$) with zebrafish in the enriched environment having higher levels of SYP compared to the barren environment (Fig. 5A). Additionally, a significant main effect of age was demonstrated ($F(1,24) = 11.351$, $p = 0.003$) with SYP declining at old age compared to young. More

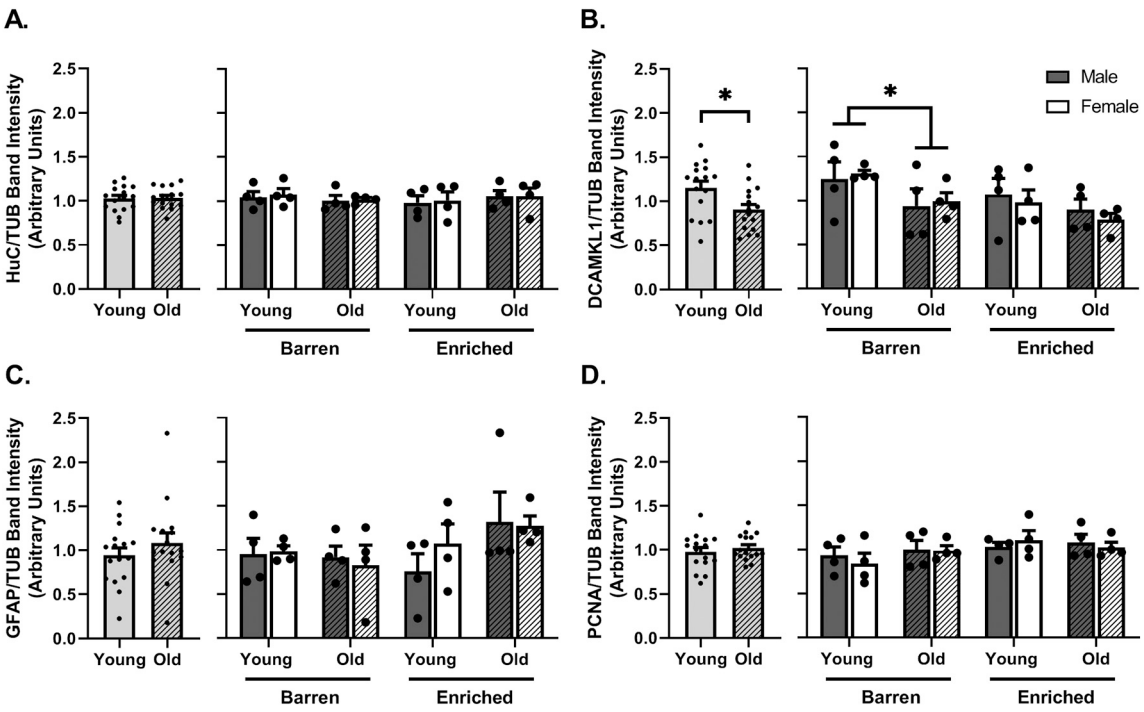

Fig. 4. Quantification of the levels of neuronal, glial and global cell proliferation markers following environmental enrichment. (A) The levels of neuronal marker HuC were not altered by age, environment and gender significantly. (B) DCAMKL1 a post-mitotic neuronal marker showed a significant decline with aging and age-related decrease was significant in barren environment. (C) GFAP, a glial marker and (D) PCNA, a global proliferation marker were not significantly changed by age, environment and gender. The continuous lines indicate significant differences between age and environment groups, *: $p < 0.05$, Error bars = +SE.

importantly, a significant age by environment interaction was revealed ($F(1,24) = 4.362$, $p = 0.048$), and this indicates that the effect of age is dependent on the environmental condition to which the animals are subjected (Fig. 5A). Pairwise comparisons demonstrated that age has an effect on animals in the barren environment and SYP levels declined significantly in old zebrafish from the barren environment as compared to the young group in barren condition ($p = 0.001$). Moreover, exposure to an enriched environment led to significant differences at old age, SYP levels are significantly higher in the old enriched animals as compared to old subjects in the barren environment ($p = 0.003$). In the SYP analysis gender effects or gender-dependent interactions were not statistically significant (gender: $F(1,24) = 0.0001$, $p = 0.990$; age by gender: $F(1,24) = 0.130$, $p = 0.722$; gender by environment: $F(1,24) = 0.110$, $p = 0.743$; age by gender by environment: $F(1,24) = 2.048$, $p = 0.165$). These results suggest that this presynaptic element can be altered by age and environment differentially and EE is maintaining the levels of SYP at older ages.

The protein levels of post-synaptic density-95 (PSD95), which is the main clustering and scaffolding protein binding directly to NMDA receptors and through Stargazin to AMPA receptors affecting excitatory function (Prange et al., 2004), were not affected by environment ($F(1,24) = 0.484$, $p = 0.493$), age ($F(1,24) = 0.019$, $p = 0.891$; Fig. 5B) or gender ($F(1,24) = 0.571$, $p = 0.457$; Fig. 5B). Also, there was no significant interaction among these factors with respect to PSD95 levels (age by gender: $F(1,24) = 0.429$, $p = 0.519$; age by environment: $F(1,24) = 0.642$, $p = 0.431$; gender by environment: $F(1,24) = 0.077$, $p = 0.784$; age by gender by environment: $F(1,24) = 0.870$, $p = 0.360$, Fig. 5B). Similarly, no significant main effect of environment ($F(1,24) = 0.259$, $p = 0.615$) or gender ($F(1, 24) = 1.232$, $p = 0.278$) was observed on the levels of the GluR2/3 subunits of AMPA receptors, which regulate calcium permeability and alters plasticity dynamics (Isaac et al., 2007). Interestingly, a significant main effect of age was revealed with respect to GluR2/3 ($F(1,24) = 8.736$, $p = 0.007$, Fig. 5C). This suggests that in older age groups levels of GluR2/3 declined significantly. Pairwise comparisons showed that the effect of age is significant in the enriched female animals, with young enriched females having significantly elevated levels of GluR2/3 as compared to old enriched female zebrafish ($p = 0.007$). Additionally, young females in the enriched environment had significantly higher levels of GluR2/3 compared to young enriched males ($p = 0.008$). The interactions between the factors when examining the GluR2/3 levels were not statistically significant (age by gender: $F(1,24) = 3.023$, $p = 0.095$; age by environment: $F(1,24) = 0.025$, $p = 0.876$; gender by environment: $F(1,24) = 3.428$, $p = 0.076$; age by gender by environment: $F(1,24) = 1.140$, $p = 0.296$). The protein levels of NR2B, which is one of the subunits of the NMDA receptor that is associated with increased synaptic plasticity (Clayton and Browning, 2001), demonstrated a significant main effect of gender ($F(1,24) = 6.294$, $p = 0.019$) with its levels increasing in females compared to males (Fig. 5D). Pairwise comparisons indicated that this gender difference was significant in old subjects exposed to an enriched environment ($p = 0.017$). Interestingly, a significant age by environment interaction was revealed with respect to NR2B ($F(1,24) = 6.450$, $p = 0.018$, Fig. 5D). Multiple comparisons showed that with advancing age NR2B levels declined significantly in subjects assigned to barren environmental conditions ($p = 0.005$), however, in the enriched environment there was no significant age-related decrease in the NR2B levels of the older subjects ($p = 0.641$). Moreover, no other significant main effects or interactions were observed with respect to NR2B levels (environment: $F(1,24) = 0.005$, $p = 0.946$; age: $F(1,24) = 3.505$, $p = 0.073$; age by gender: $F(1,24) = 3.324$, $p = 0.081$; gender by environment: $F(1,24) = 0.230$, $p = 0.636$; age by gender by environment: $F(1,24) = 1.592$, $p = 0.219$). These results imply that the plasticity-associated subunit of NMDA receptors, NR2B, can be altered in an age- and environment-dependent manner and that EE is associated with the stability and maintenance of the levels of NR2B at old age. Additionally, gender-dependent alterations were also observed in the elements comprising excitatory neurotransmission, NR2B and GluR2/3 subunit protein levels.

