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High-fat diet feeding triggers a regenerative response in the adult zebrafish brain

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Abstract

Non-alcoholic fatty liver disease (NAFLD) includes a range of liver conditions ranging from excess fat accumulation to liver failure. NAFLD is strongly associated with high-fat diet (HFD) consumption that constitutes a metabolic risk factor. While HFD has been elucidated concerning its several systemic effects, there is little information about its influence on the brain at the molecular level. Here, by using a high-fat diet (HFD)-feeding of adult zebrafish, we first reveal that excess fat uptake results in weight gain and fatty liver. Prolonged exposure to HFD induces a significant increase in the expression of pro-inflammation, apoptosis, and proliferation markers in the liver and brain tissues. Immunofluorescence analyses of the brain tissues disclose stimulation of apoptosis and widespread activation of glial cell response. Moreover, glial activation is accompanied by an initial decrease in the number of neurons and their subsequent replacement in the olfactory bulb and the telencephalon. Long-term consumption of HFD causes activation of Wnt/β-catenin signaling in the brain tissues. Finally, fish fed an HFD induces anxiety, and aggressiveness and increases locomotor activity. Thus, HFD feeding leads to a non-traumatic brain injury and stimulates a regenerative response. The activation mechanisms of a regeneration response in the brain can be exploited to fight obesity and recover from non-traumatic injuries.

Keywords High-fat diet · Brain regeneration · Zebrafish · Inflammation · Apoptosis · Proliferation · Neurogenesis · Wnt/β-catenin signaling

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common disorder and is defined as a group of conditions caused by excessive fat accumulation in the liver. NAFLD, characterized by an increase in intrahepatic lipid content, has frequently been associated with a disproportionate amount of dietary fat intake and obesity [1]. High-fat diet

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(HFD)-associated obesity is very common in patients with NAFLD and has been exploited to generate animal models of NAFLD [2]. A growing body of evidence suggests that NAFLD does not remain restricted to the liver, but acts as an early mediator of systemic diseases that affect extrahepatic organs [3, 4]. NAFLD has been reported to adversely affect various organs including those of the gastrointestinal system, the kidney, the heart, and the brain [5–9].

Numerous cross-sectional studies related to increased body mass index, weight gain, and obesity in adults aged 18–65 years have revealed that these phenomena can be related to deficits in certain cognitive functions including psychomotor performance and speed, visual construction, verbal memory, decision-making, and executive function [10–14]. Obesity has also been linked with structural and metabolic alterations in the brain that lead to impairment of cognitive functions [13, 15, 16]. These alterations span a range of potential mechanisms including compromised cerebral metabolism, elevated leptin levels, increased inflammation, and neuronal injury [17–21]. The relationship between overfeeding and obesity, and the brain has been investigated in animal models of a high-fat diet (HFD) [22–24]. Owing to the similarity of the transcriptional, metabolic, and behavioral responses to those of mammals, zebrafish has been evaluated as a valuable model to study the pathological influence of HFD in humans [6, 9, 25–27]. Accordingly, lipid accumulation in the liver has been accompanied by an increase in the expression of pro-inflammation-related genes in the zebrafish and mammalian models fed an HFD. Mouse models of HFD have revealed cognitive, microstructural, neurochemical, and metabolic alterations in the brain [28–34]. However, a comprehensive molecular analysis of the influence of HFD on the brain regions concerning the activation of cellular processes such as proliferation, cell death, and signaling pathways has not been conducted so far.

Owing to its key roles in the development and growth of tissues and organs involved in the regulation of metabolism, Wnt signaling, in particular, the β-catenin pathway (also termed the canonical Wnt pathway) has been associated with a range of metabolic diseases including obesity and NAFLD [35]. Moreover, Wnt/β-catenin signaling plays key roles in the activation of tissue repair mechanisms in a broad range of regenerating tissues and organs studied to date [36–38]. Parts of the central nervous system (CNS) including the retina, optic tectum, and spinal cord have been deciphered to activate Wnt signaling to mediate proliferation and differentiation of stem/progenitor cells in response to injury in the adult zebrafish [39–43]. Our previous study has further supported the role of Wnt/β-catenin signaling in the adult zebrafish telencephalon where the pathway becomes activated at a very early stage of regeneration, i.e., only 20 h after injury, and controls a large pool of genes involved in p53, apoptosis, MAPK, mTOR, and FoxO pathways [44]. Nevertheless, whether HFD feeding can stimulate an injury-induced regenerative response in the brain and activate Wnt/β-catenin signaling remains to be elucidated.

To address the influence of HFD feeding on the brain, we exploited the adult zebrafish that has a robust capacity to regenerate its CNS due to its constitutively active neurogenic zones of stem/progenitor cells [45, 46]. Zebrafish CNS regenerates via proliferation and differentiation of the radial glial cells (RGCs) and neuroepithelial-like progenitor cells [45–49]. This exclusive ability of zebrafish to regenerate their brain provides a platform that enables direct observation of the molecular effects of HFD on the brain in a highly regenerative context. Initially, we have validated our zebrafish model of HFD in the liver by increased fat deposition and activation of genes related to inflammation, proliferation, and apoptosis. Our results show that the consequences of fatty liver are not limited to the liver but also visible in the whole brain tissue as an increase in the expression of genes related to inflammation, apoptosis, proliferation, and neurogenesis. The HFD-induced alterations are detectable in different regions of the brain, i.e., the olfactory bulb and the telencephalon, as activation of apoptosis, glial cell response, and proliferation. While the number of neurons decreases in response to short-term HFD intake, the lost neurons are replaced by the newly forming ones after prolonged exposure to HFD. Interestingly, HFD-induced fatty liver results in the activation of Wnt/β-catenin signaling in different regions of the brain, most prominently after long-term HFD intake. Finally, HFD feeding for short- or long-term causes an increase in the anxiety, aggressiveness, and locomotor activity of the fish. Overall, HFD feeding initiates a regenerative response in the brain.

Materials and Methods

Zebrafish Husbandry

Zebrafish were supplied by Izmir Biomedicine and Genome Center (IBG) Zebrafish Core Facility and were maintained in a 14-h light/10-h dark cycle at 28.5 °C, following the guidelines of the IBG Animal Care and Use Committee. Only male zebrafish were used in the experiments to minimize the effect of female reproductive cycles and hormone fluctuations. All animal experiments were performed according to the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes and approved by the Animal Experiments Local Ethics Committee of Izmir Biomedicine and Genome Center (IBG-AELEC).

Zebrafish Feeding and Experimental Design

One-year-old male Golden zebrafish were synchronized by feeding with 5 mg artemia for 1 month before the experiment. The control group was fed a normal diet of 5 mg artemia for 2 months and a high-fat diet (HFD) group was fed 5 mg artemia and 30 mg egg yolks from chicken (59% fat, 32% proteins, 2% carbohydrates; Sigma-Aldrich, MO, USA) for 1 or 2 months [50]. The fish were fed twice a day and ensured that the meals were consumed in 1–2 min. Both control and HFD groups contained eight zebrafish. To identify the influence of HFD feeding on the brain at the molecular level, we fed zebrafish an HFD for up to two months. The experimental workflow included the generation of an HFD model, tissue preparation for immunohistochemical/immunofluorescence analysis of the liver/brain, and dissection of both liver and brain for gene expression analysis by qPCR (Fig. 1).

Tissue Preparation and Cryosectioning

The zebrafish were sacrificed in ice water, and the severed heads and bodies from four zebrafish were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 days at 4 °C as described previously [44]. The heads were then incubated in 20% sucrose-20% EDTA for 2 days at 4 °C and in 30% sucrose-20% EDTA for another 2 days at 4 °C. Next, heads were embedded in a solution of 20% sucrose-7.5% gelatin kept in a styrofoam box filled with dry ice, and stored at – 80 °C until sectioning. Transverse sections of 14 µm were cut from the olfactory bulb, telencephalon, and liver at – 25 °C using a cryostat (Leica CM1850, Wetzlar, Germany) and stored at – 20 °C until further use.

Oil Red O and Hematoxylin Staining

Stock and working solutions of oil red O (ORO) (Sigma-Aldrich, MO, USA) were prepared as described previously [51]. Frozen transverse sections of four zebrafish livers were used for ORO staining. Sections stored at – 20 °C were kept at room temperature (RT) for 30 min. Next, sections were rinsed in 50% isopropanol in distilled water for 30 s, stained with freshly prepared oil red O working solution for 5 min, rinsed for 30 s in 50% isopropanol, and dipped 5 times in hematoxylin for nuclei staining. Finally, the sections were rinsed in running water for 30 min and mounted in 70% glycerol diluted with PBS. Four different sections with no tissue loss were counted for the quantification of cells. Statistical significance was evaluated using an unpaired *t* test.

