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Environmental enrichment improves hippocampal function in aged rats by enhancing learning and memory, LTP, and mGluR5-Homer1c activity

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ABSTRACT

Previous studies from our laboratory have shown that environmental enrichment (EE) in young rats results in improved learning ability and enhanced metabotropic glutamate receptor–dependent long-term potentiation (mGluR-dependent LTP) resulting from sustained activation of p70S6 kinase. Here, we investigated whether 1-month EE is sufficient to improve hippocampus-dependent learning and memory and enhance hippocampal LTP in 23–24 month-old Fischer 344 male rats. Aged rats were housed in environmentally enriched, socially enriched, or standard housing conditions. We find that aged rats exposed to 1-month of EE demonstrate enhanced learning and memory relative to standard housed controls when tested in the Morris water maze and novel object recognition behavioral tasks. Furthermore, we find that environmentally enriched rats perform significantly better than socially enriched or standard housed rats in the radial-arm water maze and display enhanced mGluR5-dependent hippocampal LTP. Enhanced hippocampal function results from activity-dependent increases in the levels of mGluR5, Homer1c, and phospho-p70S6 kinase. These findings demonstrate that a short exposure of EE to aged rats can have significant effects on hippocampal function.

1. Introduction

A limited number of molecular targets have been identified to treat age-related memory disorders such as mild cognitive impairment during normal aging or Alzheimer's disease. Environmental enrichment (EE) preserves cognition in the senescent brain, but the genes and molecular mechanisms are only beginning to be delineated (Hu et al., 2013; Lansade et al., 2014; Paban et al., 2011; Rampon et al., 2000; Sato et al., 2013). Although humans with high cognitive activity have a lower risk for Alzheimer's disease, little is known concerning the mechanisms that give rise to the functional benefits of EE. Rodent models of aging have been used to study the effects of EE on cognition in normal aging and neurodegenerative disease (Frick et al., 2003; Kumar et al., 2012; Laviola et al., 2008; Lazarov et al., 2005). EE enhances performance in multiple well-established memory assessing behavioral tasks including the Morris water maze (MWM) and object/odor recognition; both of which are known to decline with age in humans (Evans et al., 1984; Frick et al., 2003; Sharps and Gollin, 1987; Vaucher et al., 2002). In addition to behavioral benefits, EE is also known to enhance neural plasticity and morphology in areas of the brain that are involved in mnemonic processes, such as hippocampus and cortex (Faherty et al., 2003; Foster and Dumas, 2001; Foster et al., 1996; Green and Greenough, 1986; Hullinger et al., 2015; Kumar et al., 2012; Leggio et al., 2005; Malik and Chattarji, 2012). A number of studies suggest that rodents benefit more from EE throughout their lifespan when exposed at an early age, with a short EE exposure in young rats early in life improving cognitive ability to an equal degree as when animals are exposed to EE for their entire life (Fuchs et al., 2016; Harati et al., 2011). On the other hand, when aged rats are exposed to EE late in life, they do not perform as well, suggesting EE late in life is not as beneficial (Fuchs et al., 2016). In contrast to these data, other studies have shown that initial EE

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exposure in senescent animals has appreciable benefits (Harburger et al., 2007; Kobayashi et al., 2002; Kumar et al., 2012; Speisman et al., 2013; Stein et al., 2016). In addition to time-of-enrichment exposure, there has been considerable interest in the effects surrounding the specific type of enrichment (e.g., EE vs. social enrichment [SE]). Studies have concluded that EE has a more profound effect on cognitive improvement compared with SE alone following insult or injury, but both SE and EE improve cognition beyond standard housing conditions (SC, i.e., no form of enrichment; Gajhede Gram et al., 2015; Sozda et al., 2010). We have previously shown in young rats that 4 months of EE, and not SE, is sufficient to improve learning ability and enhance metabotropic glutamate receptor–dependent long-term potentiation (mGluR-dependent LTP) via a mechanism involving activation of p70S6 kinase (p70S6K) in the hippocampus (Hullinger et al., 2015). In this study, we investigated whether late-life exposure during senescence would impact hippocampal function similar to what we observed with early-life exposure. We hypothesized that EE exposure in aged rats would be more beneficial than SE in enhancing behavioral and cellular hippocampal function. We selected a 1-month time frame for enrichment based on several studies that have provided evidence that a short EE exposure is sufficient to improve spatial memory and synaptic plasticity in aged rats, whereas a longer period is required for young animals (Harburger et al., 2007; Hullinger et al., 2015; Stein et al., 2016). Here, we investigated how EE impacts mGluR-dependent LTP and molecular pathways involved in enhanced cognition and proposed a potential model of successful cognitive aging. Three housing conditions were used for this study: EE, SE, and SC. These housing conditions were designed to highlight the specific benefits of EE by controlling for independent cognitive effects that may result from social interactions alone and/or increased activity due to animal housing in larger cages. Furthermore, our housing conditions reduce the ambiguity within some studies that term SE conditions as animals housed in pairs and in standard cages with the SC animals (i.e., animals treated in standard housing conditions) housed in isolation (Frick and Benoit, 2010; Manosevitz and Pryor, 1975; Will et al., 2004). Thus, we can determine if any mechanistic differences may exist between EE and SE, much like our previous reports in young rats. Here, we show that 23- to 24-month-old rats display enhanced learning ability and plasticity following only 1 month of EE. We find that enhancement in synaptic plasticity relies on mGluR5-Homer 1 activity, as well as phosphorylation of p70S6K. Similar to our previous findings in young rats, this enhanced plasticity and persistent p70S6K activation occur only in EE rats (i.e., rats exposed to environmental enrichment) and not in SE rats (i.e., rats exposed to social enrichment) or SC rats (Hullinger et al., 2015). These results indicate that a short exposure (1 month) to EE in aged rats (23–24 months) is sufficient to improve hippocampal function.