Gephyrin (GEP) is a scaffolding protein found at inhibitory GABAergic synapses and it clusters ionotropic $GABA_A$ receptors

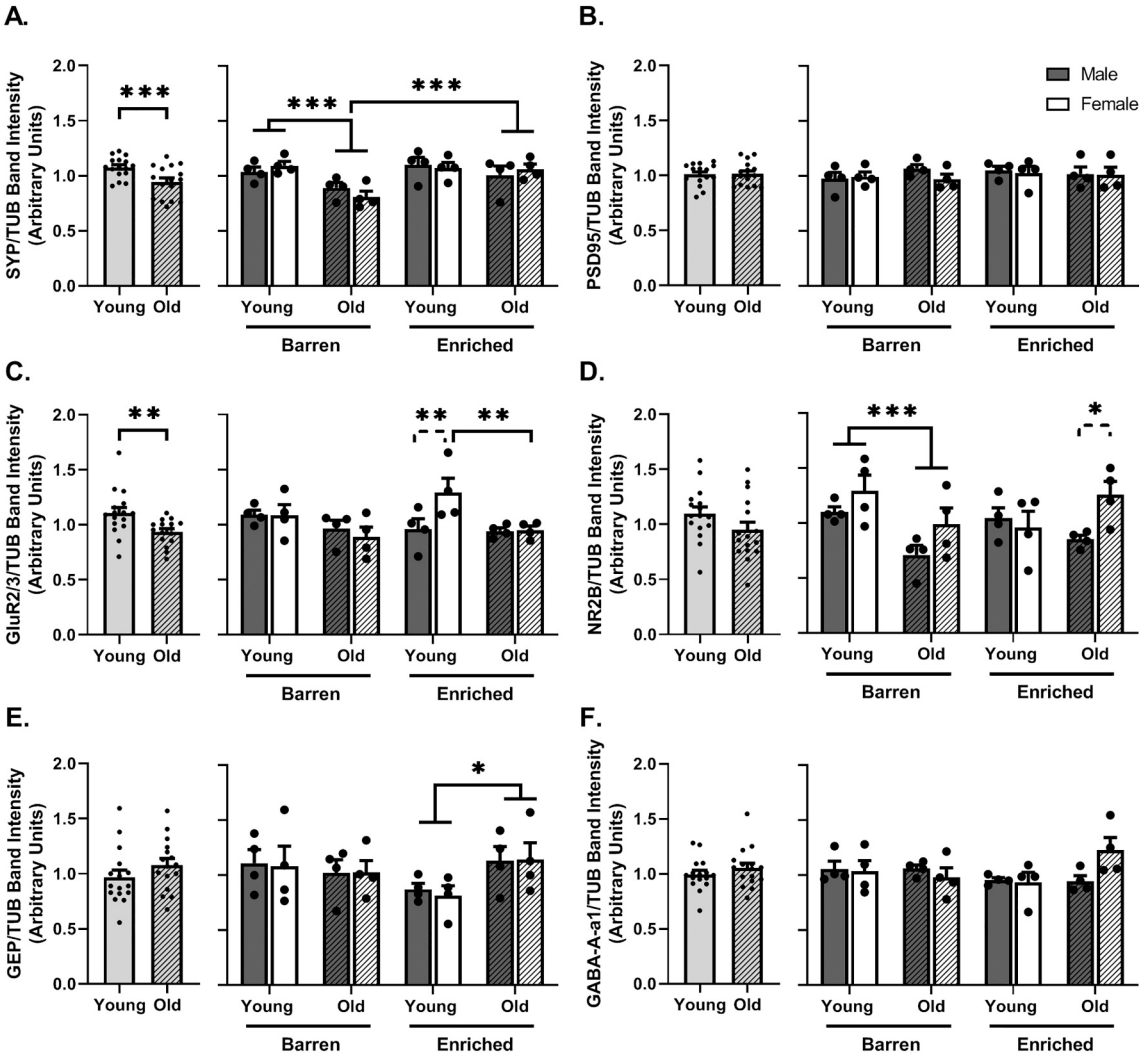

Fig. 5. Quantification of key synaptic protein levels following environmental enrichment. (A) SYP levels declined significantly with age in animals assigned to barren environmental condition. In contrast to this, there were no age-related changes in subjects with EE. Moreover, even the old zebrafish with EE have significantly higher levels of SYP compared to old animals in the barren environmental condition. (B) No age-, environment- and gender-dependent change was observed in PSD95 levels. (C) GluR2/3 levels were significantly altered by age, and gender-dependent alterations were observed in the enriched environment with young enriched females having significantly elevated levels of GluR2/3 as compared to young enriched males and old enriched females. (D) NR2B protein levels were altered by gender with females having significantly higher levels of NR2B compared to males and also a significant age by environment interaction was revealed. A significant decline in NR2B subunit levels were observed in the barren environmental condition with aging but exposure to an enriched environment prevented this decline and maintained NR2B levels with aging. (E) The protein levels of the inhibitory synaptic element, GEP, showed a significant age by environment interaction with its levels significantly increased in old enriched animals as compared to young enriched groups. (F) GABA-A-a1 was not changed significantly by the main effects of age, environment and gender. Continuous lines indicate significant differences between age and environment groups, dashed lines represent significant changes between gender groups, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$, Error bars = +SE.

(Tyagarajan and Fritschy, 2014). No significant main effect was found in GEP protein levels (environment: $F(1,24) = 0.613$, $p = 0.441$; age: $F(1,24) = 1.536$, $p = 0.227$; gender: $F(1,24) = 0.047$, $p = 0.831$). On the other hand, a significant age by environment interaction was revealed in GEP levels ($F(1,24) = 4.196$, $p = 0.05$, Fig. 5E). Post-hoc analyses showed that age was significantly altering the levels of GEP in the enriched environment, and old enriched animals had significantly elevated levels of GEP as compared to young enriched animals ($p = 0.029$). No further significant interaction was found in the levels of GEP (age by gender: $F(1,24) = 0.078$, $p = 0.782$; gender by environment: $F(1,24) = 0.004$, $p = 0.951$; age by gender by environment: $F(1,24) = 0.011$, $p = 0.917$, Fig. 5E). GABA-A-a1 is one of the major subunits of the ionotropic $GABA_A$ receptors and required for normal synaptic activity (Rissman and Mobley, 2011). In the case of GABA-A-a1, no significant main effect or interaction was observed (environment: $F(1,24) = 0.096$, $p = 0.759$; age: $F(1,24) = 1.071$, $p = 0.311$; gender: $F(1,24) = 0.456$, $p = 0.506$; age by gender: $F(1,24) = 1.233$, $p = 0.278$; age by environment: $F(1,24) = 2.277$, $p = 0.144$; gender by environment $F(1,24) = 2.672$, $p = 0.115$; age by gender by environment: $F(1,24) = 2.674$, $p = 0.115$ Fig. 5F). These data show that exposure to EE is associated with elevated levels of the inhibitory scaffolding protein GEP at older ages, which likely increases the resilience of animals against possible excitotoxic damage occurring with aging.

