

# Limited Effects of Prolonged Environmental Enrichment on the Pathology of 5XFAD Mice

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**Abstract** The environmental enrichment (EE) paradigm is regarded as a useful tool to create a physical and intellectual stimulation for laboratory rodents and has been used in a variety of Alzheimer disease (AD) mouse models. However, the results of these studies have been conflicting as EE had inconsistent effects on memory performance, A $\beta$  deposition, inflammatory status and other pathological outcomes depending on the AD model. Here, we studied the influence of a lifelong EE on the widely used 5XFAD mouse model, representing the main pathological features of AD. Although 11 months of enriched housing led to an improved survival rate and a partial rescue of motor performance, no beneficial effects in terms of anxiety phenotype, working memory performance, A $\beta$  plaque load, A $\beta$ <sub>1–42</sub> levels, endogenous APP processing and inflammatory status were observed in 5XFAD mice. Concordantly, no changes in expression levels of BACE1 or A $\beta$ -degrading enzymes like neprilysin or insulin-degrading enzyme could be detected in active mice. The 5XFAD model develops a relatively fast and aggressive pathology and therefore presents a model for early onset familial AD. Our results suggest that an intervention like EE might be too mild to counteract the fast disease progression seen in this model. Therefore, our data provide evidence that the effects of physical and cognitive

stimulation vary depending on the severity of the pathology of the model and therefore might be more beneficial in models developing a milder AD phenotype.

**Keywords** 5XFAD · Alzheimer's disease · Amyloid · Transgenic mice · Enriched environment · Physical activity · Behavior

## Introduction

Numerous epidemiological studies have been published in recent years showing a protective effect of cognitive and physical activity on the progression of neurodegenerative diseases like Alzheimer's disease (AD) [1–3]. However, the underlying molecular mechanisms leading to improved brain function and memory upon physical and mental activity seem to be multiple and are still not understood in great detail. To substantiate the outcome of epidemiological investigations, various studies employing rodent AD models subjected to environmental enrichment (EE) paradigms have been conducted and led to rather inconsistent outcomes. While some studies found a decrease in A $\beta$  deposition or levels of oligomeric A $\beta$  species [4–7], others report constant A $\beta$  plaque loads [8–11] or even an increased A $\beta$  abundance [12] following enriched housing. In addition, variable cognitive outcomes have been described upon enriched living conditions, as shown by either improved [4, 13, 14] or unchanged [9, 11] performances in spatial learning tests like the Morris water maze (MWM) or radial arm water maze (RAWM). These controversial results could be due to different experimental enrichment paradigms, as well as variations in the amount of exercise. Furthermore, differences in the animal models used could lead to contrasting outcomes as each model portrays different aspects of AD pathology and progression.

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The 5XFAD model is a well-characterized and widely used AD mouse model expressing the two human mutant transgenes amyloid precursor protein (APP) and presenilin-1 (PSEN1). A combination of five AD mutations in these transgenes acts in an additive manner and leads to an accelerated plaque formation starting as early as 2 months of age, paralleled by astrogliosis and microgliosis. 5XFAD mice develop an age-dependent motor phenotype as well as behavioral deficits accompanied by synaptic degeneration, axonal degeneration, and a regional neuron loss. Even though the model lacks clear tau pathology, 5XFAD mice recapitulate the main features of AD and therefore mimic the pathology found in humans to a great extent [15, 16].

The objective of the current study was to investigate whether environmental enrichment combined with voluntary exercise ameliorates the advanced Alzheimer-like pathology in 5XFAD mice. Therefore, 5XFAD mice were assigned to either standard housing (SH) or EE conditions with 1 month of age, when pathology is still absent, for a duration of 11 months. With 12 months of age, when the full range of AD-like pathology is present in standard housed mice, motor, behavioral, as well as quantitative analysis of A $\beta$  plaque load and inflammatory status were carried out. Surprisingly, only a partial improvement of the motor phenotype was detected, while neither the behavioral phenotype or A $\beta$  plaque load, A $\beta$ <sub>1–42</sub> levels, nor the inflammatory phenotype of 5XFAD mice were significantly affected upon EE.

## Material and Methods

### Transgenic Mice

The generation of 5XFAD mice (Tg6799) has previously been described by Oakley and colleagues [15]. Briefly, 5XFAD mice overexpress the 695 amino acids isoform of APP (APP695) which carries the Swedish, Florida, and London mutations under the control of the murine Thy-1 promoter. Additionally, human PSEN1, carrying the mutations M146L and L286V, is expressed under the control of the murine Thy-1 promoter. 5XFAD mice used in the current study were backcrossed for more than 10 generations to C57Bl/6J wild-type mice (WT) (Jackson Laboratories, Bar Harbor, ME, USA) to obtain an incipient congenic line on a C57Bl/6J genetic background [16]. Twelve-month-old 5XFAD and age-matched control C57Bl/6J WT mice were tested. Only female mice were used in the current study. All animals were handled according to German guidelines for animal care.

### Housing Conditions

With 1 month of age, 5XFAD mice were randomly distributed to either standard housing (SH,  $n = 20$ ) or enriched housing (EE,

$n = 15$ ) conditions until the age of 12 months. WT mice housed under standard conditions served as a control group ( $n = 19$ ). For SH, standard laboratory cages were used (33 cm  $\times$  18 cm  $\times$  14 cm), while for the EE living conditions, large rat cages were used (55 cm  $\times$  34 cm  $\times$  20 cm). EE cages were equipped with three running wheels, nesting material, tunnels, shelters, houses, and toys, which were changed and rearranged weekly to increase the sense of novelty (Fig. 1a). Mice were housed in groups of 4–5 to ensure social interactions. In all conditions, food and water were provided ad libitum.

## Behavioral Tasks

### Balance Beam

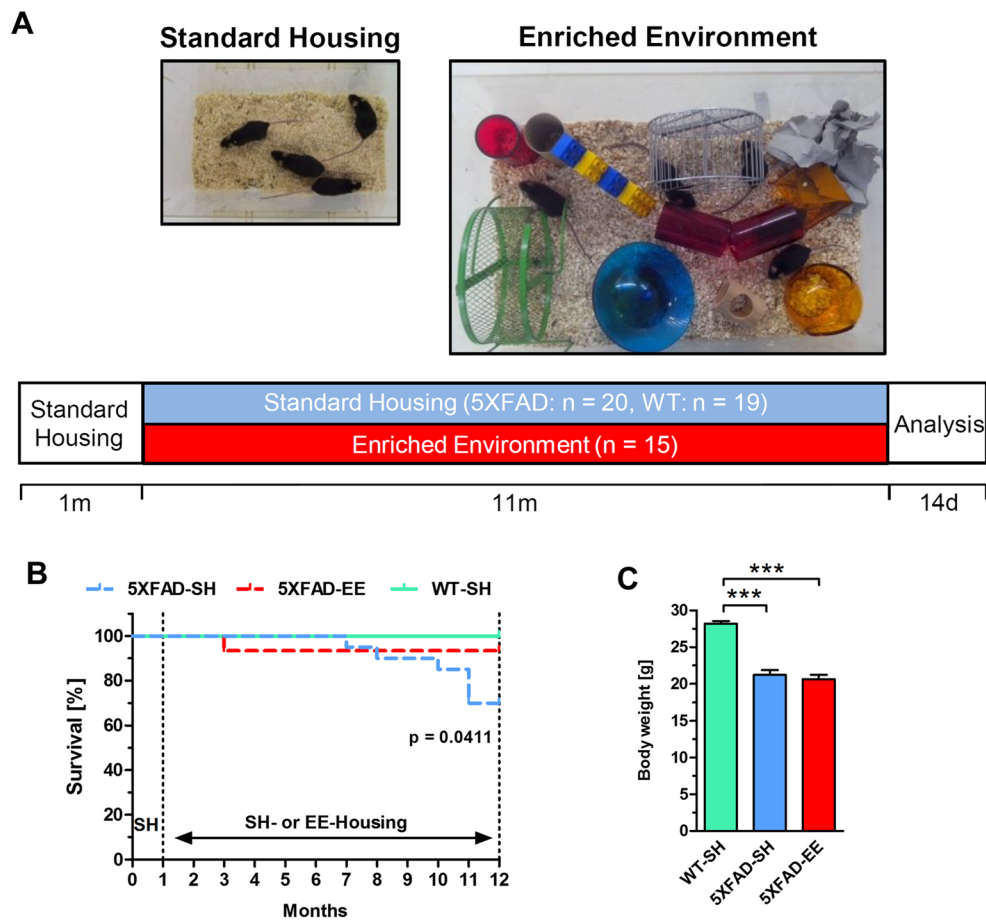
To analyze balance and general motor abilities, the balance beam test was conducted. A 1-cm-wide wooden beam is attached to two wooden support columns at a height of 44 cm. The surface is padded to protect against fall injuries. At either end of the 50-cm-long beam, a 9  $\times$  15-cm escape platform is attached. Mice are placed in the middle of the beam and released. The time for animals to fall from the beam is recorded. The test consists of 60-s trials with three consecutive trials on a single testing day. If a mouse remains on the beam for the whole 60-s trial or escapes to one of the platforms, the maximum time of 60 s is recorded.