2. Methods

2.1. Animal subjects

Twenty-two-month-old Fischer 344 (F344) rats were purchased from the National Institute of Aging (NIA) rodent colony. All animals had free access to water and food, and 12-hour dark and light cycles were maintained. Behavioral tests were performed during the dark cycle. Animals were housed as previously described (Hullinger et al., 2015). All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee and were conducted in accordance with the U.S. National Institutes of Health "Guide for the Care and Use of Laboratory Animals". Animals that became ill during the course of the enrichment and experimentation were excluded from the study. We typically observed approximately 10%–20% attrition because of health issues associated with age in this F344 rat strain (Coleman et al., 1977). Animals were monitored at least 3 times a week by the members of the laboratory in addition to the animal facility staff.

2.2. Housing conditions

The enrichment paradigm has been described in detail (Hullinger et al., 2015). Three housing conditions were used for this study: EE, SE, and SC. EE cages housed 6 rats in 60 × 60 cm plastic cages equipped with objects that included PVC pipes, plastic huts, and plastic tubes. Cages were changed weekly with the object type and location swapped to maintain novelty. SE cages housed 6 rats in 60 × 60 cm plastic cages with no objects other than normal bedding, and they served as a control to determine whether SE alone was beneficial for the animals. SC cages housed 2 rats in standard housing cages provided by the University Laboratory Animal Resources. Rats were normally housed in pairs at the Charles River NIA colony and shipped in boxes containing 5 animals each. Upon arrival to the University of Wisconsin animal facility, 22-month-old male rats were divided randomly such that each age group consisted of 6 EE rats, 6 SE rats, and 6 SC rats. The animals were housed in these conditions for 1 month before carrying out any behavioral, biochemical, or electrophysiological experiments. Behavioral experiments were carried out using a total of 3 cohorts consisting of members of each group (EE, SE, and SC; Table 1). For cohort 1, we carried out 7 days of MWM followed by a probe trial on day 7. All animals performed equally well by day 7 in both distance to find platform and probe trial on the last day of testing. Based on the data from cohort 1 indicating that there were statistical differences in behavioral ability between groups on day 6, we carried out 6 days of MWM followed by a probe trial with cohort 2. Therefore, probe trial data were only available for cohort 2 on day 6 (Table 1 and Fig. 1B). Distance to find platform data for the first 6 days of hidden platform training were pooled from cohorts 1 and 2 (Fig. 1A).

Animals were weighed on a weekly basis to determine whether levels of activity were different between groups and to monitor health (Fig. 1E). Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was used to analyze weight data. Experimenters were blind to housing conditions.

2.3. Behavior

2.3.1. Novel object recognition

This task has been described in the study by Hullinger and Burger (2015). In brief, on the first day of the paradigm (training day), rats were trained on the locations of 2 identical objects. Miniature flamingo figurines were used. Testing of object recognition memory occurred 24 hours after training. During testing, one of the flamingo figurines was replaced with a miniature figurine of finches, and rats were tested on their preference for the novel

Table 1
Cohorts of animals used for this study

Cohort	EE	SE	SC	NOR	MWM	RAWM
Cohort 1	5	5	4	X[a]	X 7 d + probe	
Cohort 2	6	6	3	X	X 6 d + probe	
Cohort 3	6	6	6			X

Uneven number of animals resulted from loss of aged rats due to death or illness during experimentation.
Key: EE, environmental enrichment; MWM, Morris water maze; NOR, novel object recognition; SC, standard housing conditions; SE, social enrichment; X, behavioral experiments carried out for a given cohort.
[a] 1 SC animal was lost before MWM testing: NOR n = 7 SC and MWM n = 6 SC.

object over the old object. Objects had been pre-tested for saliency using a different group of rats to ensure that the animals investigated both figurines equally, indicating that the objects were equally interesting to the animals. Rats were given 5 minutes to explore the objects freely.

2.3.2. Morris water maze

Morris water maze was performed as previously described with a few modifications (Hullinger et al., 2015). Animals were first tested for visual and swimming ability in a visible platform session consisting of 4 trials per day for 2 days. The hidden platform version of the MWM was performed on the day after the last visible platform training and consisted of 4 consecutive trials per day for 6 days (7 days on the first pilot experiment). At the end of trial 4 on the last day of hidden platform training, the platform was removed and a probe trial lasting 60 seconds was performed.

2.3.3. Radial-arm water maze

Radial-arm water maze (RAWM) was carried out as previously described with some modifications (Gerstein et al., 2012). The task involved 1 day of habituation, followed by 4 days of training. Rats were given 3 trials per day, with each trial lasting 90 seconds or until the animal found the platform. If the rat located the platform before the 90 seconds expired, it was allowed to sit on the platform for ~10 seconds before being removed from the maze. After performing trial 1, rats were dried and returned to their cage until all the other rats from the cohort performed trial 1 (total resting time between trials was ~30 minutes), then trials 2 and 3 were performed in this same order. Habituation training was performed with a visible platform (square-patterned flag attached to platform) and 2 of the 8 arms open. Rats were placed at the center of the maze to start each trial. Days 1–3 of training were a test of reference memory with all 8 arms open and the platform hidden in a target arm. At the start of each trial, the animals were dropped in the end of empty arms, determined before the start of each day such that a pattern of drop locations was not repeated through the remaining testing days. On day 4, reversal training was performed to test memory flexibility and perseverance, with the hidden platform relocated to a new arm (different than that of the target arm on training days 1–3) and errors calculated for incorrect arm entries (flexibility) and re-entry into the previous baited arm for days 1–3 (perseverance).

2.3.4. Behavior data analysis

Behavioral data were acquired using VideoTrack software by ViewPoint Life Sciences (Montreal, Canada) and analyzed using Prism by GraphPad (La Jolla, CA, USA). For each behavioral measure, statistical significance was determined when $p < 0.05$.

2.3.4.1. Novel object recognition. The relative exploration time was recorded for each object and expressed as a novelty score (time spent (s) investigating novel object/time spent (s) investigating both objects in total). One-way ANOVA with Tukey's multiple comparison tests was conducted to determine significance of differences in novelty score between EE, SE, and SC rats.