3.3. The activity of reactive oxygen species (ROS) and content of lipid peroxidation increases at older ages without evident effects of environmental interventions

Environment- age- and gender-mediated fluctuations in oxidative stress-related markers, which can alter the cellular and synaptic homeostasis, were assessed in the brain tissues. Acetylcholinesterase (AChE) is an element of cholinergic neurotransmission and it has non-

enzymatic neuro-modulatory effects on synaptogenesis and neurite outgrowth, both of which have significant implications for learning and memory (Bartus et al., 1982; La Torre, 1968; Zimmerman and Soreq, 2006). AChE can mediate oxidative stress-related processes differentially in both healthy and pathological brain conditions and its activity is decreased by the elevated levels of oxidative stress and free radicals (Haider et al., 2014; Melo et al., 2003). Additionally, two oxidative stress markers were investigated within the context of environment, age and gender. The first marker was ROS activity which is the indicator of free radical content. The other marker was malondialdehyde (MDA) content which shows how much lipid peroxidation results from the increased levels of ROS (Zhang et al., 2013).

The effects of environment, age and gender on AChE activity were not found to be statistically significant (environment: $\chi^2(1) = 0.427$, $p = 0.514$; age: $\chi^2(1) = 1.138$, $p = 0.286$; gender: $\chi^2(1) = 0.045$, $p = 0.832$, Fig. 6A). However, with respect to the ROS levels a significant main effect of age was observed ($\chi^2(1) = 6.554$, $p = 0.010$, Fig. 6B). At old ages, ROS activity levels were significantly elevated as compared to young age groups. Additionally, a significant main effect of gender on ROS activity ($\chi^2(1) = 7.815$, $p = 0.005$) was revealed with males having higher levels compared to females (Fig. 6B). Pairwise comparisons showed that the gender effect was significant in the enriched young group ($p = 0.033$), with young male animals having higher levels of ROS activity as compared to young females in the enriched environment. No significant environment-dependent modulations were observed in the ROS activity ($\chi^2(1) = 0.023$, $p = 0.879$). MDA levels, which are the indicators of lipid peroxidation, showed a significant main effect of age ($F(1,16) = 5.772$, $p = 0.029$, Fig. 6C), with old subjects having significantly elevated levels of MDA content as compared to young animals. Moreover, no other significant main effects or interactions were observed with respect to MDA content (environment: $F(1,16) = 0.073$, $p = 0.791$; gender: $F(1,16) = 0.014$, $p = 0.909$; age by gender: $F(1,16) = 0.001$, $p = 0.977$; age by environment: $F(1,16) = 0.149$, $p = 0.705$; gender by environment: $F(1,16) = 3.385$, $p = 0.084$; age by gender by environment: $F(1,16) = 0.275$, $p = 0.607$, Fig. 6C). Overall the data shows that the oxidative stress markers of ROS activity and lipid peroxidation content shared a similar significant age-dependent increasing pattern that was not reversed by environmental interventions.

4. Discussion

The current study demonstrated that a short-term environmental enrichment (EE) of 4 weeks, which utilizes the sensory components, did not lead to changes in the body mass indices (BMI) of the young and old animals, while at the older ages animals in the enriched environment had significantly increased brain weight as compared to their age-matched controls in the barren environment. This shows that EE exerts its effect on brain weight in an age-dependent manner without altering the overall body parameters such as BMI. One of the main aims of the current study was to demonstrate the pattern of global changes in the brain levels of neuronal, glial, proliferative and synaptic markers, as well as oxidative stress related markers, in response to an EE intervention within the context of aging and gender. Age-related declines were observed in the levels of post-mitotic neuronal marker doublecortin like kinase 1 (DCAMKL1), the presynaptic vesicle protein, synaptophysin (SYP) and excitatory post-synaptic protein subunit, *N*-methyl-D-aspartate (NMDA)-type receptor subunit 2B (NR2B) in animals of barren environmental conditions, and the current sensory EE intervention restored and maintained their levels in the old animals. Moreover, EE significantly promoted the levels of inhibitory scaffolding protein gephyrin (GEP) levels in old animals. In terms of the levels of the excitatory post-synaptic proteins, NR2B and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor subunits 2 and 3 (GluR2/3), small gender-driven effects were also observed with females having higher levels than males. Additionally, a significant increase at old age was observed in the levels of oxidative stress markers including reactive oxygen species (ROS) activity which was also altered by gender and lipid peroxidation content. Taken together, these data indicate the age-dependent positive effects of EE on the brain, with the most prominent being that EE increases brain weight at old age and restores any age-related declines in the levels of DCAMKL1, SYP and NR2B, as well as elevates GEP levels, and this may be done to protect against oxidative stress that increases in old subjects.

Body weight and length measurements of animals were utilized for

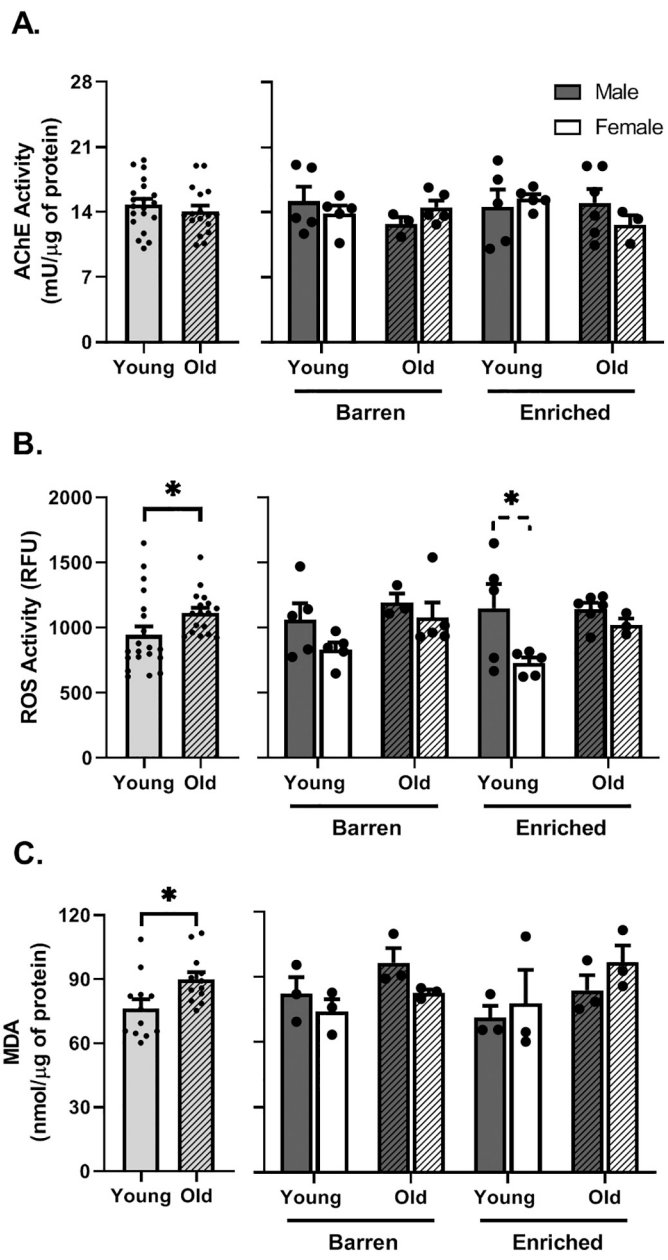

Fig. 6. The effects of environmental enrichment on oxidative stress related markers ACHE, ROS and MDA levels in the brain. (A) AChE activity did not show any significant changes with respect to age, gender and environment. (B) A significant effect of age was revealed in the ROS activity levels with old animals having significantly elevated levels of ROS activity compared to young groups. Gender-driven changes were observed in ROS activity with females having significantly lower ROS activity levels as compared to males. (C) MDA content indicating the lipid peroxidation was altered by age, with old animals having significantly higher MDA content as compared to young subjects. RFU: Relative fluorescence units. Continuous lines indicate significant differences between age and environment groups, dashed lines represent significant changes between gender groups, *: $p < 0.05$, Error bars = +SE.

the calculation of BMI, which was used to reflect possible alterations in feeding behavior or overall locomotor activity that might be induced by an environmental change. Previous studies utilizing EE have shown BMI changes and weight loss in rats exposed to an enriched environment (Pham et al., 1999). However, since these traditional EE paradigms were utilizing combined environmental components including sensory and physical exercise, environment-dependent reductions in body weight and BMI parameters would likely reflect increased physical activity. In the present study, EE was achieved through altering the sensory components without any forced physical exercise manipulations, and no differences were observed in the BMI values between the environmental conditions, which was also consistent with the previous findings obtained from young adult zebrafish (Lee et al., 2019). Additionally, an age effect was not observed on BMI levels, which is consistent with the literature, since the gain in body weight with aging is also driven by an age-related increase in the body length (Leibold and Hammerschmidt, 2015). Moreover, females had significantly higher BMIs as compared to males independent of the factors of age and environment. This observation is in good agreement with the zebrafish literature, as female animals have higher BMI values due to the fact that they constantly produce eggs and their ovaries can account for approximately 29% of the total body weight (Leibold and Hammerschmidt, 2015).