### String Suspension

To test agility and grip capacity, the string suspension task was performed. Thereby, mice are allowed to grasp a suspended cotton string only by their forepaws. During a single 60-s trial, the performance of each animal is assessed using a 0 to 5 rating system: 0 = unable to remain on the string; 1 = hangs only by fore- or hind paws; 2 = as for 1, but with attempt to climb onto string; 3 = sits on string and holds balance; 4 = four paws and tail around string with lateral movement; 5 = escape to one of the platforms.

### Accelerating Rotarod

To assess motor coordination and balance, mice were tested in the accelerating rotarod task. Testing was performed on two consecutive days with four trials per day using a computer-controlled Rotarod system (TSE Systems, Bad Homburg). Each mouse was individually placed on the rod, which accelerated from 1 to 45 rpm over a maximal trial time of 300 s. Trials were terminated when animals fell off (or the maximum time was reached) and latency to descent (s) served as an indicator of motor coordination.



**Fig. 1** Exemplary picture of standard housing (SH) and enriched environment (EE) cages used for the experiments and schematic drawing of the experimental design. Four-week-old WT and 5XFAD mice were assigned to SH cages for 11 months. Another group of 5XFAD mice was assigned to EE cages for the same amount of time. With 12 months mice underwent a battery of behavioral tests followed by body weight assessment, sacrifice, and tissue collection (a). Kaplan-Meier survival

curve of mice held under standard or enriched conditions. 5XFAD-EE mice showed a statistical tendency toward a better survival compared to 5XFAD-SH mice (b). Housing under enriched conditions had no effect on the significantly lower body weight of 5XFAD mice (c). All data were given as means  $\pm$  standard error of the mean (SEM) (\*\* $p < 0.001$ ; \* $p < 0.05$ )

### Elevated Plus Maze

The elevated plus maze tests anxiety-related behaviors in rodents. The 75-cm raised maze (arm length 18 cm, arm width 5 cm, closed wall height 15 cm) is shaped like a “+” with two open and two closed arms extending from a central platform. The test is based on the conflict of the animals desire to explore a novel environment and the avoidance of elevated open arms due to the anxiety to fall. Therefore, the time spent in the open arms is an indication of the intensity of anxiety of the animal. Mice are placed individually into the centre field to explore the maze freely for 5 min. The percentage of time spent in the open arms as well as the ratio of open arm entries to total arm entries were measured using an automatic video tracking system (ANY-maze, Stoelting). After each mouse, the maze was cleaned with 70 % ethanol to standardize odors.

### Cross Maze

Spontaneous alternation rates were determined using the cross maze test [16]. The cross maze apparatus has four arms arranged in a 90° position extending from a central space of 8 × 8 cm. Each arm is 30 cm in length, 8 cm in width, and 15-cm high. During a 10-min test session, each mouse was placed in one arm and allowed to move freely through the maze while it was tracked using an automatic video tracking system (ANY-maze, Stoelting). An alternation was defined as successive entries into the four arms in overlapping quadruple sets (e.g., 2, 3, 1, 4 or 4, 2, 1, 3 but not 2, 3, 4, 2, see also [supplemental information](#)). The alternation percentage was calculated as the percentage of actual alternations to the possible number of arm entries. To standardize odors, the maze was cleaned with 70 % ethanol after each mouse.

### Morris Water Maze

The Morris Water Maze (MWM) was carried out as described previously [17]. In brief, mice were subjected to 3 days of cued training, during which a triangular flag marked the platform position. Between different trials ( $n = 4$  per day), both the location of the platform and the position where mice were introduced into the pool was changed. Twenty-four hours after the last trial of the cued training, mice performed another 5 days of acquisition training, in which the flag was removed from the platform which remained stationary for each mouse. Proximal visual cues were attached to the outside of the pool in addition to distal cues in the testing room. A probe trial used to assess spatial reference memory was carried out 24 h after the last acquisition trial. During this test, the platform was removed from the pool, and mice were introduced from a novel entry point. Mice were then allowed to swim freely for 1 min while their swimming path was recorded using an automated video tracking system (ANY-Maze, Stoelting). Differences in quadrant abidance were compared in a within-group design.

### Immunohistochemistry on Paraffin Sections

Mice were deeply anesthetized and transcardially perfused with 4 % PFA in PBS and brains were carefully dissected. Post fixation was carried out in 4 % buffered formalin at 4 °C before the tissue was embedded in paraffin. Immunohistochemistry was performed on 4- $\mu$ m sagittal paraffin sections cut on a rotation microtome (Microm, HM 335E, Thermo Fisher Scientific, Walldorf, Germany) as described previously [18]. In brief, paraffin sections were deparaffinized in xylene and rehydrated in a series of ethanol (100, 95, 70 %). After treatment with 0.3 %  $H_2O_2$  in PBS to block endogenous peroxidases, antigen retrieval was achieved by boiling sections in 0.01 M citrate buffer pH 6.0, followed by 3-min incubation in 88 % formic acid. Non-specific binding sites were blocked by treatment with skim milk and fetal calf serum in PBS prior to the addition of the primary antibodies. The following antibodies were used in this study: 4G8 (A $\beta$ 17-14, 1:10,000, Covance, Princeton, USA) and glial fibrillary acidic protein (GFAP) (#173002, Synaptic Systems, Göttingen). The primary antibody was incubated overnight in a humid chamber at room temperature followed by incubation with a biotinylated anti-mouse secondary antibody (DAKO, Glostrup, Denmark). Staining was visualized using the ABC method using a Vectastain kit (Vector Laboratories, Burlingame, USA) and diaminobenzidine (DAB) as chromogen providing a reddish-brown color.