2.3.4.2. Morris water maze. Platform crossings in the probe trial were calculated by tallying the number of times each subject entered the platform zone during the 60-second trial. Two-way ANOVA (housing condition and day) with repeated measures and Bonferroni post hoc tests was conducted on hidden platform training to determine significance of differences between EE, SE, and SC rats on all days of training. One-way ANOVA with Tukey's multiple comparison tests was conducted on probe trial crossings to determine differences between the 3 groups.

2.3.4.3. Radial-arm water maze. Errors were defined as an incorrect entry into a nontarget arm, or lack of mobility/exploration for ≥30 seconds (in an arm or the center of maze), and were recorded for each trial. Total trial time or until target platform was located was recorded using VideoTrack software. Unpaired *t*-tests with Welch's correction were conducted for error numbers to determine differences between EE and SE groups. One-way ANOVA with Tukey's multiple comparison tests was conducted on reversal trial data to determine differences between the 3 groups.

2.4. Electrophysiology

2.4.1. Field recordings

Hippocampi were collected from rats following decapitation, and transverse hippocampal slices (400 μm) were prepared as previously described (Gerstein et al., 2012). Slices were maintained in an interface chamber at 30 °C and perfused with oxygenated artificial cerebrospinal fluid (aCSF; 124.0 NaCl, 4.4 KCl, 26.0 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, and 10 glucose [all in millimolar]). Slices were permitted to recover for at least 90 minutes before recording. Field excitatory postsynaptic potentials (EPSPs) were recorded from Schaffer collateral–CA1 synapses by placing both stimulating and recording electrodes in the *stratum radiatum*. Baseline stimuli were delivered at intensities that evoked field EPSP slopes equal to 66% of the maximum evoked response in each slice (O'Riordan et al., 2014). Baseline stimuli were delivered once every 30 seconds, and test responses were recorded for 20–30 minutes before beginning the experiment to assure stability of the response. LTP was induced using the following stimulation protocol: Half-train theta burst (0.5 TBS) consisting of a total of 5 bursts (each burst consisting of 4 stimulations at a frequency of 100 Hz) with an interburst interval of 200 ms. Field potentials were recorded, and the slope of the EPSP was calculated as a percent slope of the baseline EPSP. Data were analyzed by 2-way ANOVA (housing condition and time) with repeated measures and Bonferroni post hoc tests.

2.4.2. Drug treatment

The group I mGluR agonist(s) 3,5-dihydroxyphenylglycine (DHPG), the mGluR5-selective noncompetitive antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), the phospholipase C inhibitor (U73122), and the mGluR1α-selective competitive antagonist 2-methyl-4-carboxyphenyl glycine (LY367385) were purchased from Tocris Bioscience (Bristol, UK). The competitive mitogen-activated protein kinase inhibitor U0126 was purchased from Sigma-Aldrich (St. Louis, MO, USA). DHPG was made up as a stock solution at 5 mM and used at 10 μM, MPEP was made up as a stock solution at 5 mM and was used at 40 μM, U73122 was made up in a stock solution at 10 mM and used at 10 μM, and LY367385 was made up as a stock solution at 10 mM and used at 100 μM. U0126 was made up as a stock solution at 3 mM and used at 30 μM. DHPG and MPEP were prepared in H_2O. Stock solutions for rapamycin, LY367385, U73122, and U0126 were prepared in DMSO. Stock solutions were stored in aliquots at −20 °C for up to 2 weeks. The tat-mGluR5 peptides were synthesized at the Biotechnology Center, University of Wisconsin. Stock solutions were prepared in H_2O, stored in aliquots at −20 °C, and used within 2 weeks of preparation. Peptides were used at a final concentration of 5 μM in aCSF supplemented with 10 μM of HEPES buffer, pH 7.4, and 0.05% of bovine serum albumin. The peptide sequences have been described earlier (Ronesi et al., 2012).

For the different experiments, all drugs were applied following the baseline recording period followed by 0.5 TBS. Slices were incubated in 10 μM DHPG for 10 minutes followed by a 20-minute washout. Slices were incubated with MPEP (40 μM), LY367385

(100 μM), or U0126 for 15 minutes followed by a 40-minute washout. The tat-mGluR5 peptides were added 2 hours before 0.5 TBS. The peptides were allowed to remain for the entire recording session. Slices were incubated with rapamycin (200 nM) for 40 minutes total (20 minutes before 0.5 TBS and continuing 20 minutes after stimulation). U73122 (10 μM) was added for 10 minutes total (5 minutes before 0.5 TBS and continuing 5 minutes after stimulation).

2.5. Tissue preparation and Western blot analysis

2.5.1. Postsynaptic density preparation from stimulated hippocampal slices

Hippocampal slices (400 μM) were prepared for electrophysiology from each experimental group (SC, SE, and EE groups). For each slice, baseline recordings were obtained (20–30 minutes), stimulated (0.5 TBS), and collected immediately following stimulation (Stim-0′) or 30 minutes following stimulation (Stim-30′). Control slices were subjected to preparation technique: incubation on recording chambers with continuous aCSF flow. Control slices were then collected at various time points during the experiment and were not subjected to stimulation (NS). Slices were frozen in liquid nitrogen and stored at −80 °C until further processing. For adequate protein content, 2–3 slices were collected and pooled per treatment from 1 given animal (EE, SE, or SC). A total of 3 or 4 animals (biological replicates) were used per experimental condition for statistical analysis.