Immunofluorescence Staining and Imaging

Immunofluorescence staining was carried out as previously described [44]. The brain sections on slides were dried at RT for 30 min and washed twice in PBSTX (PBS/0.3% Triton X-100) for 10 min at RT. Following a 15-min incubation with 10 mM sodium citrate at 85 °C, the sections were washed twice with PBS for 5 min and incubated with the primary antibody in PBSTX overnight at 4 °C. The next morning, the slides were rinsed three times with PBSTX

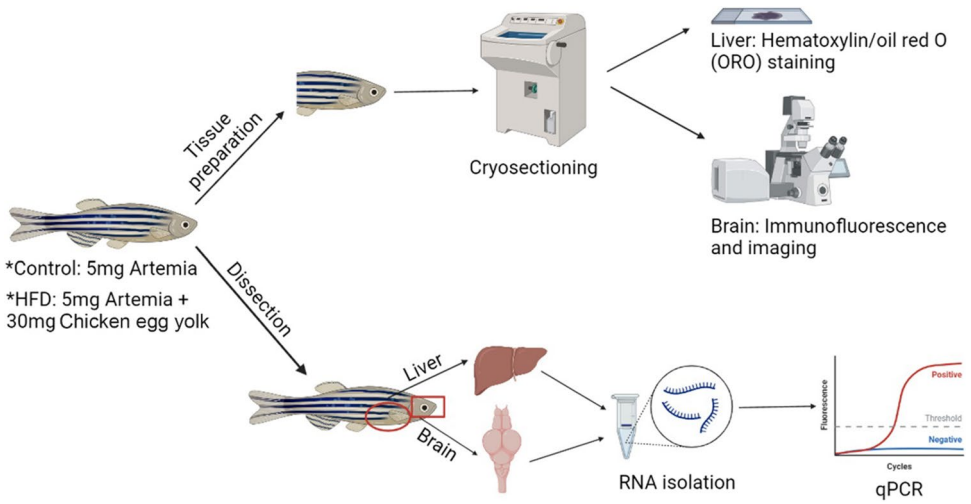

Fig. 1 The experimental workflow. The control group of zebrafish were fed with 5 mg of artemia daily. The high-fat diet (HFD) group were fed with 5 mg of artemia and 30 mg of chicken egg yolk daily. Eight fish were used in each group. Half of them were sacrificed for tissue preparation. Cryosections of livers and brains were processed for hematoxylin-oil red O (ORO) staining and immunofluorescence staining, respectively. The other half was used for dissection of the liver and brain, which were further processed for RNA isolation and quantitative PCR (qPCR) analysis of gene expression

for 10 min at RT, incubated with the secondary antibody, and washed three times with PBS for 5 min at RT. Sections were imaged by using an LSM 880 laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany). The primary antibodies are listed as follows: mouse anti-HuC/HuD (1:400, A-21271, Thermo Fisher Scientific, MA, USA), rabbit anti-cleaved-Caspase-3 (1:400, 5A1E, Cell Signaling Technology, MA, USA) mouse anti-PCNA (1:200, M0879, Dako, Agilent, CA, USA), rabbit anti-S100β (1:200, Z0311, Dako, Agilent, CA, USA) and rabbit anti-phospho-β-catenin (Ser675, 1:200, D2F1, Cell Signaling Technology, MA; USA). Secondary antibodies are listed as follows: Rhodamine (TRITC)-AffiniPure donkey anti-rabbit IgG (1:200, 711–025-152, Jackson Immunoresearch Laboratories, PA, USA) and Cy5 AffiniPure donkey anti-mouse IgG (H + L) (1:200, 712–175-150, Jackson Immunoresearch Laboratories, PA, USA). Nuclear staining was carried out by using 4′,6-diamidino-2-phenylindole (DAPI; 4083S, Cell Signaling Technology, MA, USA). Four different sections with no tissue loss were counted for the quantification of cells. Statistical significance was evaluated using an unpaired *t* test.

Western Blotting

Four zebrafish from each group (control, 1 month fed, and 2 months fed) were sacrificed according to ethical regulations. Shortly after the sacrification, total brain tissues were dissected and pooled for each group. Collected tissues were flash-frozen in liquid nitrogen and stored at – 80 °C until cell lysate extraction. For extraction, tissues were homogenized in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, MA, USA) and centrifuged at max speed for 30 min + 4 °C. Supernatants were collected and dissolved in 5X SDS loading buffer to be used for western blotting. For western blotting, samples were separated by a 12% acrylamide-bis acrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, IL, USA). Blocking was performed with 5% milk powder for 45 min at RT and incubated in the following antibodies at the indicated dilutions. Primary antibodies: rabbit anti-PARP (1:500; 9542 T, Cell Signaling Technology, MA, USA) and rabbit anti-β-Actin (1:1000; 8457S, Cell Signaling Technology, MA, USA). Secondary antibodies: anti-rabbit IgG, horseradish peroxidase (HRP)-linked (1:1000; 7074S, Cell Signaling Technology, MA, USA) and goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 546 (1:1000; A-11010, Thermo Fisher Scientific, MA, USA).

RNA Isolation and cDNA Synthesis

After 1 month or 2 months of feeding a normal diet or HFD, zebrafish were sacrificed in ice water. Heads were separated from the fish and whole brains were dissected from four zebrafish and pooled. Livers were likewise dissected from four zebrafish and pooled. Total RNAs were isolated from the brain and liver samples using a miRNeasy Mini Kit (Qiagen, Hilden, Germany). cDNAs were synthesized from total RNAs using iScript reverse transcription supermix for RT-qPCR (Bio-Rad Laboratories, Inc., CA, USA) according to the manufacturer's instructions, using a 1:1 mixture of oligodT and random primers.

Quantitative PCR (qPCR) and Statistical Analysis

qPCR was performed in triplicates using the GoTaq qPCR master mix (Promega, Madison, WI, USA) at Applied Biosystems 7500 Fast Real-Time PCR Machine (Foster City, CA, USA) as triplicates. Zebrafish *ribosomal protein L13a* (*rpl13a*) gene was used as a reference for normalization to determine relative gene expression levels. Data were analyzed using the GraphPad Prism 7 software (Graphpad Software Inc., CA, USA). The values are mean ± SEM (Standard Error of Mean) of three samples. Primer sequences for the following zebrafish genes are listed in Table 1: *rpl13a, il6st, il8, mpeg1.1, il2rga, il10, marco, caspase3a, caspase8, PCNA, GFAP, olig2, huD, neuroD1, rbfox3a, axin2,* and *lef1*. Statistical significance was evaluated using an unpaired *t* test was used for statistical calculations.

Behavioral Assays and Statistical Analysis

Behavioral tests were carried out after the fish ($n = 7$–9 per group) were fed an HFD for 1 or 2 months. The tests were carried out between 10 am and 2 pm using a trapezoidal test tank (L × W × H:27 cm × 10 cm × 17 cm) filled with 3.5 L of system water. After each fish was tested, tank water was replaced with new system water (27 ± 1 °C). Assay tanks were recorded simultaneously with two different cameras (iPhone 12 Pro Max and iPhone 13). The recordings were then analyzed with Panlab Smart v3.0. Statistical analyses were conducted by either using a parametric test (one-way ANOVA test followed by Bonferroni's multiple comparisons test) or non-parametric tests (Kruskal–Wallis tests followed by Dunn's multiple comparisons test). Statistical tests were performed using GraphPad Prism (http://www.graphpad.com/).

Novel Tank Diving Assay

The novel tank diving assay, similar to the thigmotaxis test that is used to study anxiety in rodents, is used to measure anxiety in adult zebrafish upon exposure to a new environment. In addition, it is used to measure locomotor activity.

Table 1 Sequences of forward and reverse primers

Gene name	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG
il6st	GGTCAGAACGGTCAGACGAA	AATGGCACTGGAGAATCGCA
il8	GTCGCTGCATTGAAACAGAA	AGGGGTCCAGACAGATCTCC
mpeg1.1	CCCACCAAGTGAAAGAGG	GTGTTTGATTGTTTTCAATGG
il2rga	TGGGTTCCATGTCTGTGACG	CGAGCAGGCAGGAGAGTATG
il10	CGACAGCACAGGAAATTTAGCAA	AAGAAGCGTGAGCAGAGCAG
marco	AGCGATACGACTTCCAGCAC	TGTGTGTGAACCCAAGCCTC
caspase3a	TTTGATCGCAGGACAGGCAT	AATCTGCGCAACTGTCTGGT
caspase8	AGACCAGGAACAAGGAGGCA	GTGCCAGCCGAAGAGTTTCT
PCNA	CAAGGAGGATGAAGCGGTAACA	CTGCGGACATGCTAAGTGTG
GFAP	ACCCGTGACGGAGAGATCAT	GCCAGTGTCTGAGCCTCATT
olig2	AAGAGGTGCAGATATGCGGG	TCTCGCGGCTGTTGATCTTT
huD	GGCCTATGGCGTTAAGAGGTTC	CCAGCCTGTTCCTGTGTGAC
neuroD1	CTTTCAACACACCCTAGAGTTCC	TGACACTTGTCGGTCCAGTT
rbfox3a	CCTACAGTGACAGCTATGGCA	ACTGTAACCTCCTCTGTAAAGGC
axin2	GTCTAAAGCTGGTGTGCGGA	ACTGCGTCTGTAGGACCTGT
lef1	ACCCCAAGCAAGCAGGTATG	CAATGGCTGGATGGGGGATT

The tank was filled with water to a height of 15 cm and divided into 3 horizontal zones of top, middle, and bottom, 5 cm each. The recording was started immediately after the fish were placed in the tank for 1 min at each of the 0–5, 6–10, 11–15, 16–20, 21–25, and 26–30 min intervals. Data were analyzed for the total distance (cm), mean speed (cm/s), distance in the bottom zone (cm), time spent in the bottom zone (s), and the number of entries to the top zone [52].

Mirror-Biting Assay

The mirror-biting test is a well-established fish paradigm that is widely used to study adult zebrafish's social and aggressive behavior [53, 54]. The test is conducted with a mirror placed on one side of the tank and reflects the reaction of the adult zebrafish to its mirror image. 'Biting' is defined as the fish's "tracing" behavior of its reflection as it swims quickly back and forth, and is measured by the time the fish spends in front of the mirror and touches the mirror [52]. A mirror was placed at one end of the tank, which was then divided into 2 vertical zones as the mirror-close zone (1 cm from the mirror) and the mirror-far zone (the rest of the tank). The mirror was covered with an opaque plastic sheet to prevent the fish from reacting to their reflections during the acclimatization process. After the fish were placed in the tank, they were allowed to acclimate for 5 min. Following the acclimatization, fish behavior was recorded for 15 min. Data were analyzed for the total distance (cm), mean speed (cm/s), distance in the mirror-close zone (cm), time spent in the mirror-close zone (s), and the number of entries to the mirror-close zone. calculating the Distance in the mirror-close zone (cm), total distance (cm), number of entries in the mirror-close zone, time spent in the mirror-close zone (s), and the mean speed (cm/s) [52].