### Quantification of A $\beta$ Plaque Load and GFAP Immunoreactivity

Extracellular A $\beta$  load (4G8) was evaluated in the primary motor cortex (Ctx), dentate gyrus (DG), and thalamus (Thal) using an Olympus BX-51 microscope equipped with a Moticam Pro 282A camera (Motic) and the ImageJ software package (V1.41, NIH, USA) for all animals used in the analysis. Serial images of  $\times 100$  magnification were captured on three sections per mouse which were at least 30  $\mu$ m apart from each other. Using ImageJ, pictures were binarized to 8-bit black and white images and a fixed intensity threshold was applied defining the DAB signal. Measurements were performed for a percentage area covered by DAB [19]. Accordingly, for GFAP staining quantification, images of  $\times 200$  magnification were captured and the astrocyte-covered areas were analyzed as described previously [20]. The relative A $\beta$  plaque load or GFAP immunoreactivity is expressed with 5XFAD-SH mice as the reference parameter. Five 5XFAD mice of each SH and EE group were used for the quantification. Unpaired *t* test was used to compare changes in plaque load or GFAP staining for each antibody.

### ELISA

Mice were anesthetized using  $CO_2$  asphyxiation, decapitated and brain hemispheres were dissected and immediately deep frozen. Frozen hemispheres of 12-month-old 5XFAD-SH and 5XFAD-EE mice ( $n = 8$  per group) were weighed and subsequently subjected to a sequential A $\beta$  extraction. First, brains were homogenized in 700  $\mu$ l Tris-buffered saline (TBS) buffer (120 mM NaCl, 50 mM Tris, pH 8.0 incl. Complete protease inhibitor cocktail (Roche)) per 100 mg tissue using a Dounce homogenizer (800 rpm). The resulting solution was centrifuged at 17,000 $\times g$  for 20 min at 4 °C. The TBS-soluble protein containing supernatant was stored at  $-80$  °C. The pellet was dissolved in 800  $\mu$ l of 2 % SDS and sonicated followed by a centrifugation step of 17,000 $\times g$  for 20 min at 4 °C. The supernatant, which contained SDS-soluble proteins, was transferred to a new tube containing 1  $\mu$ l of benzoylase and was rotated at room temperature (RT) for 10 min followed by storage at  $-80$  °C. Monoclonal antibody IC16 ([21]; 1:250 in PBS, pH 7.2), raised against amino acids 1–15 of the A $\beta$  sequence, served as a capture antibody. To generate standard curves, synthetic A $\beta$ 42 peptides (JPT Peptide Technologies) were used. These A $\beta$  peptides were solubilized in DMSO at 10  $\mu$ g/mL and aliquots were stored at  $-80$  °C. Ninety-six-well high-binding microtiter plates were incubated overnight at 4 °C with the capture antibody. After the capture antibody was removed, freshly diluted brain samples and freshly diluted A $\beta$  peptide standards (125–6000 pg/ml in PBS containing 0.05 % Tween-20, 1 % bovine serum albumin (BSA) were added. Subsequently, C-terminal detection antibodies specific

for A $\beta$ 42 (BAP-15; [22]) labeled with horseradish peroxidase (HRP) using the Pierce EZ-Link™ Plus Activated Peroxidase kit (Thermo Fisher Scientific) were diluted in PBS containing 0.05 % Tween-20, 1 % BSA, added to each well, and incubated overnight at 4 °C. Plates were washed three times with PBS containing 0.05 % Tween-20 and once with PBS. Then, 50  $\mu$ l of TMB ELISA peroxidase substrate (Interchim) was added and incubated for 1–10 min at RT in the dark. The reaction was stopped by adding 50  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub> and the absorbance was measured using a Paradigm microplate reader (Beckman Coulter) at 450 nm.

### SDS-PAGE

APP metabolites were analyzed by Western blotting in the TBS- and SDS-soluble brain fractions of mice housed under standard conditions or in an enriched environment. Equal amounts of protein of the TBS- or SDS-soluble fractions were mixed with 4 $\times$  SDS sample buffer, heated at 95 °C for 5 min, and resolved on 12 % Bis-Tris polyacrylamide gels. The proteins were transferred to an Immobilon-FL PVDF membrane (Millipore) by tank Western blotting. Membranes were blocked in PBS containing 3 % gelatin from cold water fish skin (Sigma-Aldrich) for 1 h and incubated overnight with the following primary antibodies diluted in TBST: polyclonal antibody CT-15 (1:3500; generous gift from Dr. Edward Koo, University of California, San Diego, USA) for the detection of full-length APP and C-terminal fragments (APP CTFs), monoclonal antibody 22C11 (1:1000; generous gift from Dr. Stefan Kins, University of Kaiserslautern, Germany) for the detection of the soluble APP ectodomain APPs, monoclonal antibody 6A1 (1:50; IBL International) against the C-terminal neo-epitope generated by  $\beta$ -secretase cleavage of human APP containing the “Swedish” mutation, and monoclonal antibody AC-74 against actin (1:2000; Sigma-Aldrich). Membranes were washed in TBST and subsequently incubated with LI-COR IRDye secondary anti-mouse and anti-rabbit antibodies (1:10,000 in TBST) for 1 h. Immunoreactive bands were visualized using a LI-COR Odyssey CLx Imager and quantified with the LI-COR Image Studio Software (version 2.1). The band intensities of full-length APP, APP CTFs, APPs, and APPs- $\beta$  were normalized to actin levels, and the values of five individual animals from either the standard housing or the enriched environment groups were averaged. The Western blotting experiments were repeated three times. For statistical analysis, the mean of the enriched environment group was expressed as percent of the control group (standard housing, set to 100) for each individual experiment. One sample *t* test was then used to compare the mean value of all three experiments of the enriched environment group to the mean of the control group (set to 100). Statistical testing was performed with GraphPad Prism V5 (GraphPad Software, San Diego, USA).

### Real-Time PCR

For real-time RT-PCR analysis, WT, 5XFAD-SH and 5XFAD-EE mice were used ( $n = 5$  per group). Mice were anesthetized and transcardially perfused with ice-cold 0.01 M PBS. Brain hemispheres were carefully dissected, snap frozen in liquid nitrogen, and stored at  $-80$  °C until further analysis. Deep frozen brain hemispheres were homogenized in 1 ml of TriFast reagent (Peqlab) per 100 mg tissue using a glass-teflon homogenizer (10 strokes, 800 rpm). RNA extraction and DNase digestion were performed according to the protocol of the manufacturer. Reverse transcription of the purified RNA samples was carried out using the First Strand complementary DNA (cDNA) Synthesis Kit (ThermoFisher) according to the protocol of the supplier. RT-PCR was performed using a Stratagene MX3000P Real-Time Cycler. The SYBR green based FastStart Universal SYBR Green (Roche) containing ROX as an internal reference dye was used for amplification. Primers were purchased from Eurofins MWG Operon as intron-spanning primer sets. Relative expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method [23]. Expression levels were normalized to housekeeping gene  $\beta$ -Actin and calibrated to average expression level of control animals for each gene [24]. The expression ratio results of the studied transcripts are tested for significance by unpaired *t* tests. The following primer sets were employed: BACE1-for: 5'-TGGTAGTAGCGATG CAGGAA-3'; BACE1-rev: 5'-ATGTGGAGATGACC GTAGGC-3'; BDNF-for: 5'-GCCTTCATGCAACC GAAGTA-3'; BDNF-rev: 5'-TGAGTCTCCAGGAC AGCAA-3'; DCX-for: 5'-TCATCTTGAGCATA GCGGAA-3'; DCX-rev: 5'-GGAAACCGGAGTTG TCAAAA-3'; GFAP-for: 5'- CTTTCTGACACGGA TTTGGT-3'; GFAP-rev: 5'-ACATCGAGATCGCC ACCTAC-3'; IDE-for: 5'-CAGGCATCGTTCAT CACATT-3'; IDE-rev: 5'-ACAGGTTTGCGCAG TTTTTC-3'; NEP-for: 5'-CCTCAGCCGAAACT ACAAGG-3'; NEP-rev: 5'-TTGCTCTCTCCAGC AAAAGC-3'; VGF-for: 5'- GTCAGACCCATAGC CTCCC-3'; VGF-rev: 5'-CTCGGACTGAAATC TCGAAGTC-3'.