Crude synaptoneurosomes were prepared as previously described (Cortese et al., 2011). Slices were homogenized in 200–300 μL of homogenization buffer (1 Tris, 1 sucrose, 0.5 EDTA, and 0.25 EGTA [all in molar]) with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA) using a glass tissue grinder with a Teflon pestle. Nuclear material and unbroken cells were removed by centrifugation at 960 × *g* for 15 minutes. The remaining supernatant was centrifuged at 15,000 × *g* for 15 minutes yielding an S2 (cytosolic) fraction and a P2 (crude synaptic) fraction. The P2 synaptic pellet was then homogenized using a 0.5 mL plastic pestle in 100 μL homogenization buffer + 0.5% SDS and sonicated. The P2 fraction is enriched for perisynaptic components including presynaptic and postsynaptic proteins, terminal mitochondria, and cytoplasm and synaptic vesicles (Booth and Clark, 1978; Whittaker, 1993). Synaptic enrichment of the P2 fraction was confirmed by Western blot analysis using antibodies against synaptophysin (1:5000; 101-011; Synaptic Systems, Goettingen, Germany) and postsynaptic density 95 (PSD95; 1:1000; 3450; Cell Signaling, Danvers, MA, USA), common synaptic markers. Protein content was quantified using the BCA protein assay (Bio-Rad, Hercules, CA, USA).

2.5.2. Western blot analysis

Samples were prepared under reducing conditions in 4× Laemmli buffer and heated at 70 °C for 5 minutes. For Western blotting, 15–30 μg of protein sample were loaded onto 4%–15% Bis-Tris SDS-polyacrylamide gels (Bio-Rad) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked in 5% milk/phosphate-buffered saline with Tween 20 (PBS-T) for 30 minutes at room temperature; all primary antibody incubations were performed at 4 °C overnight followed by 3 × 10-minute washes with PBS-T. Secondary antibody incubations were performed at room temperature for 1 hour followed by 3 × 10-minute washes with PBS-T. The following primary antibodies (and dilutions) were used: mGluR5 (1:1000; AB-5675; EMD Millipore, Temecula, CA, USA), Homer-1b/c (1:1000; sc-8923; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p70S6K (1:1000; 9206S; Cell Signaling), total p70S6K (1:1000; 9202S; Cell Signaling), pPLCγ1 (1:1000; 07-2134; Millipore, Billerica, MA, USA), and total PLCγ1 (1:1000; 05-366; Millipore, Billerica, MA, USA). Blots were probed with CaMKII (1:1000; 3357S; Cell Signaling) and PSD95 (1:500; 3450S; Cell Signaling) to validate synaptic fractions and β-tubulin (1:1000; G712A; Promega, Madison, WI, USA) as a loading control. Secondary antibodies were purchased from LI-COR (Lincoln, NE, USA) and diluted in the range of 1:10,000 to 1:15,000. Blots were then scanned using the Odyssey CLx imaging system (LI-COR). Blots were stripped using Restore Western Blot Stripping Buffer (Thermo, WI, USA) for 15 minutes and washed 3 × 10 minutes in PBS-T and subjected to standard Western blotting conditions.

Protein bands were quantified using ImageJ (NIH), and total density of each band was normalized to total protein levels for each sample as indicated by levels of β-tubulin. For phospho-p70S6K, levels were determined as a ratio of phospho to total levels of p70S6K. Unpaired *t*-test was used to determine if the level of the protein of interest in the EE group differed from the level of the protein in the other groups with respect to their poststimulation time point. The *p*-value listed for each protein (or phosphorylation state ratio) is for an unpaired *t*-test with statistical significance of $p < 0.05$.

3. Results

3.1. Aged rats exposed to 1 month of EE demonstrate enhanced learning and memory relative to SC in the MWM and novel object recognition (NOR), and EE rats perform significantly better than SE or SC rats in the RAWM

We found that after 1 month, EE animals demonstrated improved spatial and object recognition memory relative to SC animals. EE animals performed significantly better during the hidden platform phase of the MWM than SC animals (Fig. 1A, repeated measures ANOVA, $F_{(2,150)} = 21.8$, $p < 0.0001$). The main effect of group was also significant for platform crossings during the probe trial (Fig. 1B, 1-way ANOVA, $F_{(2,14)} = 3.65$, $p < 0.05$). Subsequent between-group contrasts confirmed that platform crossings were significant between EE and SC groups ($p = 0.03$).

Next, we tested the experimental groups of animals in the NOR task. The analysis of variance in NOR revealed an effect of group (Fig. 1C, 1 way ANOVA, $F_{(2,27)} = 5.24$, $p = 0.01$). Post hoc analyses confirmed that the SC group spent significantly less time exploring the novel object than the EE group (SC vs. EE: $p < 0.05$). To ensure that the NOR data represent novel object learning and not an increase in exploratory activity in EE and SE groups relative to SC groups because of their housing conditions, we examined the total time exploring both novel and old object on the testing day across groups. We found no statistically significant differences in total exploration time across groups (1-way ANOVA, $F_{(2,\ 23)} = 0.97$, $p = 0.4$). These results suggest that enhanced NOR memory observed in EE and SE rats is not due to increased exploratory opportunity outside of the home cage, rather this enhancement is attributed to learning.

Finally, we incorporated a version of the RAWM adapted for aged rats to determine any subtle behavioral differences between EE and SE groups (see Methods). This version of the RAWM includes 3 days of reference memory testing followed by 1 day of reversal training. In the reference memory test, we found that EE rats performed better than SE and SC rats, with EE rats making significantly less errors than SE rats in day 1 (Fig. 1D, unpaired *t*-test with Welch's correction $t = 2.86$, $df = 28$, $p = 0.01$) and day 2 (Fig. 1D, $t = 2.017$, $df = 31$, $p = 0.03$). Reversal training was performed on day 4 to test memory flexibility and perseverance. We did not find any statistically significant differences in errors for incorrect arm re-entry into