In addition to the BMI measurements, brain weights were also investigated among the various treatment groups. Earlier studies indicated that EE in different model organisms such as rat and mice can lead to morphological changes in the brain and increase its total weight (Bennett et al., 1969; La Torre, 1968; Rosenzweig et al., 1962). Marginal increases in the enriched groups were also evident in the present study utilizing the zebrafish model. Interestingly, it was observed that EE leads to significant increases in brain weight in the aged group. Additionally, increased brain weight values were observed just with aging, and this expansion is in parallel with the growth of the animal, and similar to previous studies that demonstrated both increases in body weight and length parameters as well as volumetric increases in the total brain during normal aging in the zebrafish (Arslan-Ergul et al., 2016; Celebi-Birand et al., 2020). Also, gender-specific changes were observed with males having higher brain weight measurements compared to females. Overall, the current data regarding the body parameters indicate that the effect of this short-term EE on the brain is exerted in an age-dependent manner without altering the overall body parameters.

One aim of the current study was to reveal possible changes in the global levels of the cellular markers in the brain with respect to EE, aging and gender. Two neuronal markers were studied. The first was embryonic lethal, abnormal vision (ELAV; Drosophila) like 3 (HuC), which is an early-differentiated neuronal marker, and the other is doublecortin like kinase 1 (DCAMKL1), which is a protein that shares high homology with doublecortin (DCX) and highly expressed in the zones of the active neurogenesis (Kim et al., 1996; Shu et al., 2006). Although, there is no direct measurement of HuC and DCAMKL1 within the context of an EE intervention, studies have shown that EE significantly increases the number of DCX-positive cells in both young and old animals (Leal-Galicia et al., 2008), and restores the declining levels of neurogenesis (Speisman et al., 2013). Similarly, our data indicated a significant age-related decline in the levels of DCAMKL1 but not in the old enriched animals. In the current study, EE prevented the age-related reduction in DCAMKL1 protein levels and maintained its levels across aging.

In addition to the neuronal markers, a glial, more specifically the astrocytic marker, glial fibrillary acidic protein (GFAP) and global proliferating cell nuclear antigen (PCNA), which is an endogenous marker of cell division and labels cycling progenitor cells (Edelmann et al., 2013), were further analyzed and compared between environmental conditions, as well as age and gender groups. No significant effect was observed on GFAP levels. Although our data indicated no significant differences, an increasing trend in the PCNA levels of the environmentally enriched group compared to barren controls was observed. Previously, in multiple model organisms it was shown that EE increases the number of PCNA-positive cells (Jeong et al., 2011; von Krogh et al., 2010; Young et al., 1999). These observations are in good agreement with our data, with an overall increasing trend with EE that was maintained, however, since we have investigated the global brain levels of GFAP and PCNA, region-specific significant changes might be masked. Future studies are being planned to examine the effects of region-specific changes in these markers. Additionally, any changes in protein levels assessed by Western blotting might not detect differences in total number but changes in the amount of protein per cell. Future studies are planning to address whether the total number and/or size of these cells change with age.

Another aim of the current study was to investigate the global changes in synaptic protein levels with respect to environment, age and gender. Synaptophysin (SYP) is a transmembrane glycoprotein that can be found in the synaptic vesicles and at the presynaptic membrane (Kwon and Chapman, 2011). Previous studies in which different model organisms were utilized have demonstrated that SYP levels declined significantly in the aging brain (Adams et al., 2008; Karoglu et al., 2017; Pinto et al., 2015; VanGuilder et al., 2010). Our data followed a similar overall age-related decrease in SYP levels, and this decline was more prominent in animals in the barren environment. Converging lines of evidence suggests that in mammalian models EE with or without physical exercise can increase the levels of SYP across different age groups (Barak et al., 2013; Frick and Fernandez, 2003; Lambert et al., 2005; Nithianantharajah et al., 2004; Saito et al., 2012). We observed a significant main effect of environment such that animals in the enriched groups have significantly higher levels of SYP, which is consistent with the previous work. Interestingly, we also have found a significant age by environment interaction in SYP levels. The data shows that its levels were maintained in old animals exposed to EE and these subjects have significantly higher levels of SYP compared to old fish in the barren environmental condition. Our results likely not only indicate that sensory EE is capable of increasing SYP levels but also this occurs in an age-dependent manner. This suggests that the more robust benefits of EE on presynaptic elements will be observed at older ages in which subtle molecular alterations underlying the biological underpinnings of cognitive decline occur in the brain, as compared to young groups in which the system is more intact and less vulnerable.

In order to understand alterations of the excitatory elements in neurotransmission, post-synaptic density 95 (PSD95), GluR2/3 and NR2B levels were assessed. PSD95 is the main clustering and scaffolding protein found in the excitatory synapses, and it anchors and localizes the main glutamatergic receptors alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl D-aspartate (NMDA) at the post-synaptic membrane (Prange et al., 2004). Our data indicated that no overall effect of environment, age and gender were observed in PSD95 levels. It was previously shown that traditional EE can elevate PSD95 levels in major brain regions (Nithianantharajah et al., 2004), but studies have also indicated that physical exercise can increase PSD95 levels (Dietrich et al., 2005; Hu et al., 2009). Therefore, the observed elevations with EE in the PSD95 levels in the literature may be associated with the exercise component of EE, and since our EE paradigm was utilizing sensory components this might be the reason why we did not observe environment-dependent changes in the PSD95 levels.

GluR2/3 are the predominant subunits of the AMPA-type glutamate receptors, and these subunits can regulate synaptic plasticity and efficacy by controlling the local calcium influx by changing the permeability of the membrane (Chetkovich et al., 2002). Our results indicated a significant age-related decline in GluR2/3 subunit levels which is consistent with previous work in which decreases in these receptor subunits were reported in mammalian models (Adams et al., 2008; Shi et al., 2007). Moreover, in the enriched environmental condition gender- and age-specific alterations were observed, with the enriched young female group having significantly elevated GluR2/3 levels compared to the enriched young males and enriched old females. EE may provide more benefits for the young female animals with respect to

GluR2/3 and elevated levels of this subunit have neuroprotective roles since the flow of calcium is limited and the levels of GluR2/3 decline in the cases of the increased oxidative stress (Carter et al., 2004; Kamat et al., 2016). In this respect ROS activity levels in the current study are complementary to the GluR2/3 levels, as we showed that in the enriched female young group there were increases in the GluR2/3 levels with accompanied decreases in the levels of ROS activity. Thus, in this group possible protective effects of this subunit were observed. Therefore, our data indicate that responsiveness to EE differs between the age and gender groups with respect to the GluR2/3 element of glutamatergic neurotransmission.