### Statistical Analysis

Differences between groups were tested with either one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post-tests or unpaired *t* tests. Survival data were calculated using the log rank test. All data were given as means  $\pm$  standard error of the mean (SEM). All calculations were performed using GraphPad Prism version 5.01 for Windows (Graph Pad Software, San Diego, USA).

## Results

### The Effect of Environmental Enrichment on the Physiological Status of 5XFAD Mice

Four-week-old 5XFAD mice were randomly exposed to standard (SH) or enriched (EE) housing conditions until the age of 12 months. WT mice housed under standard conditions served as a control group (Fig. 1a). 5XFAD-EE mice showed a better survival rate compared to 5XFAD-SH mice. Whereas 93.3 % of the enriched 5XFAD mice reached the 12-month time point, only 70 % of the standard housed 5XFAD mice survived the entire enrichment period, so that 14 mice per group completed the entire paradigm. All WT mice survived until the 12-month time point (Fig. 1b). To analyze the physiological status of the animals at the end of either housing condition, the body weight was assessed. As previously demonstrated, 12-month-old 5XFAD mice show a significantly reduced body weight compared to WT littermates ( $p < 0.001$ ) [25], which could not be rescued by EE housing ( $p < 0.001$ ) (Fig. 1c).

### Environmental Enrichment Partially Rescues the Motor Deficits in 5XFAD Mice

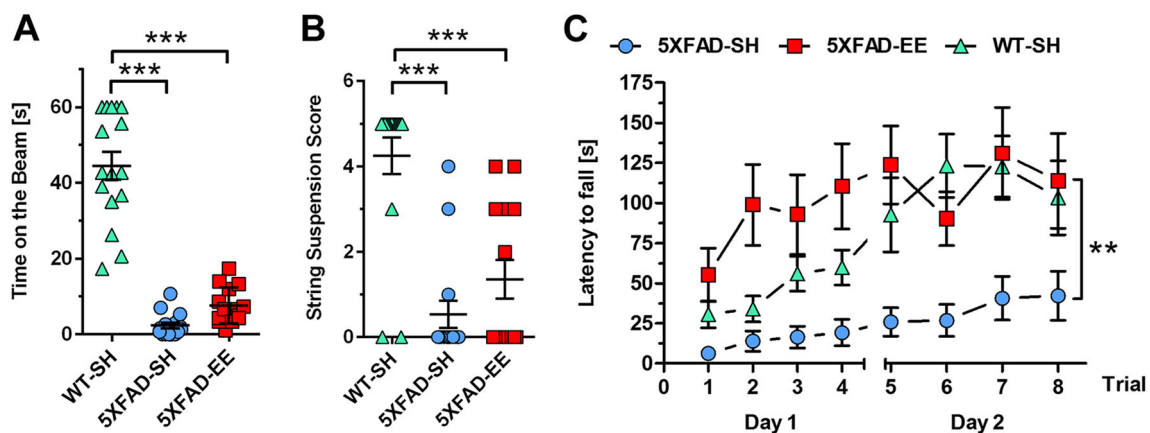
After 11 months spent in SH or EE living conditions, motor performance of the animals was analyzed using the balance beam, string suspension, and rotarod task (Fig. 2). 5XFAD-SH mice performed significantly worse than age-matched WT mice in the balance beam task ( $p < 0.001$ ). This phenotype could not be rescued after 11 months of enriched housing (Fig. 2a). Housing conditions also had no effect on the performance of 5XFAD mice in the string suspension task. Both standard and enriched housed 5XFAD mice performed poorly

on the string compared to WT mice ( $p < 0.001$  respectively) (Fig. 2b). In the rotarod task, motor coordination, as well as the typical phases of motor skill learning have been assessed. Over eight trials in 2 days, WT, 5XFAD-SH, and 5XFAD-EE improved their ability to stay on the rotarod. However, WT and 5XFAD-EE mice showed a significantly better performance on the rotarod compared to 5XFAD-SH mice as shown by overall higher latencies to fall ( $p < 0.01$  respectively) (Fig. 2c).

### Enriched Environment Fails to Restore Decreased Anxiety Levels and Working Memory Deficits in 5XFAD Mice

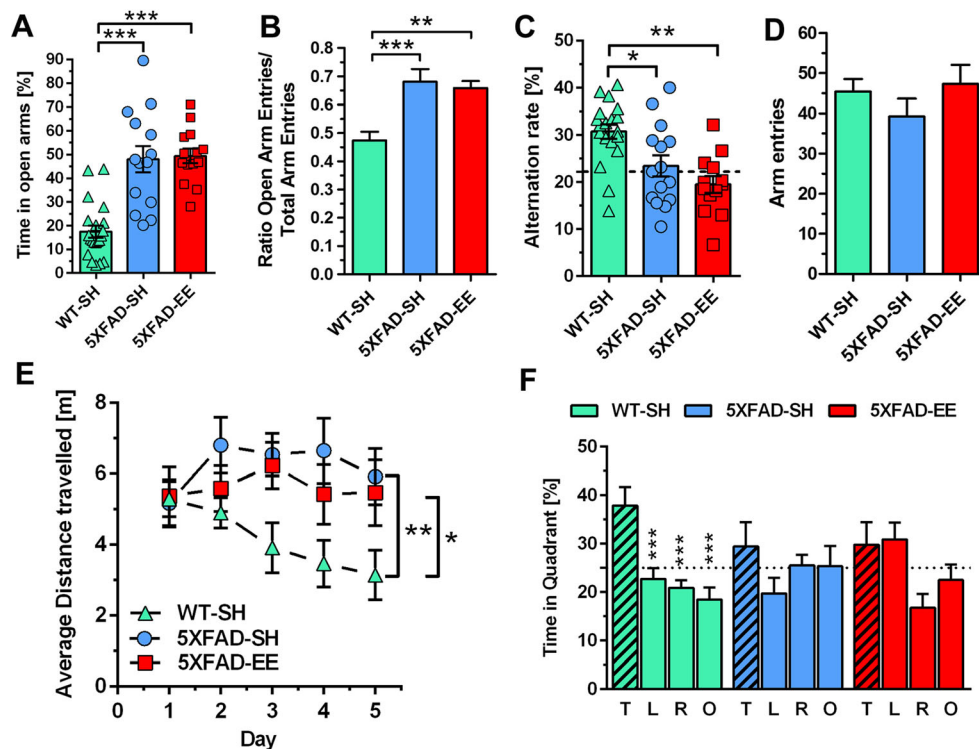
5XFAD mice show significantly low levels of anxiety in comparison with WT mice starting at the age of 6 months [16]. This disturbed anxiety phenotype persists in 5XFAD mice at 12 months of age and could not be influenced by enriched living conditions as shown by the time spent in open arms in the elevated plus maze test ( $p < 0.001$ ) (Fig. 3a). This was confirmed by calculating the ratio of open arm entries to total arm entries which revealed significant higher ratios in 5XFAD-SH and 5XFAD-EE mice compared with WT animals ( $p < 0.001$  and  $p < 0.01$ , respectively) (Fig. 3b).