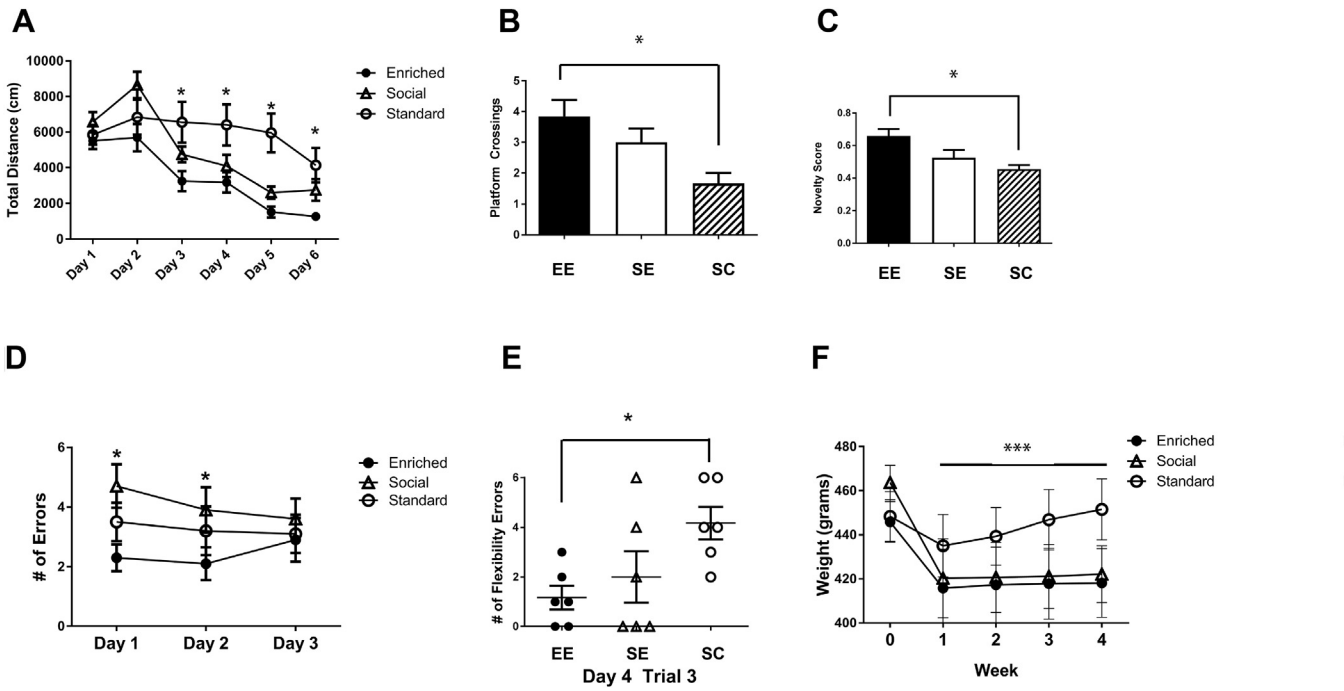

Fig. 1. One-month EE significantly improves performance in MWM, NOR, and RAWM in aged male Fischer 344 rats. (A) EE aged rats demonstrate significant improvement relative to standard housed controls during hidden platform training of the MWM (EE n = 11, SE n = 11, and SC n = 6). (B) EE rats display significantly more platform crossings during the probe trail compared with SC groups following MWM testing (EE n = 6, SE n = 6, and SC n = 3; only rats from cohort 2 were tested in the probe trial. See Methods for details). (C) Enriched groups display significant enhancements in object recognition memory on the NOR task compared with SC animals (EE n = 10, SE n = 11, and SC n = 7). (D) EE animals display significantly improved performance in the RAWM task relative to SE and SC animals on day 1 of training (EE n = 6, SE n = 6, and SC n = 6). (E) EE rats achieve the criterion faster than SC rats on the third trial of reversal learning. (F) Animal weight was recorded weekly throughout housing exposure and experimentation. EE and SE rats maintain their normal weight during enrichment relative to SC rats. Asterisks indicate statistical significance; individual *p*-values are reported in the text. Abbreviations: EE, environmental enrichment; LTP, long-term potentiation; MWM, Morris water maze; NOR, novel object recognition; RAWM, radial-arm water maze; SC, standard housing conditions; SE, social enrichment.

the previous baited arm for days 1–3 between groups (perseverance; 1-way ANOVA trial 1: $F_{(2,15)} = 0.96$, $p = 0.4$; trial 2: $F_{(2,15)} = 0.202$, $p = 0.8$; and trial 3: $F_{(2,15)} = 0.58$, $p = 0.6$). We then analyzed the number of errors to find the new location (excluding perseverative errors: flexibility errors) and found a significant effect of group on trial 3 (Fig. 1E, 1-way ANOVA, trial 3: $F_{(2,15)} = 4.17$, $p = 0.04$). Post hoc analyses confirmed that EE rats made significantly less errors than SC rats on the third trial of reversal training (EE vs. SC: $p < 0.05$).

Together, these data suggest that EE significantly improves learning and memory and that the 3-day training version of the RAWM is a sensitive test to distinguish marked enhancement of behavioral ability in EE animals compared with SE animals. To determine if EE animals might be superior because of a potential increase in activity relative to SE rats, we monitored their weight during the 1-month enrichment period.

The analysis on weight data from weeks 1–4 revealed an effect of group (Fig. 1F; 2-way ANOVA, $F_{(2, 6)} = 54.77$, $p = 0.0001$). Post hoc analyses showed no significant differences between the weight of EE and SE animals ($p = 0.4$). On the other hand, SC rats showed an increase in weight, as would be expected with an *ad libitum* diet and reduced physical activity when compared with EE and SE rats (Fig. 1F; EE vs. SC, $p = 0.0004$ and SE vs. SC, $p = 0.0002$). This suggests that the enhanced function found in EE rats is not due to an increase in exercise relative to SE rats but to the effects of the enriched environment.