Results

High-Fat Diet Induces Weight Gain and Lipid Accumulation in the Liver

To validate the influence of HFD feeding we dissected the livers from 1 and 2 months fed zebrafish. The fat build-up in the liver was considerably higher in fish fed an HFD for 2 months (long-term) than for 1 month (short-term) (Fig. 2A). Consistent with the fat accumulation, there was a significant and gradual increase in the average body-mass index (BMI) of the fish (Fig. 2B). The number and size of the lipid droplets harbored in the liver increased in fish fed HFD, as detected by hematoxylin-oil red O (ORO) staining (Fig. 2C–E). These results confirm that HFD leads to an increase in body weight and the build-up of excess fat in the liver.

HFD Causes Activation of Inflammation, Apoptosis, Proliferation, and Neurogenesis

HFD has been shown to induce the clustering of macrophages that release pro-inflammatory cytokines and chemokines in the zebrafish larval liver [55, 56]. Activation of the immune response also plays a critical role in

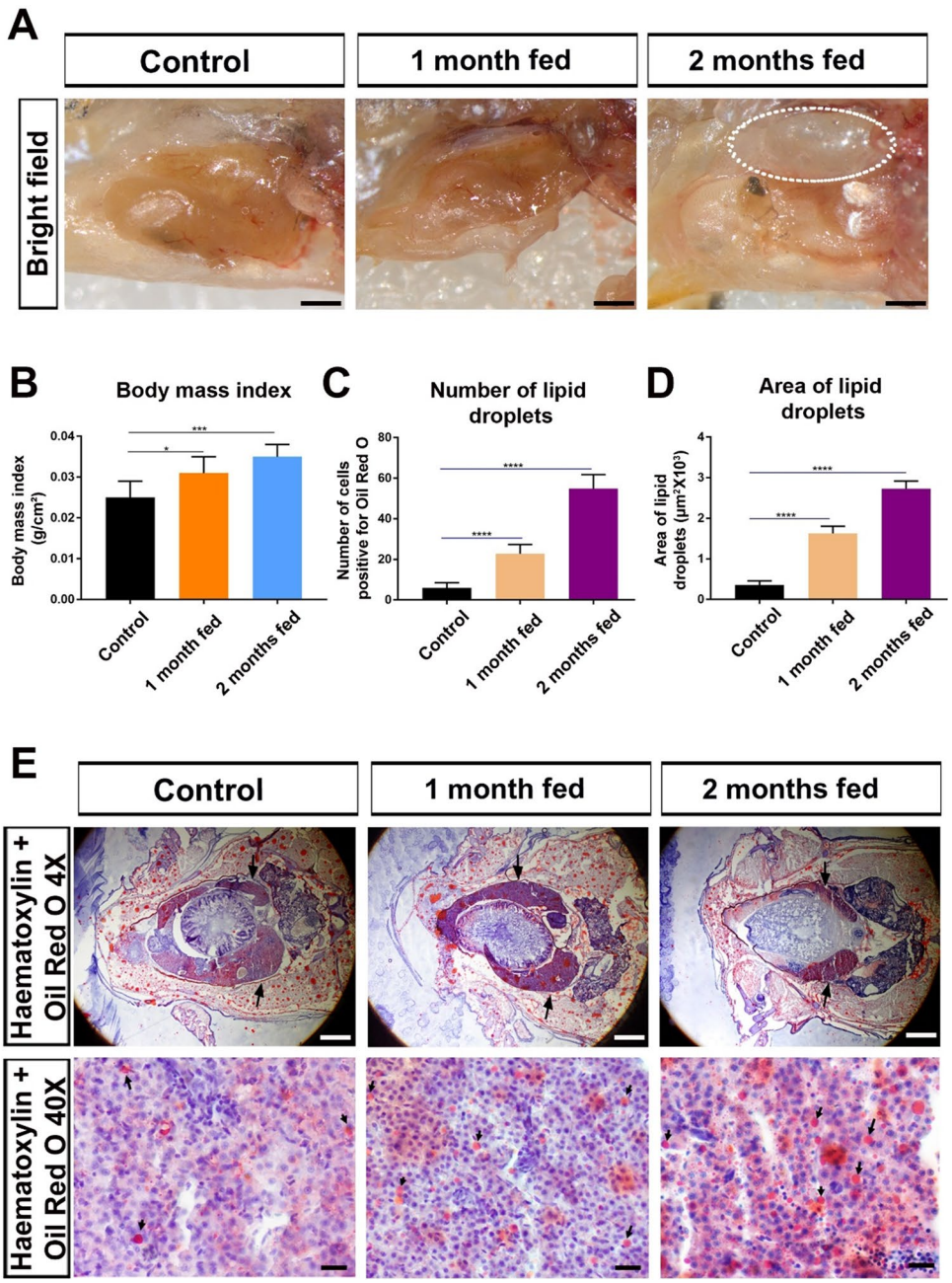

Fig. 2 High-fat diet induces weight gain and lipid accumulation in the liver. **A** Bright-field images display lipid build-up in the livers of zebrafish fed an HFD most prominently after 2 months of feeding. Dotted lines indicate large fat deposits in the liver. **B** The body-mass index (BMI) graph of zebrafish increased significantly after 1 and 2 months of HFD as compared to the control group. **C** The number of cells positive for Oil Red O in the liver of control, 1-month fed, and 2-month fed zebrafish. Statistical significance was evaluated using an unpaired *t* test. ****$p < 0.0001$. Error bars represent ± standard error of the mean (SEM, $n = 3$). **D** The area of lipid droplets in the liver of control, 1-month-fed, and 2-month-fed zebrafish. Statistical significance was evaluated using an unpaired *t* test. ****$p < 0.0001$. Error bars represent ± standard error of the mean (SEM, $n = 3$). **E** Hematoxylin (blue-purple) and oil red O (ORO, red) staining of liver sections obtained from 1-month-fed and 2 months-fed zebrafish show an increase in the number and size of the lipid droplets at × 4X and × 40 magnification. Arrows indicate the liver and the lipid droplets in × 4 and × 40, respectively. Please note that the overall number and size of the lipid droplets increase in the HFD groups. Scale bars 5 mm in **A** and upper row of **D**, 20 μM in the lower row of **E**

the promotion of neural stem cell proliferation and neuronal regeneration [57, 58]. To test whether inflammation response is activated in response to HFD, we measured the expression levels of several immune response marker genes in the liver and brain tissues. We observed that the pro-inflammatory cytokine receptor *interleukin 6 cytokine family signal transducer* (*il6st/glycoprotein 130*), the cytokine *il8*, and the macrophage marker *mpeg* significantly increased in the livers of the fish fed an HFD for short-or long-term (Fig. 3A). Activation of the immune response was accompanied by an increase in the apoptotic markers *caspase8* (*casp8*) and *caspase3a* (*casp3a*) as well as in the proliferation marker *proliferating cell nuclear antigen* (*PCNA*) (Fig. 3B). We found a similar elevation in the levels of the pro-inflammatory markers *il2rga* and *marco* and the macrophage marker *mpeg* with a concomitant decrease in the anti-inflammatory marker *il10* in the brain tissues after long-term HFD (Fig. 3C). Apoptosis-related genes *casp8* and *casp3a* and *PCNA* were significantly upregulated after short- and long-term feeding (Fig. 3D). There was a concomitant increase in the expression of the glial marker *glial fibrillary acidic protein* (*GFAP*), which is known to be strongly upregulated in response to traumatic brain injury (Fig. 3E, [59]).