To investigate if the housing condition has an effect on hippocampus-related spatial working memory, mice were tested in the cross maze task. As previously published, 5XFAD mice showed a significant impairment in the cross maze at 12 months of age compared to WT animals ( $p < 0.05$ ) [16]. 5XFAD-EE mice showed no improvement in spontaneous alternation behavior as they displayed even lower alternation rates compared to WT mice than 5XFAD-SH mice ( $p < 0.01$ ). The number of arm entries was unchanged, confirming that the reduced alternation percentage was not caused by a decrease in overall explorative behavior of 5XFAD mice



**Fig. 2** Environmental enrichment had a limited effect on the motor performance of 12-month-old 5XFAD mice. The balance beam (a) and the string suspension test (b) revealed no improvement in 5XFAD mice after EE. Only the rotarod test (c) showed a complete rescue of the

phenotype with 5XFAD-EE mice performing even better than WT mice. All data were given as means  $\pm$  standard error of the mean (SEM) (\*\*\*)  $p < 0.001$ ; \*\*)  $p < 0.01$



**Fig. 3** Environmental enrichment does not change the anxiety phenotype of 5XFAD mice. The elevated plus maze revealed that 5XFAD-SH and 5XFAD-EE mice show equally reduced anxiety levels, as time spent in open arms was significantly higher compared to WT animals in both groups (a). The ratio of total open arm entries to total arm visits showed the same pattern (b). Evaluation of working memory using the cross maze demonstrated that 5XFAD mice subjected to EE showed no improvement in spontaneous alternation behavior (*dotted line* indicates chance level)

(c). There was no difference in total arm entries between WT, 5XFAD-SH, and 5XFAD-EE mice (d). Both 5XFAD-SH and 5XFAD-EE mice showed deficits in spatial reference memory during acquisition training (e) and the probe trial of the Morris water maze task (f) compared to standard housed WT controls (*dotted line* indicates chance level). All data were given as means  $\pm$  standard error of the mean (SEM) (\*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ); T target, L left, R right, O opposite quadrants

(Fig. 3d). In order to investigate the effect of EE on spatial reference memory, the Morris water maze test was performed. 5XFAD mice showed deficits in the acquisition training (Fig. 3e) and the probe trial of the Morris water maze (Fig. 3f), as previously published [26], which could not be rescued by housing under EE conditions. Recognition memory was also tested using the novel object recognition test. However, all tested groups performed well in this task suggesting that 12-month-old 5XFAD mice harbor no impairment in object recognition memory (Supplemental Fig. S1).

### Enriched Environment Does Not Influence Amyloid Deposition and APP Processing in Brains of 5XFAD Mice

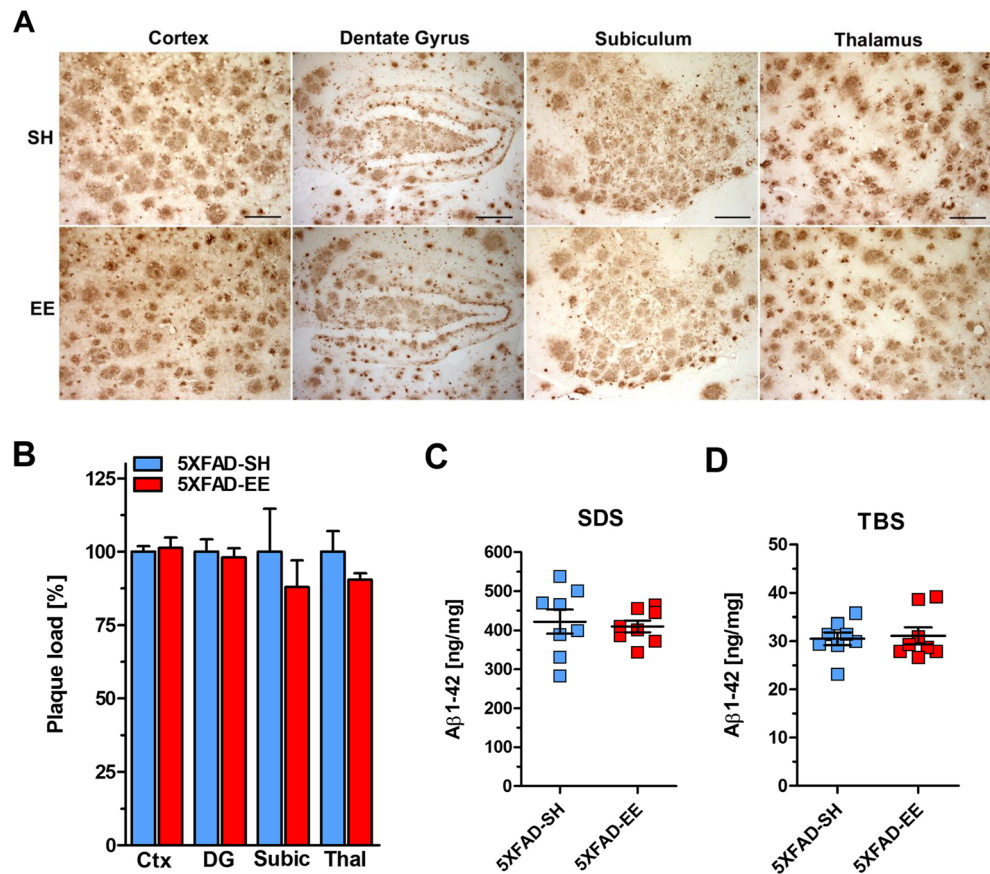
5XFAD mice show a massive A $\beta$  plaque load in various brain areas with 12 months of age [15, 16]. In order to determine whether amyloid deposition would be affected by voluntary exercise, a plaque load quantification was performed in cortex, dentate gyrus, subiculum, and thalamus of 5XFAD-SH and EE brains. Therefore, immunohistochemical analyses using an A $\beta$  (4G8) antibody to quantitatively examine A $\beta$  deposition were conducted. Brains of SH and EE 5XFAD

mice at 12 months of age showed a similar A $\beta$  plaque load in all analyzed areas (Fig. 4a, b).

As previously shown, A $\beta_{1-42}$  is the dominant A $\beta$  peptide present in brains of 5XFAD mice [27]. To analyze whether housing conditions influence its levels, A $\beta_{1-42}$  contents in TBS-soluble and insoluble (SDS-soluble) brain fractions were quantified using ELISA. No differences in A $\beta_{1-42}$  levels could be detected between standard and enriched housed 5XFAD mice (Fig. 4c, d).

In order to analyze whether long-term voluntary exercise has an effect on APP processing in vivo, SDS-Page and Western blots were performed. APP processing in the brains of 5XFAD-EE mice were compared to standard housed control animals. Full-length APP and APP CTFs were detected in the SDS-soluble fraction (Fig. 5a, b) and APPs- $\beta$  as well as APPs total were detected in the TBS-soluble fraction (Fig. 5c, d). Levels of full-length APP, APP CTFs, APPs, and APPs- $\beta$  remained unchanged upon long-term enrichment in 5XFAD mice. Therefore, physical activity does not seem to affect APP processing in the mouse brain.