3.2. EE enhances LTP in aged rats

We have previously shown that young EE rats display enhanced synaptic plasticity when compared with both SE and SC rats following 4 months of enrichment and that SE and SC animals display similar properties of synaptic plasticity (Hullinger et al., 2015). For this study, we wanted to determine if these differences could be detected with a brief exposure of 1-month enrichment and whether the same molecular mechanisms underlying the enhancement of LTP in young EE rats were also taking place in aged rats (Hullinger et al., 2015; Stein et al., 2016). Therefore, we examined the synaptic plasticity properties of aged rats after 1 month of enrichment. EE appears to have a stronger effect on learning and memory than SE alone, as shown in the RAWM task. For this reason, we focused on synaptic plasticity differences between EE and SE to understand the molecular differences between these 2 forms of enrichment. Our results show that aged enriched rats demonstrate increased LTP relative to SE controls (Fig. 2A; % baseline: enriched = 149.2 ± 25% and social = 121.5 ± 29%; $F_{(2,30)} = 8.33$, $p < 0.05$) and that application of DHPG enhances the synaptic response in SE animals (Fig. 2A; % baseline: enriched = 149.2 ± 2% and social + DHPG = 149.4 ± 1.5%; $p = 0.8$ and % baseline: social = 121.5 ± 29% and social + DHPG = 149.4 ± 26%; $F_{(2,30)} = 4.84$, $p < 0.05$). We also found that DHPG did not further enhance LTP in EE rats. Next, the mGluR5-selective noncompetitive antagonist, MPEP, and mGluR1α-selective competitive antagonist LY367385 were used to block LTP in EE rats. Preincubation of EE hippocampal slices with the MPEP (Fig. 2B; % baseline: enriched = 149.2 ± 24% and enriched + MPEP = 124 ± 26%; $F_{(2,30)} = 6.6$, $p < 0.05$) but not LY367385 (Fig. 2B; % baseline: enriched = 149.2 ± 2% and enriched + LY367385 = 149.6 ± 5%; $F_{(2,30)} = 1.78$, $p = 0.1$) selectively blocked LTP. This confirms previous results in mice and young EE rats that this form of LTP is mGluR5 dependent (Hullinger et al., 2015; O'Riordan et al., 2014). Next, we wanted to see which downstream effector of mGluR5 was responsible for the enhanced LTP function in EE rats. The canonical

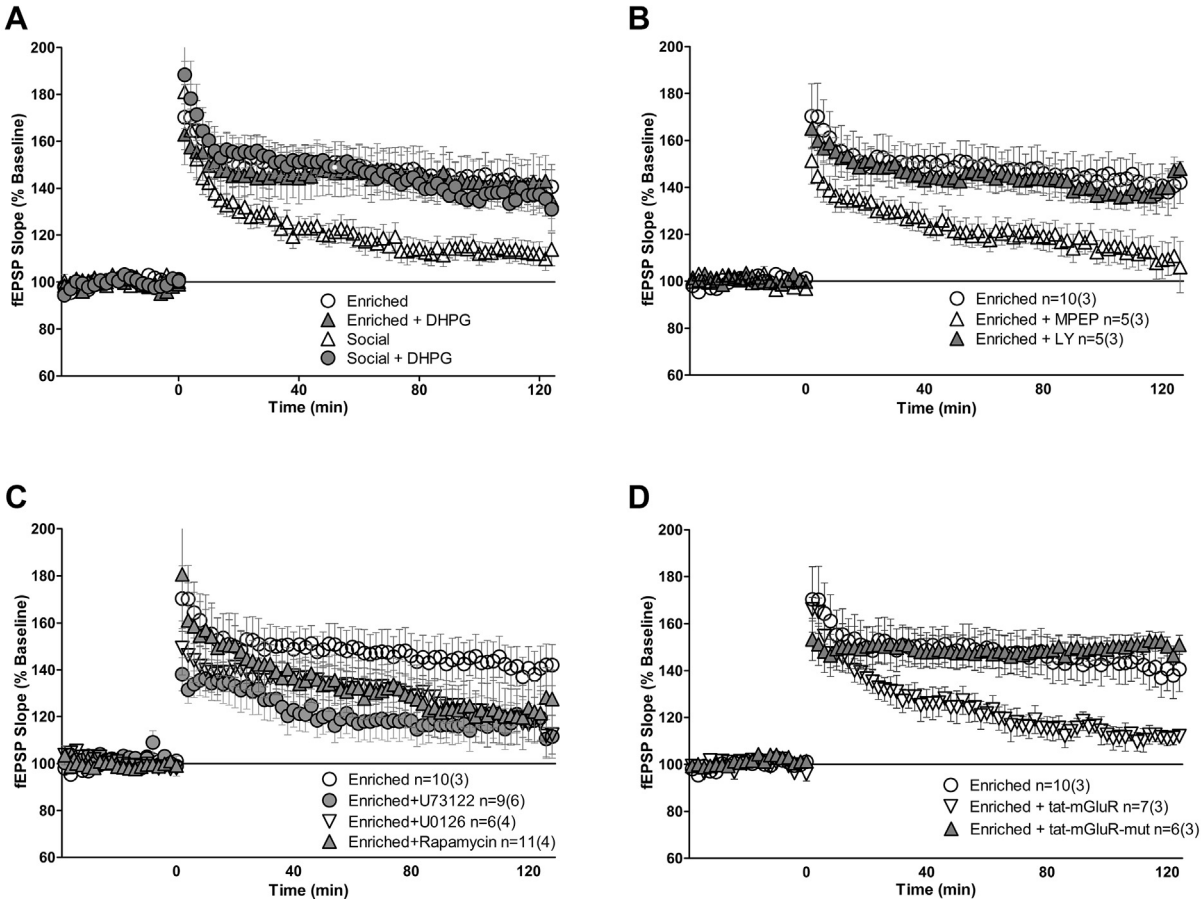

Fig. 2. Enrichment enhances LTP in aged rats. (A) Enriched animals show LTP expression in the absence of DHPG priming. n = 10(3) indicates 10 slices from 3 animals. (B) LTP expression in enriched animals is dependent on mGluR5, but not mGluR1 activation. (C) LTP function in enriched animals is dependent on ERK, mTOR, and PLCγ signaling. (D) Homer1c-mGluR5 interactions are necessary for LTP maintenance in enriched rats. Abbreviations: DHPG, 3,5-dihydroxyphenylglycine; fEPSP, field excitatory postsynaptic potential; LTP, long-term potentiation; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; PLC, phospholipase C.