NR2B is one of the predominant subunits of the NMDA receptors in the brain, and its expression can regulate maturation, synaptic plasticity and enhancement of long-term potentiation (LTP) (Clayton and Browning, 2001; Philpot et al., 2001). Importantly we have observed that in the barren environmental condition, the NR2B subunit significantly declines with age, and this observation was consistent with the literature utilizing mammalian models of aging (Adams et al., 2008; Shi et al., 2007). It was also shown that application of EE prevents this age-related reduction of the NR2B levels and maintains the levels of this subunit at older ages, which likely increases the brain resilience of the animals (Cao et al., 2007). Additionally, a significant effect of gender was observed in our data showing that females have significantly higher NR2B levels compared to males. It was previously shown that sex hormones such as estradiol increase the expression of NR2B receptors and estradiol strengthens LTP mediated by changes in NR2B levels (Smith and McMahon, 2006). Consistent with those findings is the fact that female zebrafish have higher levels of estrogen compared to males (von Hofsten and Olsson, 2005), and this might modulate the increased expression of the NR2B levels in female animals.

Gephyrin (GEP) and the Gamma-Aminobutyric acid type A alpha 1 subunit (GABA-A-a1) were investigated to estimate the effects of EE within the context of aging and gender on inhibitory neurotransmission. GEP is a major scaffolding protein of inhibitory synapses and it clusters $GABA_A$ receptors (Tyagarajan and Fritschy, 2014). GABA-A-a1 is one of the major subunits of the $GABA_A$ receptors that forms a high-affinity binding site for GABA and its expression regulates resilience against extrinsic factors such as stress (Ardi et al., 2016). Studies have shown that GEP levels can decrease in specific brain regions with aging (Pinto et al., 2015), and in the current study an age by environment interaction was shown in the GEP levels with old animals in the enriched environment having significantly increased the levels of GEP as compared to their younger counterparts. In the EE group an age-specific elevation of GEP may manifest as a higher compensation against excitotoxic damage that could occur with aging and increase the resilience of subjects. Consistent with this hypothesis, in pathological aging conditions increased excitotoxic damage lowers the GEP levels (Agarwal et al., 2008). GABA-A-a1 levels were relatively stable with respect to factors of age and environment, previous studies indicated GABA-A-a1 levels tend to be stable across different age groups (Palpagama et al., 2019).

Oxidative stress-related markers were further investigated since increased oxidative damage can lead to disruptions in the local calcium dynamics, synaptic degeneration and alterations in key synaptic and cellular proteins and is modulated by age and environmental interventions (Mattson and Liu, 2002). ROS activity and the content of malondialdehyde (MDA), which is a highly reactive component produced by lipid peroxidation, were evaluated as oxidative stress markers. Our results indicated significant elevations of both ROS activity and lipid peroxidation at old ages, these observations were in conjunction with previous studies utilizing the mammalian models (for review see Mattson and Magnus, 2006). However, in the current study EE did not modulate these detrimental markers of the oxidative stress. It has been shown that short-term EE can reduce ROS activity after an exposure to factors such as stress and brain insults (Cechetti et al., 2012; Marcon et al., 2018). However, studies have demonstrated that ROS activity levels tend to be stable between enriched and control environments when an additional stressor is not applied (Marcon et al., 2018; Prado Lima et al., 2018). Our data showed similar patterns in parallel with these recent findings since no additional stressor like unpredictable chronic stress or induction of a brain insult was introduced within our EE setup (Marcon et al., 2018; Prado Lima et al., 2018). Interestingly, we have observed a significant main effect of gender on ROS activity levels with males having higher levels of ROS activity compared to females. It has been reported that gender-dependent susceptibilities to oxidative stress have been found in the brain, and females are more resistant to oxidative stress through sex hormone-mediated regulatory pathways (Giordano et al., 2013). Although, the effect of age had similar impacts on the oxidative stress markers ROS and lipid peroxidation, no gender-driven change was observed with respect to MDA content. In a recent study it was demonstrated that different oxidative stress markers are differentially altered by age and gender (Pinchuk et al., 2019).

Acetylcholinesterase (AChE) activity levels were determined to investigate any possible changes in the cholinergic system since this enzyme is differentially altered by increased oxidative stress in both normal aging and pathological conditions. In normal aging, increased oxidative stress markers are generally associated with lower levels of AChE, while in the pathological conditions amyloid plaque formation can lead to aberrant increases in AChE levels (Haider et al., 2014; Melo et al., 2003). Our data showed no significant age- or environment-driven changes in the AChE activity. Pioneering studies in the EE literature have shown that EE can increase the AChE activity levels as compared to control groups maintained in isolation (Bennett et al., 1964; La Torre, 1968). Another recent study has compared the effects of short-term sensory EE with a control group in which the animals were maintained in groups rather than isolation showed no significant changes in hippocampal AChE levels (Prado Lima et al., 2018). Likewise, our AChE data did not show any environment-dependent alterations and this observation might result from our control group, which was also maintained in groups and their social hierarchy to eliminate isolation stress.

5. Conclusion

Previous studies along with the current work have shown that environmental enrichment (EE) leads to age-dependent changes in the brain and these subtle molecular alterations are associated with increased brain resilience. The current study focused on sensory EE and our data have shown that short-term EE increases the brain weight in old animals without affecting general body parameters like BMI. Key cellular elements including doublecortin like kinase 1, and synaptic elements such as synaptophysin and NMDA-type receptor subunit NR2B declined with aging in barren environmental conditions and EE reverses these age-related declines and lead to maintenance of the levels of these proteins at older ages. Moreover, the results from EE showed gender-driven patterns, as shown by its differential effects on AMPA-type glutamate receptor subunits 2 and 3 and NMDA-type receptor subunit NR2B. Age-related elevations were shown in the levels of oxidative stress markers, but EE did not alter this trend. The current study gives more insight about the effects of sensory EE on the brain with respect to factors of both age and gender in the zebrafish model. Finally, these data provide information about the biological underpinnings of successful aging achieved through EE and provide targets, which can be manipulated and used for future translational research, as well as help to establish further the zebrafish model for studying age-related changes with respect to EE.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2021.111346.

CRediT authorship contribution statement

Elif Tugce Karoglu-Eravsar: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Original Draft, Visualization.

Melek Umay Tuz-Sasik: Formal Analysis, Investigation, Writing – Original Draft, Visualization. **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 competing interests.

Acknowledgements

The authors would like to thank Tulay Arayici for excellent technical assistance with the experiments in the zebrafish facility and Dursun Emre Karoglu for his help with preparation of figures.

This work was supported by an EMBO Installation Grant (Funds provided by TUBITAK) to Michelle M. Adams. The authors ETK-E and MUT-S were supported by the TUBITAK 2211 – National Graduate Scholarship Programme (BIDEB).

References

Adams, M.M., Smith, T.D., Moga, D., Gallagher, M., Wang, Y., Wolfe, B.B., Rapp, P.R., Morrison, J.H., 2001. Hippocampal dependent learning ability correlates with N-methyl-D-aspartate (NMDA) receptor levels in CA3 neurons of young and aged rats. J. Comp. Neurol. 432, 230–243. https://doi.org/10.1002/cne.1099.

Adams, M.M., Shi, L., Linville, M.C., Forbes, M.E., Long, A.B., Bennett, C., Newton, I.G., Carter, C.S., Sonntag, W.E., Riddle, D.R., Brunso-Bechtold, J.K., 2008. Caloric restriction and age affect synaptic proteins in hippocampal CA3 and spatial learning ability. Exp. Neurol. 211, 141–149. https://doi.org/10.1016/j.expneurol.2008.01.016.

Agarwal, S., Tannenberg, R.K., Dodd, P.R., 2008. Reduced expression of the inhibitory synapse scaffolding protein gephyrin in Alzheimer's disease. J. Alzheimers Dis. 14, 313–321. https://doi.org/10.3233/jad-2008-14305.