**Fig. 4** Plaque load quantification in cortex (Ctx), dentate gyrus (DG), subiculum (Subic) and thalamus (Thal) using a pan A $\beta$  antibody (4G8) showed that living condition had no effect on A $\beta$  plaque load in any of the regions (a, b). Quantification of A $\beta_{1-42}$  using ELISA showed no differences in SDS (c) and TBS (e) fractions in standard or enriched housed 5XFAD mice. All data were given as means  $\pm$  standard error of the mean (SEM). Scale bar = 200  $\mu$ m



### Housing Conditions Did Not Change the Inflammatory Phenotype of 5XFAD Mice

5XFAD mice start to develop amyloid deposits and exhibited neuroinflammation as early as 2 months of age [15]. To determine whether long-term voluntary exercise prevents activation of inflammatory pathways, immunohistochemical stainings and subsequent quantifications with the reactive astrocyte marker GFAP were performed. No differences in GFAP signal could be detected in cortex, dentate gyrus and thalamus between standard and enriched housed 5XFAD mice with 12 months of age (Fig. 6a, b). RT-PCR analysis using primers against GFAP were carried out in 12-month-old WT, 5XFAD-SH, and 5XFAD-EE mice. 5XFAD-SH showed significantly increased GFAP messenger RNA (mRNA) levels compared to WT littermates ( $p < 0.01$ ). Surprisingly, 5XFAD mice housed under enriched conditions showed even higher GFAP mRNA levels (Fig. 6c,  $p < 0.001$  compared to WT and  $p < 0.05$  compared to 5XFAD-SH respectively).

### Gene Expression Changes in 5XFAD-EE Mice

Numerous studies have shown that the induction of brain-derived neurotrophic factor (BDNF) mRNA and protein is one of the crucial events that underlies cognitive enhancement

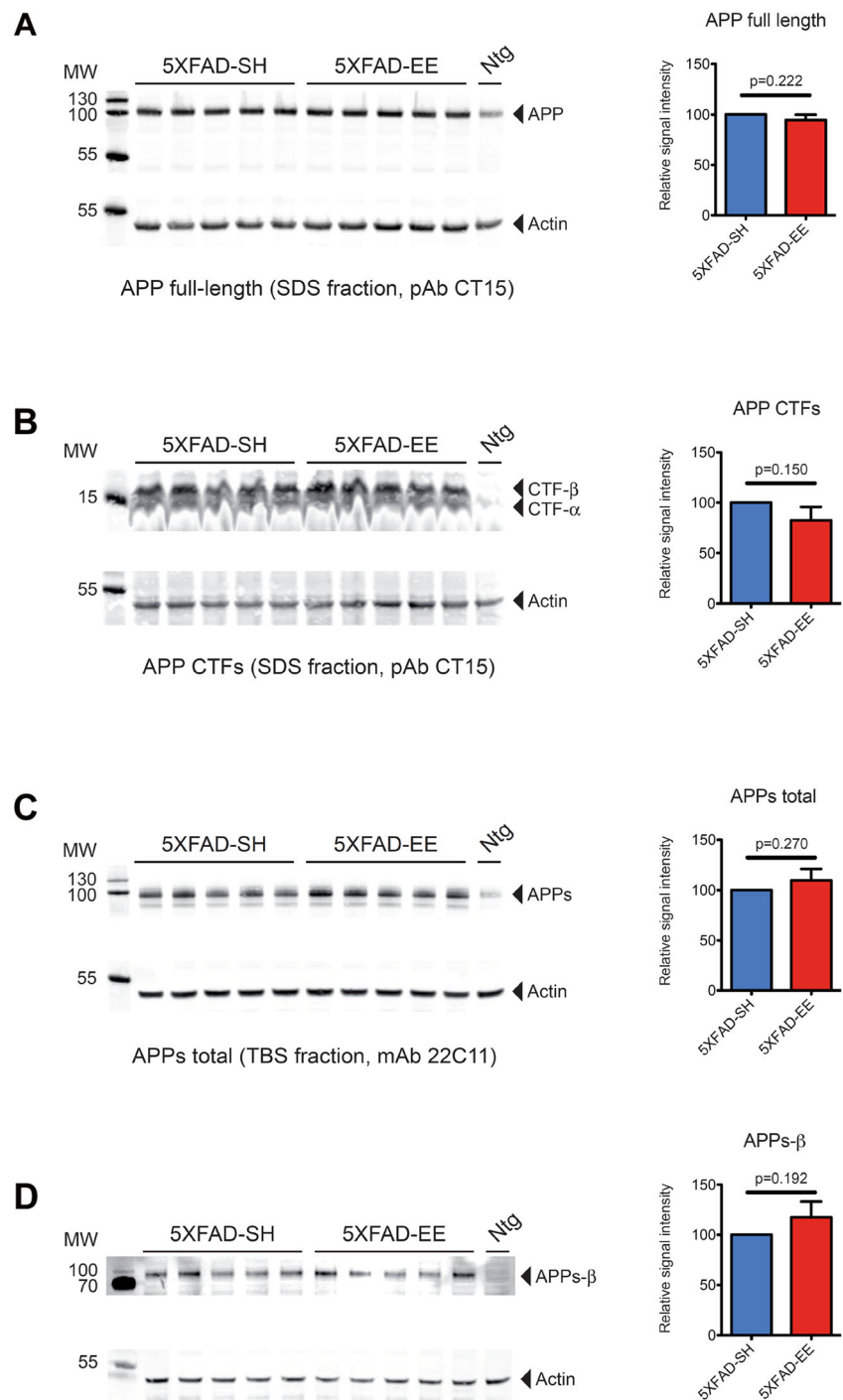
in response to physical activity [28, 29]. In order to test whether BDNF mRNA levels are changed in the brains of enriched housed 5XFAD mice, RT-PCR analysis of whole brain hemispheres of WT, 5XFAD-SH, and 5XFAD-EE mice were performed. BDNF expression levels were slightly decreased in 5XFAD-SH mice compared to WT controls, as previously published. However, upon environmental enrichment, expression levels of BDNF were significantly increased in 5XFAD-EE mice reaching WT levels (Fig. 7).

Next to the induction of neurotrophins, it has been reported that enriched environment in combination with voluntary exercise enhances the proliferation, survival, and maturation of newborn neurons in the dentate gyrus [30, 31]. To examine whether EE induces neurogenesis in 5XFAD mice, mRNA levels of doublecortin (DCX), a marker for immature neurons, have been analyzed. However, no significant differences in DCX levels could be detected in WT, 5XFAD-SH, and 5XFAD-EE mice.

Another neurogenesis-related gene which has been shown to be upregulated upon exercise is VGF nerve growth factor inducible (VGF) [32]. We therefore assessed the expression levels of VGF in WT as well as standard and enriched housed 5XFAD mice. Interestingly, 5XFAD-SH mice displayed significantly reduced VGF levels compared to WT controls. This phenotype could not be reversed by



**Fig. 5** Analysis of APP processing in 5XFAD mice after prolonged enriched environment. No changes were seen in the levels of full-length APP or APP C-terminal fragments (CTFs) in SDS fractions between standard and enriched housed 5XFAD mice using antibody CT-15 directed against the APP C-terminus (**a**, **b**). The same holds true for total secreted APP in the TBS fraction using antibody 22C11 (**c**) or APPs- $\beta$  using antibody 6A1 (**d**)



EE, as 5XFAD-EE mice showed similar VGF expression levels like 5XFAD-SH mice.