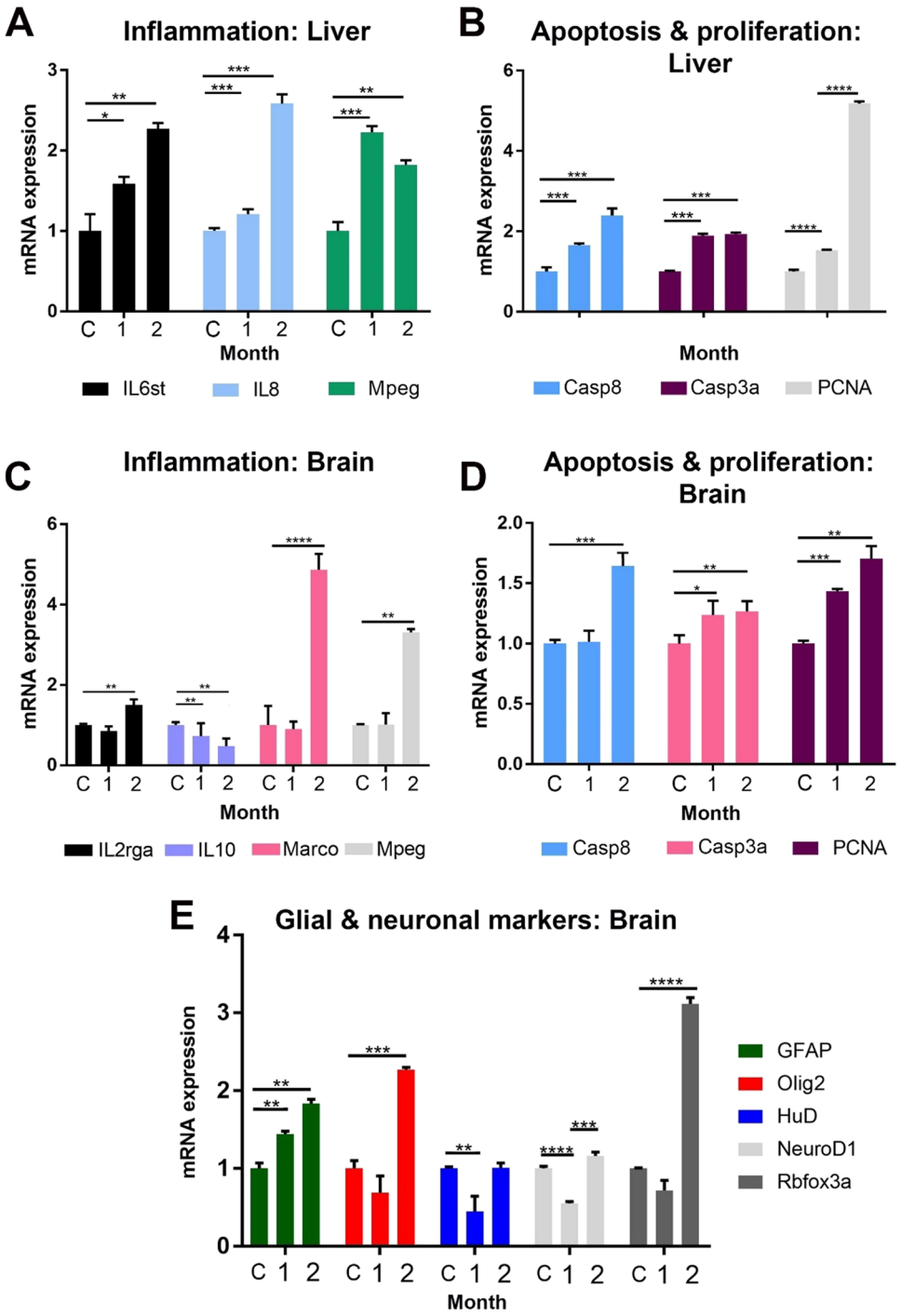

Fig. 3 HFD causes activation of inflammation, apoptosis, proliferation, and neurogenesis in the liver and the brain expression levels of **A** inflammation-related genes in the liver, **B** apoptosis- and proliferation-related genes in the liver, **C** inflammation-related genes in the brain, **D** apoptosis- and proliferation-related genes in the brain and **E** glial and neuronal markers in the brain determined by qPCR in 1 month fed and 2 months fed fish relative to control fish fed a normal diet. Statistical significance was evaluated using an unpaired *t* test. $*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$. Error bars represent ± standard error of the mean (SEM, $n=3$). Three independent experiments were conducted. C: control

Interestingly, there was a decrease in the oligodendrocyte marker *oligodendrocyte transcription factor* (*olig2*) and the neuronal markers *neuronal differentiation 1* (*neuroD1*), *ELAV-like neuron-specific RNA binding protein 4* (*HuD*), and *Rbfox3a* (*neun*) in response to short-term HFD and a remarkable recovery after long-term HFD, proposing that the dying oligodendrocytes and neurons were replaced with the newborn ones (Fig. 3E). Since these genes are expressed in mature neurons in the adult zebrafish brain [59–62], our data suggest that HFD results in neuronal cell death, glial cell activation and induction of a regenerative response in the brain. Together, these data indicate that HFD triggers inflammation, apoptosis, and proliferation not only in the liver but also in the brain and reactive neurogenesis in the brain.

Long-Term Exposure to HFD Stimulates Apoptotic Cell Death, Glial Cell Activation, and Proliferation in the Adult Brain

Since expression of apoptosis-related genes in the brain was downregulated after 1 month of HFD feeding and upregulated after 2 months of feeding, next, we aimed to examine the detailed response of different brain regions to HFD feeding concerning the activation of apoptosis. Immunofluorescence staining for cleaved-Caspase 3 (C-Caspase3) showed that apoptosis increased in the olfactory bulb after short-term feeding and was further boosted after prolonged feeding (Fig. 4A compare middle and right panels to left panel, Fig. 4C). A parallel upregulation of C-Caspase3 was detectable in the telencephalon after 2 months of feeding (Fig. 4B, C). Cleaved PARP, another marker of apoptosis, also increased in the total brain extracts of zebrafish fed a long-term HFD (Fig. 4D).

Activation of RGCs upon injury is a hallmark of regeneration in the adult zebrafish brain [63]. RGCs are characterized by a few markers including S100β and express the cell proliferation marker PCNA. To unravel the regenerative response of RGCs to HFD feeding, we performed immunofluorescence staining of the proliferating RGCs for S100β and PCNA. We detected a gradual increase in glial cell proliferation, marked by S100β and PCNA double-positive

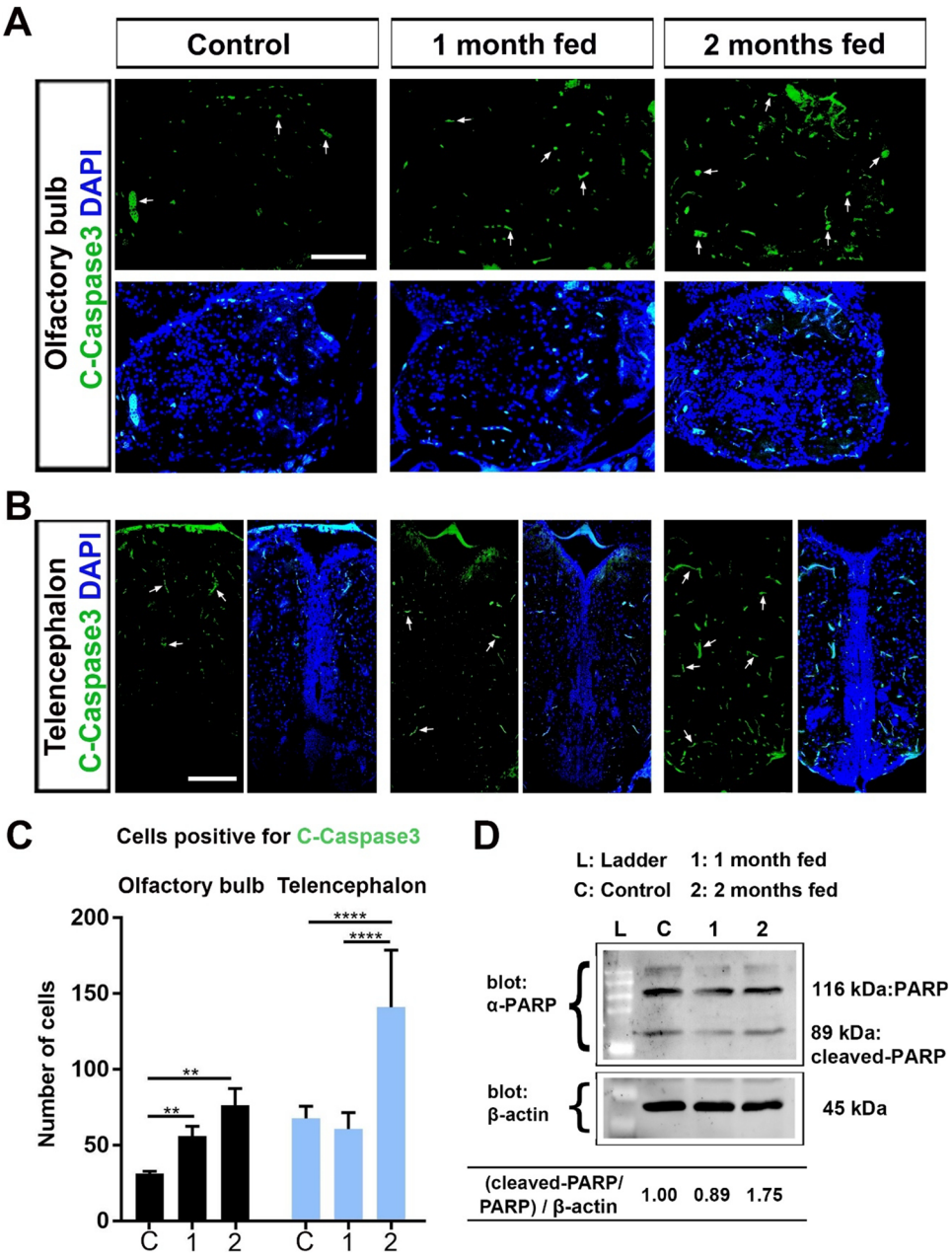

Fig. 4 Long-term exposure to HFD stimulates apoptotic cell death in the adult brain. Anti-cleaved-Caspase-3 (green) staining of brain sections obtained from the **A** olfactory bulb and **B** telencephalon regions of control, 1 month fed, and 2 months fed zebrafish. Arrows indicate several apoptotic cells. Sections are counterstained for DAPI. Scale bars 200 µm. **C** The number of cells positive for C-Caspase3 in the brain sections obtained from the olfactory bulb and telencephalon regions of control (C), 1 month fed and 2 months fed zebrafish. Statistical significance was evaluated using an unpaired *t* test. $^{**}p < 0.01$ and $^{****}p < 0.0001$. Error bars represent ± standard error of the mean (SEM, $n = 3$). **D** Western blot of whole brain tissue lysates collected from control, 1 month fed and 2 months fed to fish. Cleaved-PARP/PARP to β-actin ratios were calculated