Ardi, Z., Albrecht, A., Richter-Levin, A., Saha, R., Richter-Levin, G., 2016. Behavioral profiling as a translational approach in an animal model of posttraumatic stress disorder. Neurobiol. Dis. 88, 139–147. https://doi.org/10.1016/j.nbd.2016.01.012.

Arslan-Ergul, A., Adams, M.M., 2014. Gene expression changes in aging Zebrafish (Danio rerio) brains are sexually dimorphic. BMC Neurosci. 15, 29 https://doi.org/10.1186/1471-2202-15-29.

Arslan-Ergul, A., Erbaba, B., Karoglu, E.T., Halim, D.O., Adams, M.M., 2016. Short-term dietary restriction in old zebrafish changes cell senescence mechanisms. Neuroscience 334, 64–75. https://doi.org/10.1016/j.neuroscience.2016.07.033.

Barak, B., Shvarts-Serebro, I., Modai, S., Gilam, A., Okun, E., Michaelson, D.M., Mattson, M.P., Shomron, N., Ashery, U., 2013. Opposing actions of environmental enrichment and Alzheimer's disease on the expression of hippocampal microRNAs in mouse models. Transl. Psychiatry 3, e304. https://doi.org/10.1038/tp.2013.77.

Bartus, R.T., Dean 3rd, R.L., Beer, B., Lippa, A.S., 1982. The cholinergic hypothesis of geriatric memory dysfunction. Science (80-.) 217, 408–414.

Bennett, E.L., Diamond, M.C., Krech, D., Rosenzweig, M.R., 1964. Chemical and anatomical plasticity of brain. Science (80-.) 146, 610 LP–619. https://doi.org/10.1126/science.146.3644.610.

Bennett, E.L., Rosenzweig, M.R., Diamond, M.C., 1969. Rat brain: effects of environmental enrichment on wet and dry weights. Science (80-.) 163, 825 LP–826. https://doi.org/10.1126/science.163.3869.825.

Bennett, J.C., McRae, P.A., Levy, L.J., Frick, K.M., 2006. Long-term continuous, but not daily, environmental enrichment reduces spatial memory decline in aged male mice. Neurobiol. Learn. Mem. 85, 139–152. https://doi.org/10.1016/j.nlm.2005.09.003.

Cao, X., Cui, Z., Feng, R., Tang, Y.-P., Qin, Z., Mei, B., Tsien, J.Z., 2007. Maintenance of superior learning and memory function in NR2B transgenic mice during ageing. Eur. J. Neurosci. 25, 1815–1822. https://doi.org/10.1111/j.1460-9568.2007.05431.x.

Carter, T.L., Rissman, R.A., Mishizen-Eberz, A.J., Wolfe, B.B., Hamilton, R.L., Gandy, S., Armstrong, D.M., 2004. Differential preservation of AMPA receptor subunits in the hippocampi of Alzheimer's disease patients according to Braak stage. Exp. Neurol. 187, 299–309. https://doi.org/10.1016/j.expneurol.2003.12.010.

Cechetti, F., Worm, P.V., Lovatel, G., Moysés, F., Siqueira, I.R., Netto, C.A., 2012. Environmental enrichment prevents behavioral deficits and oxidative stress caused by chronic cerebral hypoperfusion in the rat. Life Sci. 91, 29–36. https://doi.org/10.1016/j.lfs.2012.05.013.

Celebi-Birand, D., Ardic, N.I., Karoglu-Eravsar, E.T., Sengul, G.F., Kafaligonul, H., Adams, M.M., 2020. Dietary and pharmacological interventions that inhibit mammalian target of rapamycin activity alter the brain expression levels of neurogenic and glial markers in an age-and treatment-dependent manner. Rejuvenation Res. XX, 1–13. https://doi.org/10.1089/rej.2019.2297.

Chetkovich, D.M., Chen, L., Stocker, T.J., Nicoll, R.A., Bredt, D.S., 2002. Phosphorylation of the postsynaptic density-95 (PSD-95)/discs large/zona occludens-1 binding site of stargazin regulates binding to PSD-95 and synaptic targeting of AMPA receptors. J. Neurosci. 22, 5791–5796. https://doi.org/10.1523/JNEUROSCI.22-14-05791.2002.

Clayton, D.A., Browning, M.D., 2001. Deficits in the expression of the NR2B subunit in the hippocampus of aged fisher 344 rats. Neurobiol. Aging 22, 165–168. https://doi.org/10.1016/S0197-4580(00)00196-2.

Clemenson, G.D., Deng, W., Gage, F.H., 2015. Environmental enrichment and neurogenesis: from mice to humans. Curr. Opin. Behav. Sci. 4, 56–62. https://doi.org/10.1016/j.cobeha.2015.02.005.

Dietrich, M.O., Mantese, C.E., Porciuncula, L.O., Ghisleni, G., Vinade, L., Souza, D.O., Portela, L.V., 2005. Exercise affects glutamate receptors in postsynaptic densities from cortical mice brain. Brain Res. 1065, 20–25. https://doi.org/10.1016/j.brainres.2005.09.038.

Edelmann, K., Glashauser, L., Sprungala, S., Hesl, B., Fritschle, M., Ninkovic, J., Godinho, L., Chapouton, P., 2013. Increased radial glia quiescence, decreased reactivation upon injury and unaltered neuroblast behavior underlie decreased neurogenesis in the aging zebrafish telencephalon. J. Comp. Neurol. 521, 3099–3115. https://doi.org/10.1002/cne.23347.

Frick, K.M., Fernandez, S.M., 2003. Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. Neurobiol. Aging 24, 615–626.

Gamberger, D., Lavrac, N., Srivatsa, S., Tanzi, R.E., Doraiswamy, P.M., 2017. Identification of clusters of rapid and slow decliners among subjects at risk for Alzheimer's disease. Sci. Rep. 7, 6763 https://doi.org/10.1038/s41598-017-06624-y.

Garcia, A.D.R., Doan, N.B., Imura, T., Bush, T.G., Sofroniew, M.V., 2004. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat. Neurosci. 7, 1233–1241. https://doi.org/10.1038/nn1340.

Giordano, G., Tait, L., Furlong, C.E., Cole, T.B., Kavanagh, T.J., Costa, L.G., 2013. Gender differences in brain susceptibility to oxidative stress are mediated by levels of paraoxonase-2 expression. Free Radic. Biol. Med. 58, 98–108. https://doi.org/10.1016/j.freeradbiomed.2013.01.019.

Grimm, A., Eckert, A., 2017. Brain aging and neurodegeneration: from a mitochondrial point of view. J. Neurochem. 143, 418–431. https://doi.org/10.1111/jnc.14037.

Gur, R.E., Gur, R.C., 2002. Gender differences in aging: cognition, emotions, and neuroimaging studies. Dialogues Clin. Neurosci. 4, 197–210.

Haider, S., Saleem, S., Perveen, T., Tabassum, S., Batool, Z., Sadir, S., Liaquat, L., Madiha, S., 2014. Age-related learning and memory deficits in rats: role of altered brain neurotransmitters, acetylcholinesterase activity and changes in antioxidant defense system. Age (Omaha) 36, 9653. https://doi.org/10.1007/s11357-014-9653-0.

Hu, S., Ying, Z., Gomez-Pinilla, F., Frautschy, S.A., 2009. Exercise can increase small heat shock proteins (sHSP) and pre- and post-synaptic proteins in the hippocampus. Brain Res. 1249, 191–201. https://doi.org/10.1016/j.brainres.2008.10.054.

Isaac, J.T.R., Ashby, M.C., McBain, C.J., 2007. The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. Neuron 54, 859–871. https://doi.org/10.1016/j.neuron.2007.06.001.