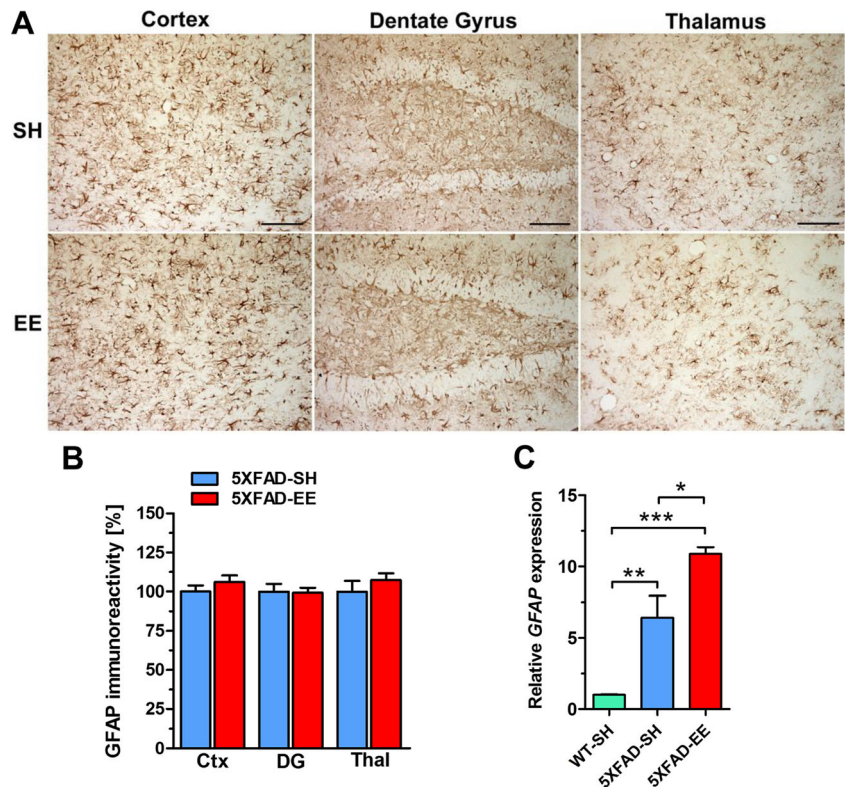
Several investigations have reported increased levels of A $\beta$ -degrading enzymes like neprilysin (NEP) upon EE [33, 34]. It has been previously shown that 5XFAD mice show decreased levels of NEP compared to age-matched WT controls, which could be confirmed in our study. However, 5XFAD-EE mice showed equally low levels of NEP like 5XFAD-SH mice. Accordingly, other proteases known to

influence A $\beta$  levels such as insulin-degrading enzyme (IDE) and the  $\beta$ -secretase BACE1 did not show expression level changes upon prolonged enriched living in 5XFAD mice.

## Discussion

The aim of the present study was to examine whether an enriched environment paradigm combined with voluntary

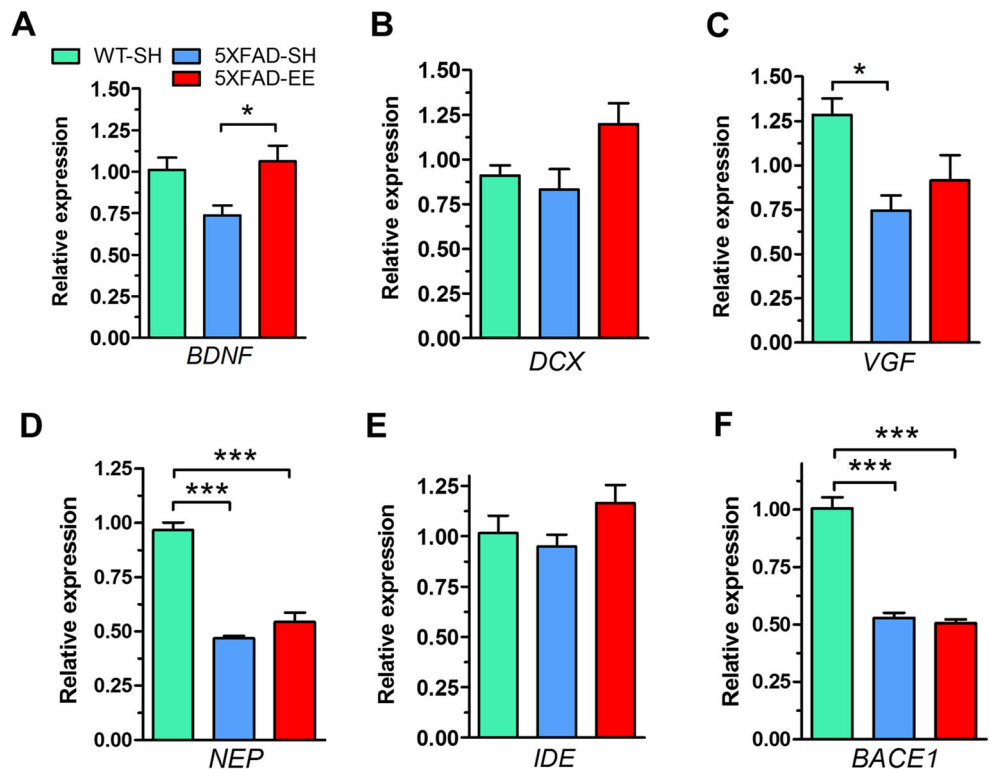
**Fig. 6** Using an antibody against the astrocytic marker GFAP, 5XFAD-SH, and 5XFAD-EE mice showed comparable immunoreactivity in all three brain regions analyzed (a, b). Real-time PCR analysis revealed increased *GFAP* mRNA levels in 5XFAD-SH and 5XFAD-EE mice compared to WT controls. Compared to 5XFAD-SH mice, enriched animals show even higher *GFAP* levels. *Ctx* cortex, *DG* dentate gyrus, *Thal* thalamus. All data were given as means  $\pm$  standard error of the mean (SEM) (\*\* $p < 0.001$ ; \*\* $p < 0.01$ ;  $p^* < 0.05$ ). Scale bar = 100  $\mu$ m



exercise is effective in an AD mouse model showing a relatively early and aggressive presentation of AD-related

phenotypes. The enrichment paradigm started prior AD pathology progression, mimicking a preventative approach, and

**Fig. 7** Gene expression changes following long-term enriched environment in 5XFAD mice. WT-SH, 5XFAD-SH, and 5XFAD-EE mice showed similar levels of *DCX* (b) and *IDE* (e). *BDNF* levels are increased in 5XFAD-EE mice (a), while *VGF* (c), *NEP* (d), and *BACE1* (f) expression are decreased in standard and enriched housed 5XFAD mice compared to WT controls. Levels of significance were labeled as follows: \*\*\* $p < 0.001$ ; \* $p < 0.05$



was carried out over 11 months. At the end of the treatment period, 5XFAD-SH mice display a strong motor phenotype, severe amyloid pathology accompanied by massive inflammation in various brain areas, as well as behavioral deficits. Despite abundant studies showing that environmental enrichment in combination with physical activity can slow down or even prevent the progression of Alzheimer-like pathologies, the 5XFAD mouse model fails to show significant improvement after a lifelong continuous enrichment.

AD mouse models frequently show reduced survival rates which can possibly be explained by cerebral vascular damage caused by heavy amyloid deposition [35, 36], but also early life stress has been suggested as a confounding factor [37]. As reported previously, we could confirm a premature death phenotype of 5XFAD mice compared to WT animals [38]. These authors also reported an even reduced survival when 5XFAD mice were crossed with Tg30 Tau transgenic mice, together with an aggravated motor phenotype. In the present study, the reduced survival could be rescued upon enriched conditions as 5XFAD-EE mice showed significantly higher survival rates than sedentary 5XFAD mice. One possible explanation might be the mitigated motor phenotype as shown by an improved rotarod performance. This is consistent with findings from human studies showing that individuals diagnosed with AD display prolonged survival the more physically active they are [39].

Severe sensory-motor impairments in the string suspension task and beam walking have been previously reported for 5XFAD mice starting with 9 months of age [16]. This phenotype could not be rescued by a lifelong cognitive and physical stimulation. However, with 12 months of age, 5XFAD mice display an abnormal rotarod performance, which was completely reversed upon enrichment. The significant rescue of the rotarod performance points to a regular interaction of 5XFAD mice with the enriched environment, specifically the running wheels, and therefore, together with the prolonged survival rate, confirms the effectiveness of the intervention performed in this study.