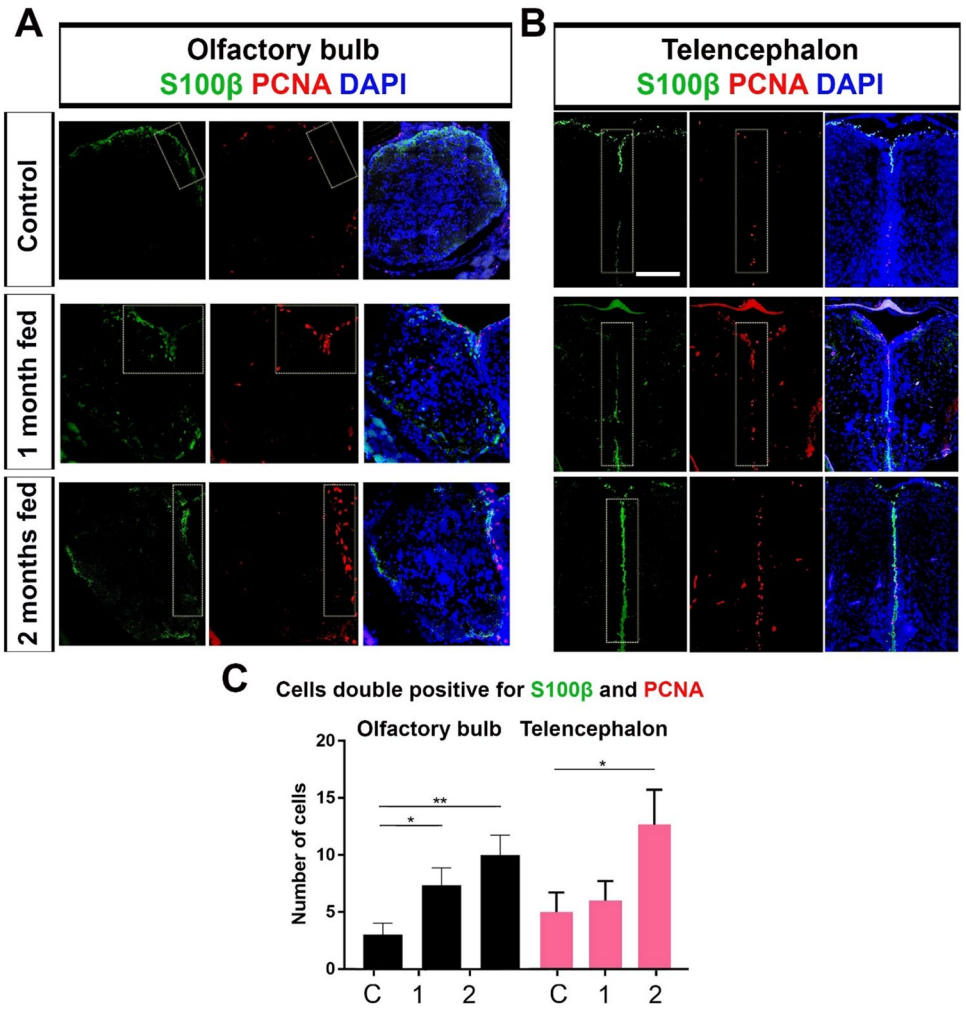

Fig. 5 Long-term exposure to HFD stimulates glial cell activation and proliferation. Anti-s100β (green) and anti-PCNA (red) staining of brain sections obtained from the **A** olfactory bulb and **B** telencephalon of control, 1 month fed, and 2 months fed zebrafish. Dotted rectangles indicate periventricular zones where radial glial cells proliferate. Dotted ellipses indicate brain parenchyma. Sections are counterstained for DAPI. Scale bars 200 µm. **C** The number of cells double positive for S100β and PCNA in the brain sections obtained from the olfactory bulb and telencephalon regions of control (**C**), 1 month fed and 2 months fed zebrafish. Statistical significance was evaluated using an unpaired t-test. $*p < 0.05$ and $**p < 0.01$. Error bars represent ± standard error of the mean (SEM, $n = 3$)

cells, which became prominent in the olfactory bulb and telencephalon after HFD feeding (Fig. 5A, B). The number of double-positive cells in both CNS tissues increased concurrently, marking a substantial part of the glial cells located at the periventricular zones (Fig. 5A, B compare green and red cells in boxes, Fig. 5C). Strikingly, proliferating cells marked by PCNA were not restricted to the ventricular zones in the telencephalon but were also detected deeper in the brain parenchyma (Fig. 5B). These results collectively suggest that brain tissue is highly responsive to HFD feeding concerning activation of apoptosis, glial cell response, and proliferation.

HFD Intake Results in Initial Loss and Subsequent Recovery of Neurons

An increase in apoptosis and concomitant reduction of mRNA levels of the neuronal markers *neuroD1* and *HuD* in the brain suggests that HFD could promote neuronal cell death. To test this hypothesis, we examined the expression of HuC/D by antibody staining on the sections of the olfactory bulb and the telencephalon obtained from 1 and 2 months fed zebrafish. There was a significant reduction in neuron densities in the olfactory bulb of the fish fed a short-term HFD as compared to the control (Fig. 6A middle column, Fig. 6C). However, long-term HFD resulted in a considerable recovery in the number of HuC/D-positive neurons (Fig. 6A right column, Fig. 6C). Strikingly, following a similar decrease at 1 month of HFD, the neuron densities were substantially restored in the telencephalon at 2 months of HFD (Fig. 6B compare middle columns to right columns, Fig. 6C). Thus, short-term HFD induces neuron loss, which is compensated by the new neurons emerging in the course of prolonged exposure to HFD.

HFD Feeding Activates Wnt/β-Catenin Signaling in the Brain

Wnt/β-catenin signaling pathway becomes activated in the regenerating zebrafish telencephalon at the early wound healing stage, supporting the tissue-wide regeneration-promoting role of the pathway [44]. Immunofluorescence

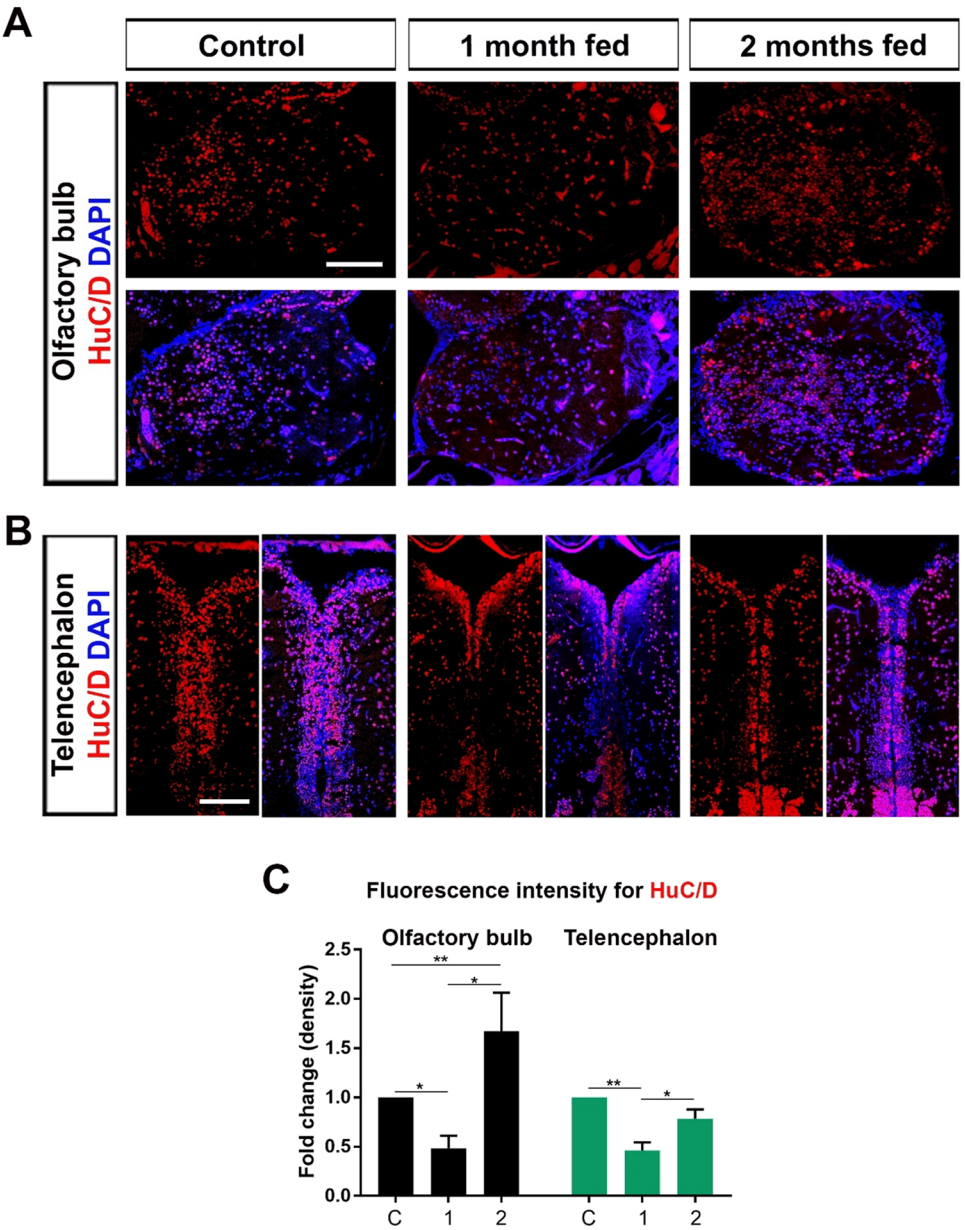

Fig. 6 HFD intake results in loss and subsequent recovery of neurons in the brain. Anti-HuC/D (green) staining of brain sections obtained from the **A** olfactory bulb **B** telencephalon of control, 1 month fed, and 2 months fed zebrafish. Sections are counterstained for DAPI. Scale bars 200 µm. **C** The fluorescence intensity of cells positive for HuC/D in the brain sections obtained from the olfactory bulb and telencephalon regions of control (**C**), 1 month fed and 2 months fed zebrafish. Statistical significance was evaluated using an unpaired *t* test. $*p < 0.05$ and $**p < 0.01$. Error bars represent ± standard error of the mean (SEM, $n = 3$)

staining of the olfactory bulb and the telencephalon revealed elevation of phospho-β-catenin (phosphorylated at Ser675, increasing nuclear localization and transcriptional activation of β-catenin) in the brains of the fish fed HFD for short-term and a further increase in response to prolonged HFD feeding (Fig. 7A, B). The increase was reflected in the number of cells positive for β-catenin (Fig. 7C). Finally, qPCR results confirmed canonical Wnt signaling activation as evidenced by significant upregulation of the Wnt/β-catenin target genes *axin2* and *lef1*, as well as β-catenin in the total brain tissues of fish, fed an HFD for 2 months (Fig. 7D). Thus, we conclude that Wnt/β-catenin signaling is activated in the brain in response to HFD.