Jeong, Y.H., Kim, J.M., Yoo, J., Lee, S.H., Kim, H.-S., Suh, Y.-H., 2011. Environmental enrichment compensates for the effects of stress on disease progression in Tg2576 mice, an Alzheimer's disease model. J. Neurochem. 119, 1282–1293. https://doi.org/10.1111/j.1471-4159.2011.07514.x.

Kamat, P.K., Kalani, A., Rai, S., Swarnkar, S., Tota, S., Nath, C., Tyagi, N., 2016. Mechanism of oxidative stress and synapse dysfunction in the pathogenesis of Alzheimer's disease: understanding the therapeutics strategies. Mol. Neurobiol. 53, 648–661. https://doi.org/10.1007/s12035-014-9053-6.

Karoglu, E.T., Halim, D.O., Erkaya, B., Altaytas, F., Arslan-Ergul, A., Konu, O., Adams, M. M., 2017. Aging alters the molecular dynamics of synapses in a sexually dimorphic pattern in zebrafish (Danio rerio). Neurobiol. Aging 54, 10–21. https://doi.org/10.1016/j.neurobiolaging.2017.02.007.

Kim, C.-H., Ueshima, E., Muraoka, O., Tanaka, H., Yeo, S.-Y., Huh, T.-L., Miki, N., 1996. Zebrafish elav/HuC homologue as a very early neuronal marker. Neurosci. Lett. 216, 109–112. https://doi.org/10.1016/0304-3940(96)13021-4.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310. https://doi.org/10.1002/aja.1002030302.

Kishi, S., Uchiyama, J., Baughman, A.M., Goto, T., Lin, M.C., Tsai, S.B., 2003. The zebrafish as a vertebrate model of functional aging and very gradual senescence. Exp. Gerontol. 38, 777–786.

Kwon, S.E., Chapman, E., 2011. Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. Neuron 70, 847–854. https://doi.org/10.1016/j.neuron.2011.04.001.

La Torre, J.C., 1968. Effect of differential environmental enrichment on brain weight and on acetylcholinesterase and cholinesterase activities in mice. Exp. Neurol. 22, 493–503. https://doi.org/10.1016/0014-4886(68)90144-1.

Lambert, T.J., Fernandez, S.M., Frick, K.M., 2005. Different types of environmental enrichment have discrepant effects on spatial memory and synaptophysin levels in female mice. Neurobiol. Learn. Mem. 83, 206–216. https://doi.org/10.1016/j.nlm.2004.12.001.

Leal-Galicia, P., Castañeda-Bueno, M., Quiroz-Baez, R., Arias, C., 2008. Long-term exposure to environmental enrichment since youth prevents recognition memory decline and increases synaptic plasticity markers in aging. Neurobiol. Learn. Mem. 90, 511–518. https://doi.org/10.1016/j.nlm.2008.07.005.

Lee, C.J., Paull, G.C., Tyler, C.R., 2019. Effects of environmental enrichment on survivorship, growth, sex ratio and behaviour in laboratory maintained zebrafish Danio rerio. J. Fish Biol. 94, 86–95. https://doi.org/10.1111/jfb.13865.

Leibold, S., Hammerschmidt, M., 2015. Long-term hyperphagia and caloric restriction caused by low- or high-density husbandry have differential effects on zebrafish postembryonic development, somatic growth, fat accumulation and reproduction. PLoS One 10, 1–31. https://doi.org/10.1371/journal.pone.0120776.

Manuel, R., Gorissen, M., Stokkermans, M., Zethof, J., Ebbesson, L.O.E., van de Vis, H., Flik, G., van den Bos, R., 2015. The effects of environmental enrichment and age-related differences on inhibitory avoidance in zebrafish (Danio rerio Hamilton). Zebrafish 12, 152–165. https://doi.org/10.1089/zeb.2014.1045.

Marcon, M., Mocelin, R., Benvenutti, R., Costa, T., Herrmann, A.P., de Oliveira, D.L., Koakoski, G., Barcellos, L.J.G., Piato, A., 2018. Environmental enrichment modulates the response to chronic stress in zebrafish. J. Exp. Biol. 221 https://doi.org/10.1242/jeb.176735.

Mattson, M.P., Liu, D., 2002. Energetics and oxidative stress in synaptic plasticity and neurodegenerative disorders. NeuroMolecular Med. 2, 215–231. https://doi.org/10.1385/NMM:2:2:215.

Mattson, M.P., Magnus, T., 2006. Ageing and neuronal vulnerability. Nat. Rev. Neurosci. 7, 278–294. https://doi.org/10.1038/nrn1886.

Mattson, M.P., Duan, W., Lee, J., Guo, Z., 2001. Suppression of brain aging and neurodegenerative disorders by dietary restriction and environmental enrichment: molecular mechanisms. Mech. Ageing Dev. 122, 757–778. https://doi.org/10.1016/S0047-6374(01)00226-3.

McCarrey, A.C., An, Y., Kitner-Triolo, M.H., Ferrucci, L., Resnick, S.M., 2016. Sex differences in cognitive trajectories in clinically normal older adults. Psychol. Aging 31, 166–175. https://doi.org/10.1037/pag0000070.

McDonald, M.W., Hayward, K.S., Rosbergen, I.C.M., Jeffers, M.S., Corbett, D., 2018. Is environmental enrichment ready for clinical application in human post-stroke rehabilitation? Front. Behav. Neurosci. 12, 135. https://doi.org/10.3389/fnbeh.2018.00135.

Melo, J.B., Agostinho, P., Oliveira, C.R., 2003. Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. Neurosci. Res. 45, 117–127. https://doi.org/10.1016/s0168-0102(02)00201-8.

Middeldorp, J., Hol, E.M., 2011. GFAP in health and disease. Prog. Neurobiol. 93, 421–443. https://doi.org/10.1016/j.pneurobio.2011.01.005.

Mora, F., Segovia, G., del Arco, A., 2007. Aging, plasticity and environmental enrichment: structural changes and neurotransmitter dynamics in several areas of the brain. Brain Res. Rev. 55, 78–88. https://doi.org/10.1016/j.brainresrev.2007.03.011.

Mora-Gallegos, A., Rojas-Carvajal, M., Salas, S., Saborío-Arce, A., Fornaguera-Trías, J., Brenes, J.C., 2015. Age-dependent effects of environmental enrichment on spatial memory and neurochemistry. Neurobiol. Learn. Mem. 118, 96–104. https://doi.org/10.1016/j.nlm.2014.11.012.

Nithianantharajah, J., Levis, H., Murphy, M., 2004. Environmental enrichment results in cortical and subcortical changes in levels of synaptophysin and PSD-95 proteins. Neurobiol. Learn. Mem. 81, 200–210. https://doi.org/10.1016/j.nlm.2004.02.002.

Palpagama, T.H., Sagniez, M., Kim, S., Waldvogel, H.J., Faull, R.L., Kwakowsky, A., 2019. GABA_a receptors are well preserved in the hippocampus of aged mice. eneuro 6. https://doi.org/10.1523/ENEURO.0496-18.2019. ENEURO.0496-18.2019.

Pavlidis, M., Digka, N., Theodoridi, A., Campo, A., Barsakis, K., Skouradakis, G., Samaras, A., Tsalafouta, A., 2013. Husbandry of zebrafish, Danio rerio, and the cortisol stress response. Zebrafish 10, 524–531. https://doi.org/10.1089/zeb.2012.0819.

Pham, T.M., Söderström, S., Winblad, B., Mohammed, A.H., 1999. Effects of environmental enrichment on cognitive function and hippocampal NGF in the non-handled rats. Behav. Brain Res. 103, 63–70. https://doi.org/10.1016/S0166-4328(99)00019-4.

Pham, T.M., Winblad, B., Granholm, A.-C., Mohammed, A.H., 2002. Environmental influences on brain neurotrophins in rats. Pharmacol. Biochem. Behav. 73, 167–175. https://doi.org/10.1016/S0091-3057(02)00783-9.