effector of mGluR5 activity is phospholipase C (PLC), but mTOR and ERK are also shown to be activated by mGluR5 function (Mao and Wang, 2016; Matta et al., 2011; Menard and Quirion, 2012; Ronesi and Huber, 2008). Here, we show that disruption of ERK, mTOR, or PLCγ signaling reduces the enhanced LTP seen in enriched animals, but LTP is not completely blocked (Fig. 2C). Acute hippocampal slices were pretreated with rapamycin to block mTOR (% baseline: enriched = 149.2 ± 11% and enriched + rapamycin = 136 ± 15%; $F_{(2,30)} = 10.14$, $p < 0.05$), U0126 to disrupt ERK (% baseline: enriched = 149.2 ± 13% and enriched + U0126 = 134.7 ± 16%; $F_{(2,30)} = 2.47$, $p = 0.01$), or U73122 to disrupt PLCγ (% baseline: enriched = 149.2 ± 25% and enriched + U73122 = 122.3 ± 28%; $F_{(2,30)} = 8.16$, $p < 0.05$). Each of these treatments diminished EE-dependent enhancement of hippocampal LTP in aged slices (Fig. 2C). Finally, because mGluR-dependent LTP requires intact Homer1c/mGluR5 interactions, we studied the effects of disrupting these interactions using a peptide that mimics the binding domain of mGluR5 to Homer1c. We found that disruption of mGluR5/Homer1c scaffolds with the tat-mGluR5 peptide prevented LTP enhancement observed in enriched animals (Fig. 2D; % baseline: enriched = 149.2 ± 23% and enriched + tat-mGluR5 = 124.6 ± 26%; $F_{(2,30)} = 10.57$, $p < 0.05$). In addition, when slices were treated with a tat-mGluR5-mutant scramble, we did not suppress enhanced LTP (Fig. 2D; % baseline: enriched = 149.2 ± 1% and enriched + tat-mGluR5-mut = 148.6 ± 2%; $F_{(2,30)} = 1.7$, $p = 0.2$).

3.3. Enhanced LTP in EE rats results in activation of Homer1c, mGluR5, and their downstream effector p70S6K

We have previously reported that the Homer1c-mGluR5–associated signaling is increased in hippocampal slices prepared from young animals following EE (Hullinger et al., 2015). Here, we wanted to determine if SE and/or EE increases Homer1c-mGluR5 protein expression and the mGluR5-associated downstream phosphorylation of p70S6K in aged animals. To address this, we measured protein levels in synaptoneurosomes prepared from hippocampal slices immediately following 0.5 TBS stimulation (Stim-0′) or 30 minutes following 0.5 TBS stimulation (Stim-30′). In aged SC rats, synaptic levels of mGluR5 (Stim-0′: unpaired *t*-test, t = 0.36, df = 4, $p = 0.7$ and Stim-30′: t = 0.977, df = 4, $p = 0.4$), Homer1c (Stim-0′: t = 1.680, df = 6, $p = 0.1$ and Stim-30′: t = 0.99, df = 5, $p = 0.4$), and p-p70S6K (Stim-0′: t = 1.418, df = 6, $p = 0.2$ and Stim-30′: t = 1.25, df = 5, $p = 0.3$) were unchanged immediately following 0.5 TBS stimulation or 30 minutes after stimulation compared with nonstimulated (NS) controls (Fig. 3A; left panel, Fig. 3B top). We found that synaptic protein levels of mGluR5 (t = 3.19, df = 4, $p = 0.03$), Homer1c (t = 6.432, df = 5, $p = 0.001$), and p-p70S6K (t = 2.861, df = 6, $p = 0.03$) were significantly increased in EE slices at 30 minutes after 0.5 TBS compared with NS EE slices (Fig. 3A, right panel and Fig. 3B, bottom). EE also results in increased levels of Homer1c (t = 2.8, df = 6, $p = 0.03$) immediately following