HFD Feeding Causes an Increase in Anxiety, Aggressiveness, and Locomotor Activity in Zebrafish

HFD feeding is known to exert neurobehavioral alterations including anxiety/depressive-like behaviors and aggressiveness in adult vertebrates [6, 64, 65]. Moreover, obesity has been associated with an increase in systemic inflammation condition that is accompanied by dysregulation of brain homeostasis [66, 67]. Owing to the increase in inflammation and apoptosis, we set out to examine relevant changes in the behavior of zebrafish fed an HFD.

Similar to most other species, exposure to novelty gives rise to robust anxiety responses in zebrafish. Thus, zebrafish exhibit bottom-dwelling behavior when

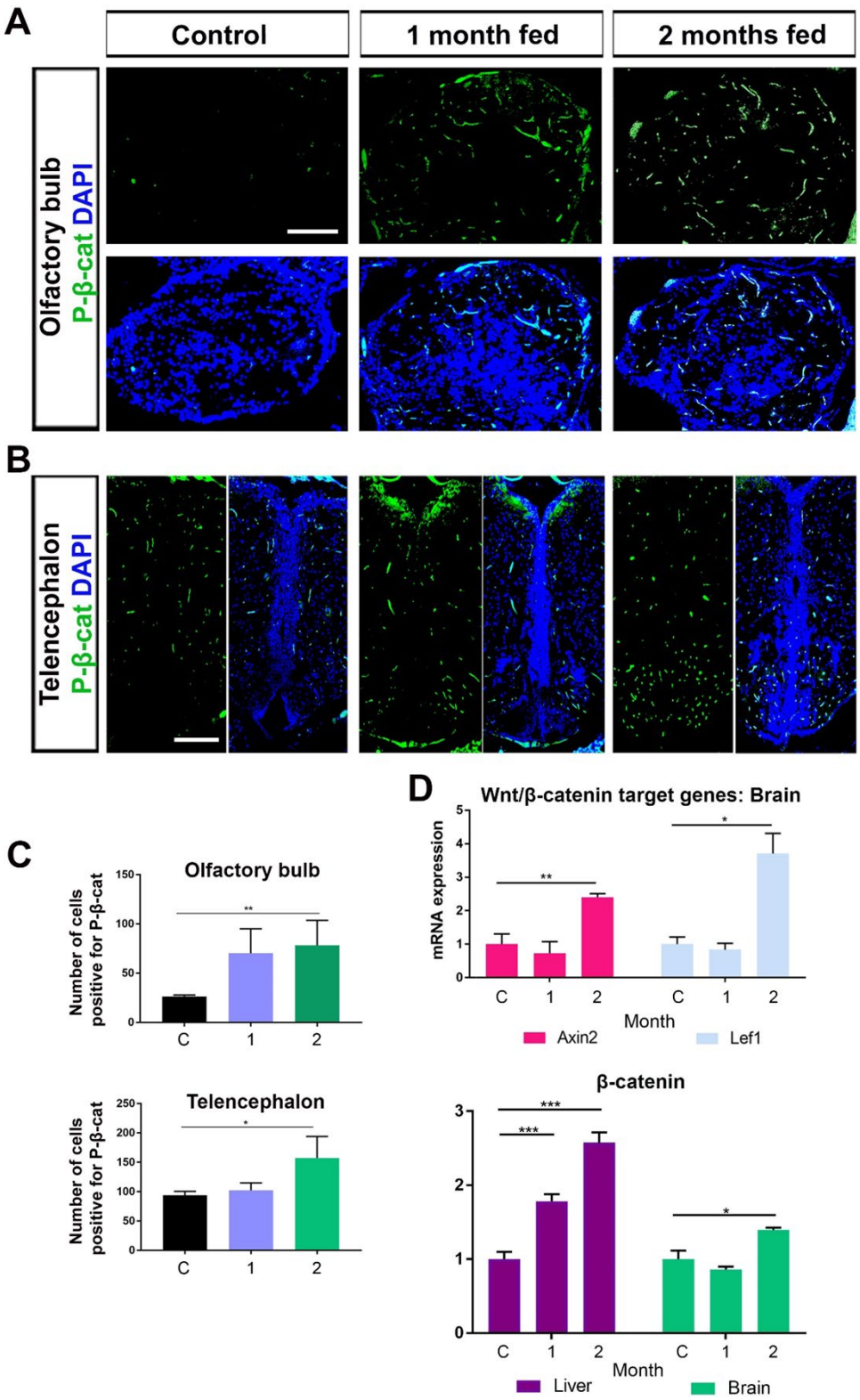

Fig. 7 HFD feeding activates Wnt/β-catenin signaling in the brain. Anti-phospho-β-catenin (green) staining of brain sections obtained from the **A** olfactory bulb and **B** telencephalon of control, 1-month fed, and 2-month fed to zebrafish. Sections are counterstained for DAPI. Scale bar = 100 µm. **C** The number of cells positive for phospho-β-catenin in the brain sections obtained from the olfactory bulb and telencephalon regions of control (**C**), 1 month fed and 2 months fed zebrafish. Statistical significance was evaluated using an unpaired *t* test. $*p < 0.05$ and $**p < 0.01$. Error bars represent ± standard error of the mean (SEM, $n = 3$). **D** Relative expression levels of canonical Wnt target genes Axin2 and Lef1 in the brain were determined by qPCR in 1-month-fed and 2-month-fed fish relative to control (**C**) fish fed a normal diet. Statistical significance was evaluated using an unpaired *t* test. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Error bars represent ± standard error of the mean (SEM, $n = 3$). Three independent experiments were conducted

placed in a new environment and begins to explore the area only after a few minutes of adaptation [68, 69]. To explore whether HFD feeding influences the anxiety level of the zebrafish, we used the novel tank diving assay, a behavioral test based on measuring the instinctive behavior of zebrafish when placed in an unfamiliar environment as well as the locomotor activity. First, the total distance traveled in the first 15 min and mean speed in this period

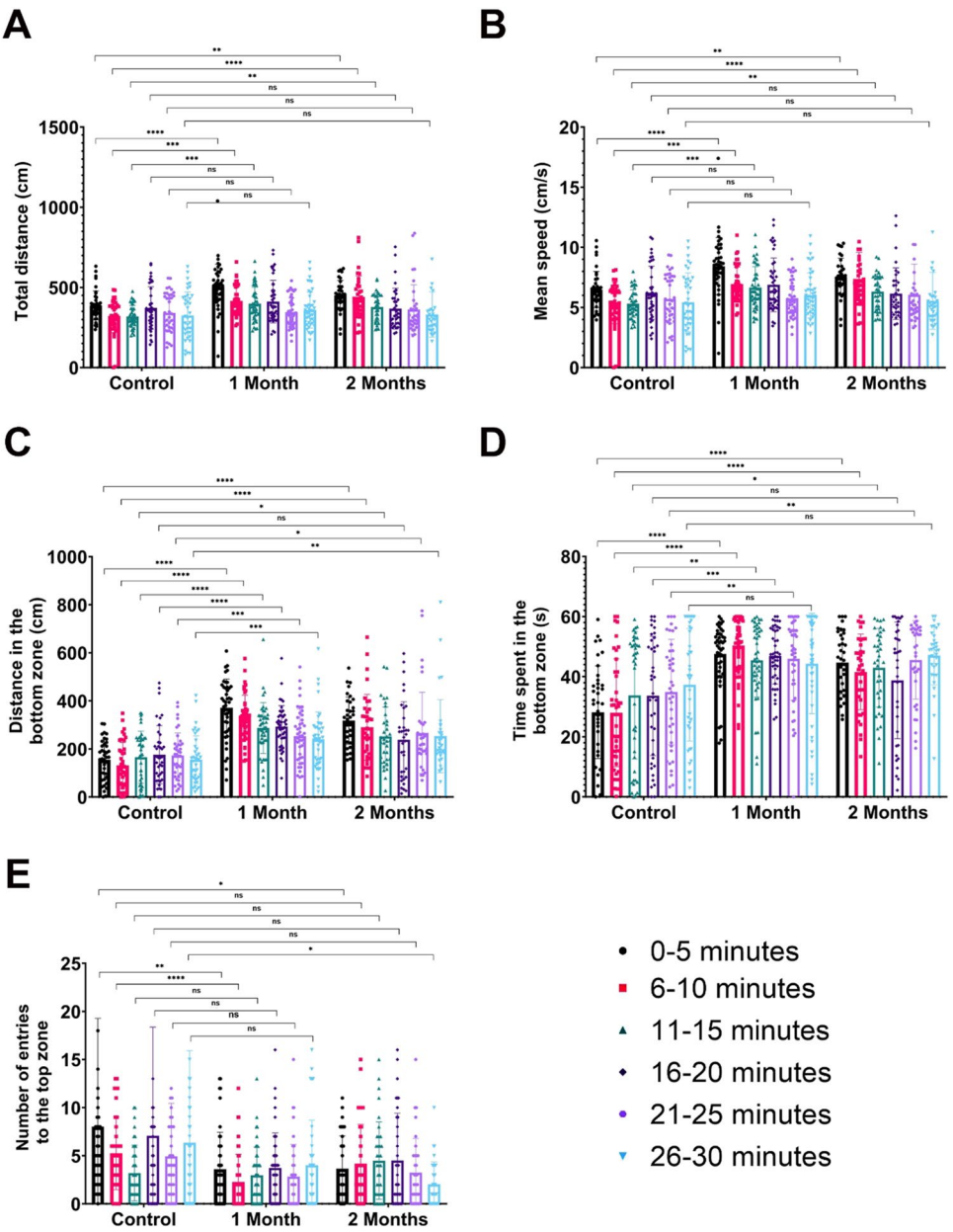

Fig. 8 HFD feeding causes an increase in anxiety in zebrafish. **A** Total distance (cm), **B** mean speed (cm/s), **C** distance in the bottom zone (cm), **D** time spent in the bottom zone (s), and **E** number of entries to the top zone measured for fish fed a control diet or HFD for 1 month or 2 months. Statistical significances were evaluated using a one-way ANOVA test or Kruskal–Wallis test, continued with Bonferroni's test or Dunn's multiple comparisons test as post hoc, respectively. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$, and ns non-significant. Error bars represent ± standard deviation of the mean (SD, $n=7$–9)

significantly increased in fish fed with an HFD for 1 or 2 months compared to the control (Fig. 8A, B). These results show that HFD feeding results in an early increase in locomotor activity. Moreover, fish fed an HFD for short- or long-term traveled longer distances and spent significantly longer time at the bottom zone of the tank, and displayed longer latency to enter the top zone in almost all periods (Fig. 8C, D). In several periods including the first 5 min, the number of entries to the top zone of the tank also decreased in HFD groups compared to the control (Fig. 8E).