Philpot, B.D., Sekhar, A.K., Shouval, H.Z., Bear, M.F., 2001. Visual experience and deprivation bidirectionally modify the composition and function of NMDA receptors in visual cortex. Neuron 29, 157–169. https://doi.org/10.1016/S0896-6273(01)00187-8.

Pinchuk, I., Weber, D., Kochlik, B., Stuetz, W., Toussaint, O., Debacq-Chainiaux, F., Dollé, M.E.T., Jansen, E.H.J.M., Gonos, E.S., Sikora, E., Breusing, N., Gradinaru, D., Sindlinger, T., Moreno-Villanueva, M., Bürkle, A., Grune, T., Lichtenberg, D., 2019. Gender- and age-dependencies of oxidative stress, as detected based on the steady state concentrations of different biomarkers in the MARK-AGE study. Redox Biol. 24, 101204 https://doi.org/10.1016/j.redox.2019.101204.

Pinto, J.G.A., Jones, D.G., Williams, C.K., Murphy, K.M., 2015. Characterizing synaptic protein development in human visual cortex enables alignment of synaptic age with rat visual cortex. Front. Neural Circuits 9, 3. https://doi.org/10.3389/fncir.2015.00003.

Prado Lima, M.G., Schimidt, H.L., Garcia, A., Daré, L.R., Carpes, F.P., Izquierdo, I., Mello-Carpes, P.B., 2018. Environmental enrichment and exercise are better than social enrichment to reduce memory deficits in amyloid beta neurotoxicity. Proc. Natl. Acad. Sci. 115, E2403 LP–E2409. https://doi.org/10.1073/pnas.1718435115.

Prange, O., Wong, T.P., Gerrow, K., Wang, Y.T., El-Husseini, A., 2004. A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. Proc. Natl. Acad. Sci. U. S. A. 101, 13915–13920. https://doi.org/10.1073/pnas.0405939101.

Rissman, R.A., Mobley, W.C., 2011. Implications for treatment: GABAA receptors in aging, down syndrome and Alzheimer's disease. J. Neurochem. 117, 613–622. https://doi.org/10.1111/j.1471-4159.2011.07237.x.

Rosenzweig, M.R., Krech, D., Bennett, E.L., Diamond, M.C., 1962. Effects of environmental complexity and training on brain chemistry and anatomy: a replication and extension. J. Comp. Physiol. Psychol. 55, 429.

Saito, R., Smoot, M.E., Ono, K., Ruscheinski, J., Wang, P.-L., Lotia, S., Pico, A.R., Bader, G.D., Ideker, T., 2012. A travel guide to Cytoscape plugins. Nat. Methods 9. https://doi.org/10.1038/nmeth.2212.

Shi, L., Adams, M.M., Linville, M.C., Newton, I.G., Forbes, M.E., Long, A.B., Riddle, D.R., Brunso-Bechtold, J.K., 2007. Caloric restriction eliminates the aging-related decline in NMDA and AMPA receptor subunits in the rat hippocampus and induces homeostasis. Exp. Neurol. 206, 70–79. https://doi.org/10.1016/j.expneurol.2007.03.026.

Shin, E., Kashiwagi, Y., Kuriu, T., Iwasaki, H., Tanaka, T., Koizumi, H., Gleeson, J.G., Okabe, S., 2013. Doublecortin-like kinase enhances dendritic remodelling and negatively regulates synapse maturation. Nat. Commun. 4, 1440 https://doi.org/10.1038/ncomms2443.

Shu, T., Tseng, H.C., Sapir, T., Stern, P., Zhou, Y., Sanada, K., Fischer, A., Coquelle, F.M., Reiner, O., Tsai, L.H., 2006. Doublecortin-like kinase controls neurogenesis by regulating mitotic spindles and M phase progression. Neuron 49, 25–39. https://doi.org/10.1016/j.neuron.2005.10.039.

Smith, C.C., McMahon, L.L., 2006. Estradiol-induced increase in the magnitude of Long-term potentiation is prevented by blocking NR2B-containing receptors. J. Neurosci. 26, 8517 LP–8522. https://doi.org/10.1523/JNEUROSCI.5279-05.2006.

Smith, T.D., Adams, M.M., Gallagher, M., Morrison, J.H., Rapp, P.R., 2000. Circuit-specific alterations in hippocampal synaptophysin immunoreactivity predict spatial learning impairment in aged rats. J. Neurosci. 20, 6587–6593.

Speisman, R.B., Kumar, A., Rani, A., Pastoriza, J.M., Severance, J.E., Foster, T.C., Ormerod, B.K., 2013. Environmental enrichment restores neurogenesis and rapid acquisition in aged rats. Neurobiol. Aging 34, 263–274. https://doi.org/10.1016/j.neurobiolaging.2012.05.023.

Tuz-Sasik, M.U., Karoglu-Eravsar, E.T., Kinali, M., Arslan-Ergul, A., Adams, M.M., 2020. Expression levels of SMAD specific E3 ubiquitin protein ligase 2 (Smurf2) and its interacting partners show region-specific alterations during brain aging. Neuroscience 436, 46–73. https://doi.org/10.1016/j.neuroscience.2020.04.003.

Tyagarajan, S.K., Fritschy, J.-M., 2014. Gephyrin: a master regulator of neuronal function? Nat. Rev. Neurosci. 15, 141–156. https://doi.org/10.1038/nrn3670.

van Praag, H., Kempermann, G., Gage, F.H., 2000. Neural consequences of environmental enrichment. Nat. Rev. Neurosci. 1, 191–198. https://doi.org/10.1038/35044558.

VanGuilder, H.D., Yan, H., Farley, J.A., Sonntag, W.E., Freeman, W.M., 2010. Aging alters the expression of neurotransmission-regulating proteins in the hippocampal synaptoproteome. J. Neurochem. https://doi.org/10.1111/j.1471-4159.2010.06719.x.

Volgin, A.D., Yakovlev, O.V., Demin, K.A., Abreu, M.S. de, Rosemberg, D.B., Meshalkina, D.A., Alekseeva, P.A., Friend, A.J., Amstislavskaya, T.G., Kalueff, A.V., 2018. Understanding the role of environmental enrichment in Zebrafish neurobehavioral models. Zebrafish 15, 425–432. https://doi.org/10.1089/zeb.2018.1592.

von Hofsten, J., Olsson, P.-E., 2005. Zebrafish sex determination and differentiation: involvement of FTZ-F1 genes. Reprod. Biol. Endocrinol. 3, 63. https://doi.org/10.1186/1477-7827-3-63.

von Krogh, K., Sorensen, C., Nilsson, G.E., Overli, O., 2010. Forebrain cell proliferation, behavior, and physiology of zebrafish, Danio rerio, kept in enriched or barren environments. Physiol. Behav. 101, 32–39. https://doi.org/10.1016/j.physbeh.2010.04.003.

Young, D., Lawlor, P.A., Leone, P., Dragunow, M., During, M.J., 1999. Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat. Med. 5, 448–453. https://doi.org/10.1038/7449.

Yu, L., Tucci, V., Kishi, S., Zhdanova, I.V., 2006. Cognitive aging in zebrafish. PLoS One 1, e14. https://doi.org/10.1371/journal.pone.0000014.

Zhang, D.L., Hu, C.X., Li, D.H., Liu, Y.D., 2013. Lipid peroxidation and antioxidant responses in zebrafish brain induced by Aphanizomenon flos-aquae DC-1 aphantoxins. Aquat. Toxicol. 144–145, 250–256. https://doi.org/10.1016/j.aquatox.2013.10.011.

Zimmerman, G., Soreq, H., 2006. Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. Cell Tissue Res. 326, 655–669. https://doi.org/10.1007/s00441-006-0239-8.