5XFAD mice demonstrate reduced anxiety levels starting with 6 months, which are aggravated in an age-dependent manner [16]. Environmental enrichment in combination with exercise was not capable to compensate anxiety-related and risk assessment behavioral deficits. This is consistent with recent data from our group showing no changes in lower levels of anxiety-like behavior in the APP/PS1KI mouse model after 4 months of EE [8]. In contrast, Verret and colleagues recently reported a restoration of abnormally low anxiety levels in Tg2576 mice when EE occurred early in the animal's lifespan [40]. Interestingly, the disturbed anxiety phenotype of Tg2576 mice could not be re-established when the EE paradigm was started at a later time point in the animal's lifespan, when AD pathology was already more severe.

Many reports on enriched environment or increased physical activity in AD mouse models displayed clearly improved

performances in cognitive tests [10, 41, 42]. However, we were unable to detect an improvement in working memory upon enriched living conditions in 5XFAD mice using either cross maze or MWM tasks. This is again consistent with results from a previous study employing an AD model with severe pathological alterations, where standard- and enriched housed APP/PS1KI mice showed equally bad performances in the Y-maze task compared to age-matched WT controls [8]. APP/PS1KI mice, as well as the 5XFAD mice employed in the current study, differ from most other AD mouse models in terms of their early and aggressive phenotype. While other models like TgCRND8 or APP/PS1ENdE9 mice also display an early onset of amyloid deposition and a reduced amyloid burden following enriched housing [33, 43], the presence of intraneuronal A $\beta$  and neuron loss in APP/PS1KI [44] and 5XFAD [16, 45] might be a key distinctive feature [46].

In the current study, we were also unable to detect any effect on amyloid plaque pathology upon enriched environment living conditions. Previous reports on the influence of EE on extracellular plaque pathology in transgenic AD mouse models are inconsistent. While some studies are in good agreement with our observations, reporting no effect of enriched living conditions on amyloid deposition [8, 10, 47–49], others found a reduction [33, 43] or even increased A $\beta$  plaque deposition [12, 50]. 5XFAD mice harbor a robust plaque pathology which is preceded by intraneuronal accumulation of A $\beta$  starting at the age of 6 weeks. With 2 months of age, first plaques appear in deep layers of the cortex and in the subiculum, which progresses during aging to the entire cortex, subiculum, and hippocampus. To a lesser extent, plaques also become apparent in thalamus, olfactory bulb, and brainstem [15]. The EE starting time point of the current study was chosen before the onset of plaque deposition, as there are studies showing that reduced plaque pathology can be only observed when exercise starts in the presymptomatic phase [9, 13]. We were not able to detect differences in A $\beta$  plaque load in cortex, hippocampus, subiculum or thalamus between enriched and sedentary 5XFAD mice at 12 months of age. Notably, it has been reported earlier that there is a rapid increase in amyloid pathology in 5XFAD mice until the age of 6 months, which subsequently becomes less severe and reaches a certain plateau level at the age of 10 to 14 months, depending on the sex of the animal [25, 51]. Therefore, one cannot exclude that the enrichment paradigm leads to a certain delay in plaque deposition at earlier time points during disease progression, which, by the end of the treatment period is not detectable due to the reached plateau level of amyloid plaques. To test this hypothesis, plaque pathology could be analyzed at earlier ages during the EE treatment period in a time-dependent manner.

Beauquis and colleagues recently reported reduced A $\beta_{1-40}$  and A $\beta_{1-42}$  levels upon exposure to environmental enrichment despite of an unchanged number and size of A $\beta$  plaques

[52]. This is consistent with findings from Rao et al., showing significantly decreased levels of soluble and insoluble  $A\beta_{1-40}$  and  $A\beta_{1-42}$  levels in both cortex and hippocampus upon voluntary exercise [53]. There are multiple mechanisms described which could possibly be involved in lowering  $A\beta$  levels due to exercise and thereby improving cognitive outputs. For example, Rao et al. found reduced protein levels of  $\beta$ -secretase (BACE1) in active APP/PSEN mice compared to non-active mice, suggesting reduced APP processing. Moreover, increased levels of  $A\beta$ -degrading enzymes (ADEs) like neprilysin (NEP) and insulin-degrading enzyme (IDE) after EE paradigms have been reported, supposedly contributing to an increased  $A\beta$  peptide degradation [33, 34]. However, in our study, no differences in soluble and insoluble  $A\beta_{1-42}$  levels could be detected. Accordingly, BACE1, NEP, and IDE levels were unchanged between active and inactive 5XFAD mice and no differences in APP processing could be detected.

Neuroinflammation, characterized by activation of astrocytes and microglia, is one of the major hallmarks of AD and develops in parallel to extracellular amyloid deposition in the 5XFAD mouse model [15]. Many reports describe a decline in inflammatory markers upon increased exercise accompanied by reduced  $A\beta$  levels [54–56]. In good agreement with unchanged amyloid plaque load, our findings show no changes in the inflammatory status between enriched and sedentary 5XFAD mice in the analyzed brain areas cortex, hippocampus, and thalamus. Surprisingly, exercised 5XFAD mice displayed even higher *GFAP* mRNA levels compared to standard housed animals. We were not able to detect changes in *GFAP* on the protein level by means of immunohistochemistry; however, recent reports describe a significant increase in the surface and volume of *GFAP*-positive profiles upon enriched housing or exercise in both control and 3xTg AD mice [57], while the age-dependent increase in microglia density could be reduced in 3xTg mice in enriched mice [58].

In agreement with a previous investigation, we detected decreased *BDNF* levels in standard housed transgenic animals compared to WT controls. Also, other AD-like mouse models were described to display lowered *BDNF* levels, which is linked to their observed pathology including synaptic and cellular loss [59]. Moreover, AD patients show significantly reduced *BDNF* brain levels when compared to healthy individuals. In active 5XFAD mice, *BDNF* levels increased significantly but were not significantly elevated compared to WT animals. This finding is in good agreement with numerous studies showing elevated expression levels of the neurotrophic factor *BDNF* following voluntary exercise in both humans and rodents [60] and consequently further indicates a successful EE protocol and proper use of the running wheels throughout the enrichment period. Increased *BDNF* levels were also found in C57Bl6 WT mice which have been subjected to long-term enriched living conditions using exactly the same

paradigm [17]. An increasing amount of literature supports the hypothesis that *BDNF* plays an important role in the neuroprotective effects seen upon physical activity, but the precise mechanisms underlying the positive effects remain uncertain [61]. However, in our study, increased *BDNF* levels in 5XFAD-EE mice were not associated with beneficial effects of exercise on cognitive impairment. Accordingly, Liu and colleagues even found an exercise induced decrease of *BDNF* mRNA levels in APP/PS1 mice paralleled by a better cognitive performance and LTP [62]. Hence, further studies will be needed to determine the influence of growth factors like *BDNF* on neuronal changes upon exercise.

In conclusion, the effect of a permanent enriched environment combined with voluntary exercise was analyzed in the 5XFAD model, which progressively develops numerous pathological hallmarks of AD and therefore represents a robust and aggressive AD mouse model mainly reflecting familial AD. Despite partial benefits on motor performance and a prolonged survival, no effects on anxiety levels, working memory performance, plaque deposition,  $A\beta_{1-42}$  levels, APP processing, or inflammatory markers could be observed. These results suggest that a lifelong cognitive and physical stimulation did not exert therapeutic benefits with regard to AD-like pathological changes in 5XFAD mice, although positive effects have been found in numerous other familial AD mouse models.

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