Next, to test whether HFD feeding induces any aggressive behavior, we exploited the mirror-biting assay, which is used to assess the level of aggressiveness in the social behavior of adult zebrafish [53, 70]. We observed a significant increase in the total distance traveled and mean speed of the fish fed an HFD for 1 month compared to the control, similar to the novel tank diving assay (Fig. 9A, B). Short- or long-term HFD feeding led to an increase in the distance traveled, but not time spent, in the mirror-close zone (Fig. 9C, D). Besides, short-term HFD-fed fish displayed a tendency to enter more frequently in the mirror-close zone (Fig. 9E). These data together indicate that HFD feeding likely triggers anxiety-like behavior and aggressiveness in zebrafish, evidenced by delayed adaptation to the new environment and increased tendency to attack their reflection in the mirror, respectively.

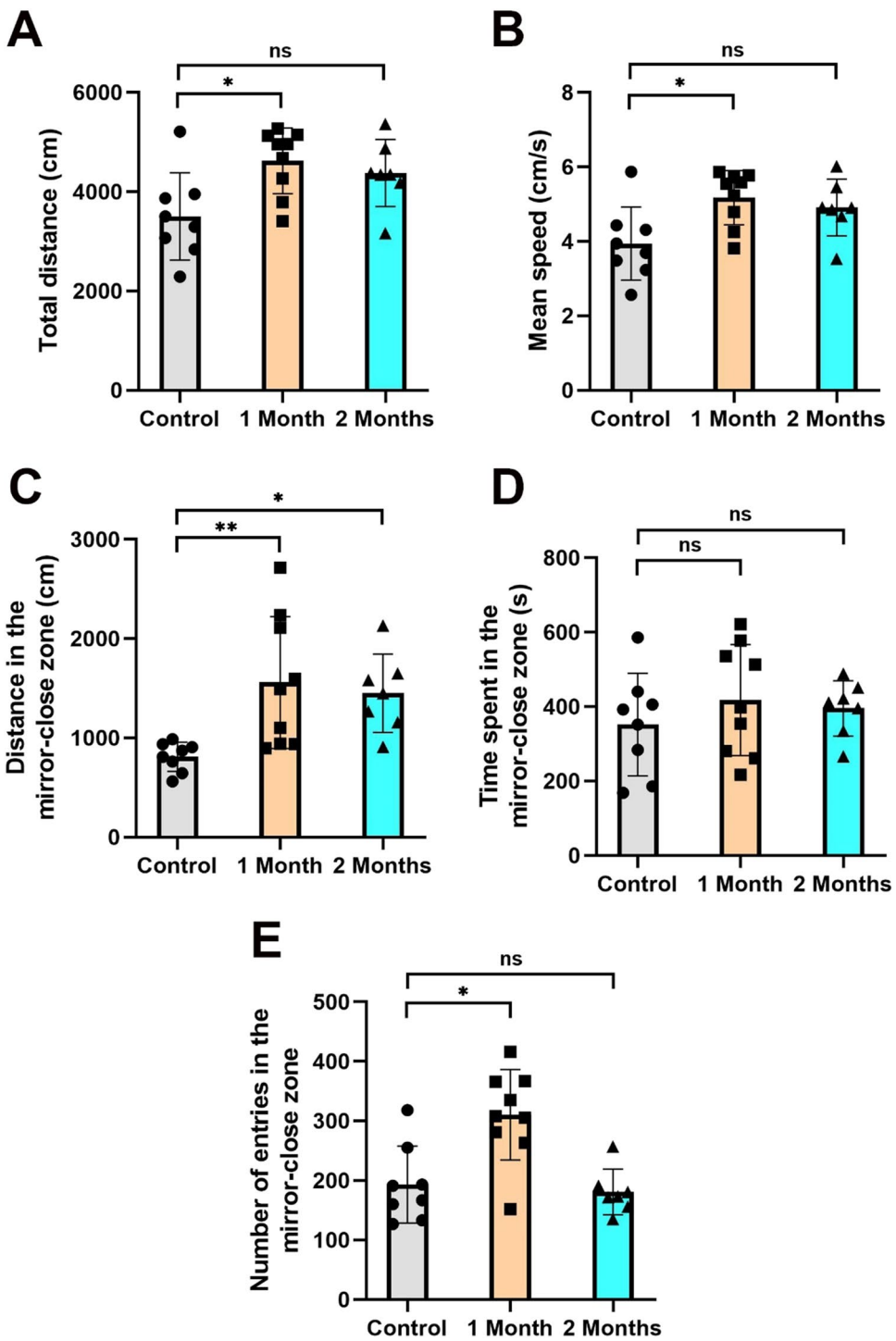

Fig. 9 HFD feeding causes an increase in aggressiveness in zebrafish. **A** Total distance (cm), **B** mean speed (cm/s), **C** distance in the mirror-close zone (cm), **D** time spent mirror-close zone, and **E** number of entries in the mirror-close zone measured for fish fed a control diet or HFD for 1 month or 2 months. Statistical significances were evaluated using a one-way ANOVA test or Kruskal–Wallis test, continued with Bonferroni's test or Dunn's multiple comparisons test as post hoc, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and ns non-significant. Error bars represent ± standard deviation of the mean (SD, $n = 7–9$)

Discussion

NAFLD is characterized by the build-up of excess fat in the liver cells that is not caused by alcohol and is mostly linked to HFD feeding-induced obesity. NAFLD can have a systemic effect on other organs over time. In this study, we used an adult zebrafish model of HFD feeding to unravel how HFD affects the brain. Our data show that HFD feeding (i) is detectable as weight gain and abnormal lipid deposition in the liver, (ii) is accompanied by an increase in the expression levels of the genes associated with inflammation, apoptosis, and proliferation in the liver and brain tissues, (iii) stimulates glial cell activation and neuronal turnover in the forebrain (olfactory bulb and telencephalon), (iv) activates canonical Wnt signaling pathway in these brain regions and

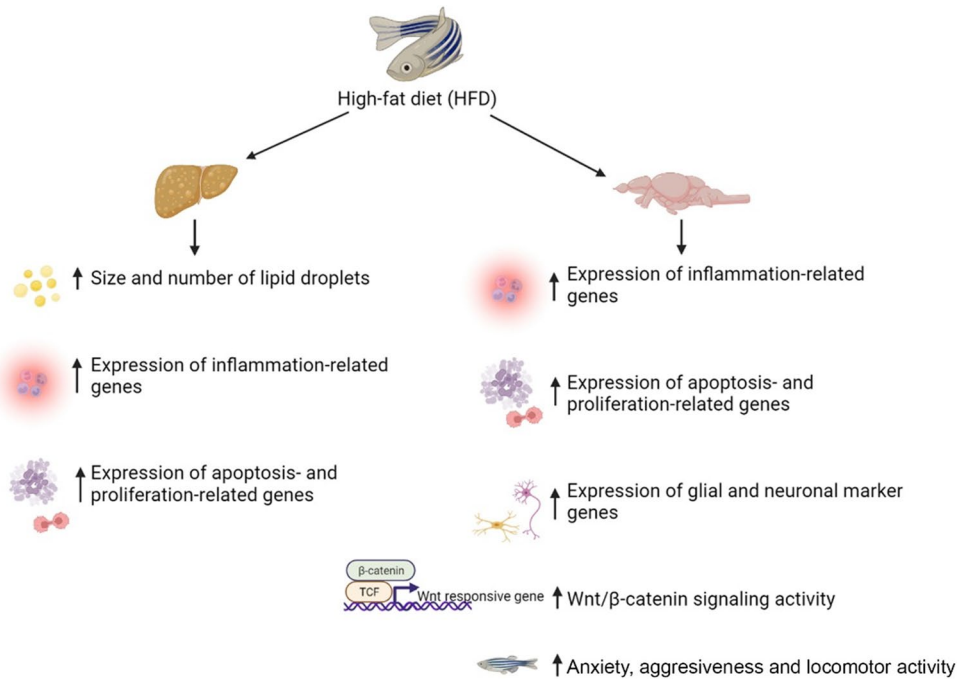

Fig. 10 Summary of the influence of HFD feeding in the liver and the brain. (Left) HFD increases the size and number of lipid droplets, the elevation of inflammation-, apoptosis-, and proliferation-related genes' expression. (Right) HFD feeding leads to changes in the expression of genes related to inflammation, apoptosis, proliferation, neurogenesis, and Wnt/β-catenin signaling pathway in the brain as well as neurobehavioral effects

(v) increases anxiety, aggressiveness and locomotor activity (Fig. 10).

Zebrafish constitute a useful model to study mammalian diet-related pathological conditions as HFD elicits similar biological responses in mammals and zebrafish [6, 9, 25–27]. Our results showed that, in parallel to the considerable increase in body weight, fish fed a long-term HFD exhibited fat accumulation in the liver, similar to obesity phenotypes [50, 71, 72]. In addition to its adverse effects on the liver [2], dietary changes including HFD have been associated with brain disorders including dementia, Alzheimer's disease, and Parkinson's disease [73–76]. Thus, our results further support the relationship between HFD-induced obesity and the brain, by showing for the first time that HFD feeding induces a regeneration response accompanied by activation of the canonical Wnt signaling pathway in the brain of the highly regenerative zebrafish.

HFD-induced obesity and NAFLD have been revealed to cause hepatic inflammation [1, 77–79]. Our data showed gradually increasing upregulation of the pro-inflammatory cytokines and their receptors *il6st* and *il8* in the liver and a relatively mild upregulation of *il2rga* and *il6st* (data not shown but similar to *il2rga*) in the brain, in response to prolonged HFD. IL-6 has been identified as a regulatory factor that plays a role in blood coagulation and platelet activation in zebrafish and mammalian obesity [72]. IL-6 levels are significantly increased in the hippocampus of rats fed an HFD [80]. IL-6 receptor IL6st, a member of the Janus kinase 1/Signal transducer and activator of transcription 3 (Jak1/Stat3) pathway is activated in an injury-dependent manner during regeneration of the heart, optic tectum, retina, and telencephalon [44, 81–83]. Another pro-inflammatory marker IL-2R has been found to increase in neuropsychiatric disorders including schizophrenia, depression, and post-traumatic stress disorder [84]. IL-8 and Marco are also essential for the modulation of the innate immune response during neuronal injury and neuroinflammation [85–89]. The macrophage-expressed gene *mpeg*, which is upregulated during bacterial infection, is likewise upregulated during the regenerative response in the liver and the central nervous system [44, 56, 90, 91]. On the other hand, high BMI levels have been associated with reduced levels of the anti-inflammatory cytokine IL-10 in the human frontal cortex [92]. Thus, differential regulation of these early immune response elements upon HFD feeding suggests that they coordinate activation of an injury-induced inflammatory response in both the liver and the brain. Several studies in rodents assert that consumption of an HFD is sufficient to induce an inflammatory response in the brain and increase the permeability of the blood–brain barrier (BBB), disrupting its integrity, especially in the hippocampus, and leading to impaired cognition [32, 76, 93–97]. The enhanced permeability of the BBB allows the passage of reactive oxygen species and pro-inflammatory cytokines from the blood to the brain, leading to neuroinflammation [95, 98]. Therefore, it is likely that the inflammatory response caused by HFD in the liver, results in neuroinflammation triggered by non-traumatic brain damage.

Resolution of inflammation is essential for the restoration of tissue integrity and function and includes apoptosis as a key component to stop inflammation and initiate the healing process [99, 100]. We observed transcriptional activation of

apoptosis-related genes in the liver after short-term HFD and in the brain after long-term HFD. Increased levels of apoptosis in the liver have been observed in liver injury models generated via feeding HFD [101, 102]. High fat consumption is also associated with the induction of apoptosis-related genes such as Bcl2 and Bax in the mammalian hippocampus [32, 93]. We detected cleaved-Caspase-3 positive apoptotic cells in the olfactory bulb and telencephalon of the forebrain. The distribution of apoptotic cells in the glomerular layer of the olfactory bulb is consistent where mitral cells are abundant [103]. Mitral cells have been reported to be sensitive to metabolic changes such as HFD-induced obesity [104, 105]. Moreover, cleaved-Caspase-3-positive cells in the telencephalon were concentrated in the parenchyma, suggesting that they correspond to not only the neurons but also the oligodendrocytes, endothelial cells or macrophage/microglia [59]. Our results are in line with a previous study showing enhanced expression of *casp9* in the telencephalon of zebrafish fed an HFD [27]. Strikingly, both transcriptome analyses of brain tissues and histological examination of brain sections have revealed activation of apoptosis signaling pathways at different stages of regeneration (12 h post-lesion (hpl), 20 hpl, and 3 days-post lesions) after telencephalic and optic tectum injuries [44, 59, 62, 106]. Thus, the concomitant increase of inflammatory signals and apoptotic gene expression in our HFD model could point out that the brain gives similar molecular responses to long-term HFD feeding and traumatic brain injury to initiate a healing process. It is also noteworthy that the inflammation response of the whole brain and the apoptotic response of the three different brain tissues is indistinct after short-term HFD feeding but becomes pronounced after long-term HFD feeding, suggesting that neuroinflammation and neural apoptosis are activated in response to prolonged feeding [107].

A key reaction of the zebrafish brain to inflammation is the activation of RGCs, which upregulate proliferation and result in increased neurogenesis [44, 58, 59, 108]. Reactive gliosis marked by elevated GFAP expression in astrocytes has also been revealed to accompany brain inflammation as a response to HFD consumption in mouse and rat models [76, 109, 110]. These observations are in coherence with our findings where expression of the RGC markers GFAP and S100β and the proliferation marker PCNA were enhanced in different regions of the brain after short- or long-term HFD feeding. Proliferating RGCs were mostly detected at the periventricular zones in the telencephalon. Most strikingly, we found a significant downregulation of the neuronal markers NeuroD1 and HuC/D after short-term HFD consumption and a recovery in their expression after prolonged feeding. This proposes that HFD feeding induces neuronal cell death in the early stage and the newborn neurons replenish the lost neurons over time. Collectively, similar to a traumatic brain injury, HFD stimulates glial cell proliferation and neurogenesis in the adult zebrafish brain.

Owing to its large share in the body's oxygen and glucose consumption, brain damage has multiple metabolic and systemic effects, partly due to disruption of the blood–brain barrier [111]. Zebrafish, which has a strong capacity to regenerate its CNS and a BBB to maintain brain homeostasis as in mammals, could be a particularly useful model to explore these effects in both the brain and other organ systems [47, 112]. For example, traumatic brain injury results in the activation of various metabolic processes in different regions of the zebrafish brain [44, 62]. Moreover, transcriptome analyses from larval and adult zebrafish models of early-onset familial or late-onset Alzheimer's disease have revealed significant changes in the energy metabolism-related genes and signaling pathways [113–115]. Interestingly, fatty acid metabolism was among the most significantly altered pathways in these models, implying impairment of energy production. Thus, injury- or disease-related brain damage appears to be associated with alterations in energy metabolism.

The Wnt signaling pathway is involved in brain development and function with key roles in the regulation of neurogenesis, synaptogenesis, synaptic plasticity, and neuronal plasticity/repair [116–119]. Moreover, Wnt signaling is deciphered to play a key role in the activation of the innate immune system and neuroinflammation in neurodegenerative diseases and mental disorders [35, 120–123]. Wnt/β-catenin signaling is activated in the early stages of brain regeneration, with a regeneration-promoting role [44, 124–127]. Our results display a prominent activation of Wnt/β-catenin signaling in the total brain tissue as well as in the olfactory bulb and the telencephalon in the brain, after prolonged exposure to HFD. To our knowledge, this is the first evidence of active canonical Wnt signaling in the brain in response to HFD feeding. Thus, Wnt/β-catenin signaling appears to be a primary pathway with a key role in the regulation of the regenerative response of the brain to different types of damage including traumatic brain injury and HFD feeding-induced brain damage.

In humans, obesity and overweight are associated with aggressive behaviors and anxiety/depression [128, 129]. HFD feeding of mice, rats, or zebrafish results in a similar increase in aggression and anxiety [6, 64, 65, 130]. Moreover, the offspring of rats fed an HFD during lactation exhibited delayed neurobehavioral development and an increase in depressive and aggressive behaviors (Giriko et al. 2013). Another study on mice has proposed that an HFD-induced increase in anxiety-like behavior may be associated with dopamine dysregulation (Han et al. 2020). We observed a similar increase in the anxiety and aggressiveness of zebrafish fed an HFD for the short- or long-term. Interestingly, there was a parallel increase in the locomotor activity

of fish fed an HFD, shown by both the novel tank diving and mirror-biting assays. Picolo et al. have recently reported that HFD feeding for the short term did not affect the locomotor activity in adult zebrafish [6]. However, those experiments were terminated on the 16th day of feeding, meaning that the fish were fed for a quarter to half the time we used. Elevated levels of anxiety have been associated with increased locomotor activities in zebrafish [70, 131–133]. Thus, HFD-induced anxiety may be accompanied by an increase in locomotion.

HFD-induced inflammation, apoptosis, and reactive neurogenesis in two different regions of the forebrain, i.e., olfactory bulb and telencephalon, appear to increase the anxiety and aggressiveness in zebrafish. Deficits occurring in the olfactory bulb have been associated with anxiety-like behaviors in humans and rodents, likely because the olfactory bulb is densely populated with glucocorticoid receptors responsible for stress resilience [134–136]. Impaired regeneration of the mammalian hippocampus, which has a homolog structure within the pallium of zebrafish telencephalon, likewise caused an increase in anxiety-related behaviors [137]. Further studies have uncovered a potent link between anxiety and disrupted regulation of neuronal excitability in the hippocampus [138]. Collectively, by triggering neuronal damage in the forebrain, HFD feeding appears to cause behavioral changes including anxiety and aggressiveness. Further research unveiling the levels of plasma neurotransmitters and expression of neurotransmitter receptors in different brain regions is needed to elucidate the impact of HFD feeding on these and other behavioral changes.

Conclusion

To sum up, our data strongly suggest that consumption of HFD triggers a non-traumatic regenerative response in the zebrafish brain. Since the regenerative capacity of the mammalian brain is very restricted, HFD feeding could be highly detrimental to the mammalian brain. Thus, revealing the molecular mechanisms that initiate the regeneration-like program of the zebrafish brain to HFD exposure may offer a solution to not only fight obesity but also heal non-traumatic injuries of the brain.

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Authors' Contributions GO, YA, and GC designed the experiments. YA, YKP, OO, DN, and DI performed the experiments. YA, YKP, OO, DN, and GC drafted the manuscript. GO wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability Not applicable.

Declarations

Ethics Approval and Consent to Participate The animal study protocol was approved by the Animal Experiments Local Ethics Committee of Izmir Biomedicine and Genome Center (IBG-AELEC) (2020–003, 12/02/2020).

Consent for Publication Not applicable.

Conflict of Interest The authors declare that they have no conflict of interest.

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