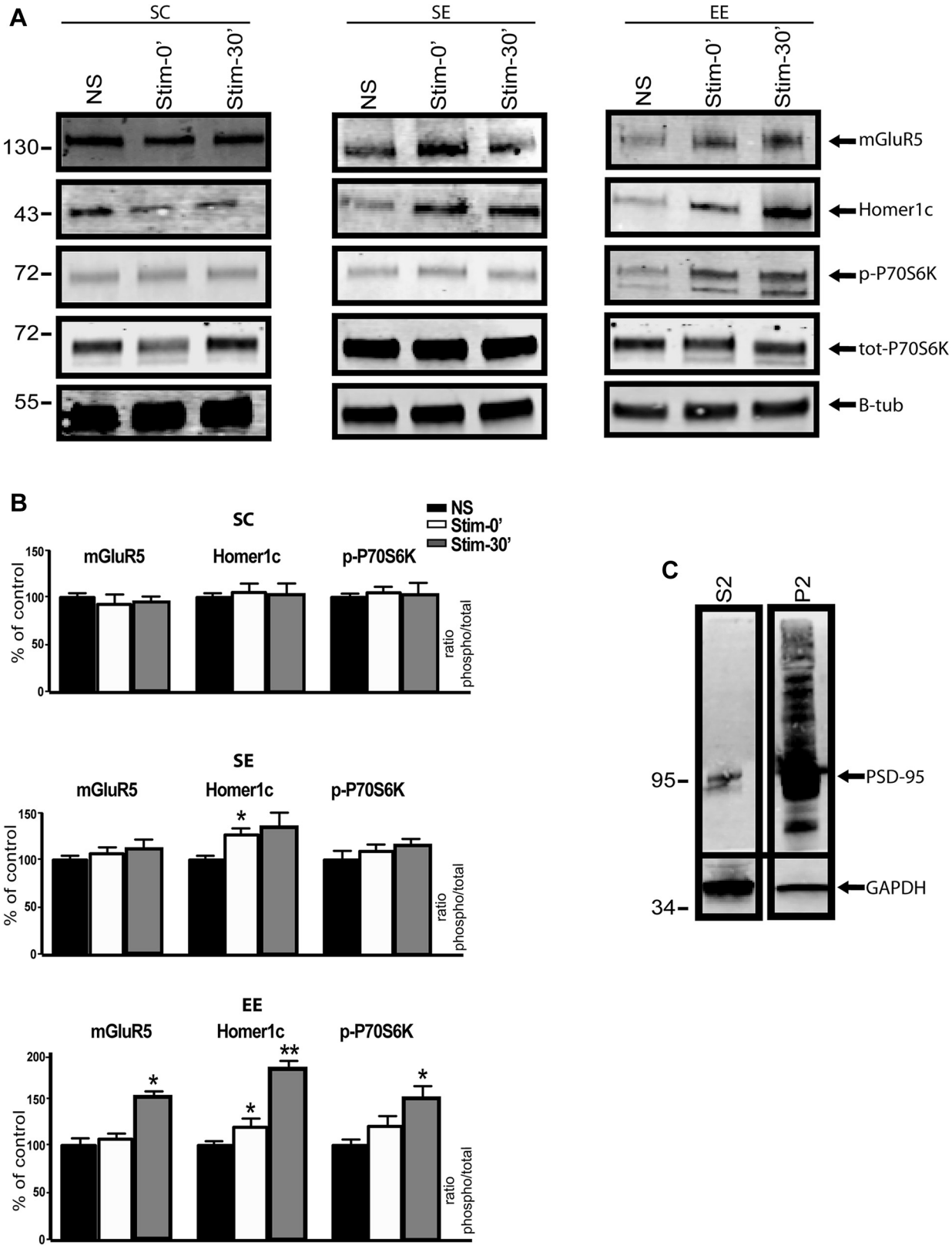

Fig. 3. One-month EE in aged rats results in activity-dependent activation of Homer1c, mGluR5, and their downstream signaling effector p70S6K. (A) Representative immunoblots from acute hippocampal slices prepared from EE, SE, and SC aged rats collected at various time periods after 0.5 TBS stimulation. (B) Quantified group data for proteins expressed as a percentage of that in control NS protein. Phospho-p70S6K levels were normalized to total p70S6K protein levels. Levels of Homer1c and mGluR5 were normalized to β-tubulin. Levels of mGluR5 ($p = 0.03$), Homer1c ($p = 0.001$), and p-P70S6K ($p = 0.03$) were significantly increased 30 minutes in EE slices following 0.5 TBS stimulation EE compared NS slices (lower panel). Error bars indicate standard error of the mean. All graphs represent 3 or 4 animals per group (see Methods). Asterisks indicate statistical significance ($^*p < 0.05$, $^{**}p < 0.005$); individual *p*-values are reported in the text. (C) Synaptic enrichment of the P2 synaptic fraction was confirmed by Western blot analysis using antibodies against PSD95, a common postsynaptic marker. GAPDH was used as a loading control. Abbreviations: EE, environmental enrichment; NS, nonstimulated; p70S6K, p70S6 kinase; PSD95, postsynaptic density 95; SC, standard housing conditions; SE, social enrichment; Stim-0′, immediately following stimulation; Stim-30′, 30 minutes after stimulation.

stimulation (Fig. 3A, right panel and Fig. 3B, bottom). Moreover, we found that 1-month SE produces modest increases in Homer1c and mGluR5 compared with NS controls, with significant increases in levels of Homer1c ($t = 3.31$, $df = 4$, $p = 0.03$) immediately after stimulation (Fig. 3A; middle panel, middle lane and Fig. 3B middle panel). Levels of p-p70S6K were unchanged in stimulated slices following SE. To support Homer1c specificity, we measured levels of the Homer1a protein isoform and found no change across enrichment exposures and stimulation paradigms (data not shown). Synaptic enrichment of the P2 fraction was confirmed by Western blot analysis using antibodies against PSD95, a common postsynaptic marker (Fig. 3C).

4. Discussion

We have previously demonstrated that a 4-month exposure to EE produces profound increases in hippocampus-dependent memory and mGluR5-dependent LTP in young, 2-month-old F344 rats (Hullinger et al., 2015). Furthermore, we found EE promotes a sustained activity-dependent phosphorylation of p70S6K (Hullinger et al., 2015). Here, we have extended these observations, examining the effects of EE on hippocampus-dependent memory, hippocampal LTP, and activity-dependent phosphorylation of p70S6K in the aging, 23- to 24-month-old F344 rats. In addition, we wanted to further highlight any differential effects that arise from exposure to SE and EE conditions independently. Our key findings are that 1-month exposure to EE in aging rats is superior to SE by (1) increasing behavioral performance on RAWM; (2) enhancing mGluR5-dependent LTP; and (3) increasing expression levels of mGluR5, Homer1c, and phospho-p70S6K in an activity-dependent manner.

4.1. A brief EE exposure can improve spatial memory in aged rats when experienced later in life

It is known that EE improves cognition; however, it is not clear whether the positive benefits associated with enrichment rely on the time and duration of exposure. Studies have found that both brief (3–5 weeks) and prolonged (>3 months) exposure to EE improve cognitive function in aged animals (>20 months) (Kobayashi et al., 2002). Furthermore, exposure to EE early in life appears to create a cognitive reserve that is characterized by improved spatial learning and memory that is continuously expressed as the animal reaches an aged state (≥24 months) (Fuchs et al., 2016). What is interesting about the report by Fuchs et al. is that EE exposure in young female Long-Evans rats early in life (8 weeks–18 months) improves cognitive ability later in life to an equal degree as when animals are exposed for their entire life (8 weeks–24 months). On the other hand, aged rats exposed to EE late in life (18–24 months) do not perform as well, suggesting EE late in life is not as beneficial (Fuchs et al., 2016). In contrast to the report by Fuchs et al., our new data demonstrate that a brief exposure (only 1 month) of aged male F344 rats to EE produces significant cognitive benefits in the aging brain—benefits that specifically influence memory-related processes in the hippocampus. The discrepancies between Fuchs et al.'s study and our present study could be due to differences in rat strain and sex (Andrews, 1996). Although it is important to investigate sex differences following EE, which are known to exist in the cognitive domain (Chamizo et al., 2016), we maintained consistency between our previous work using young male rats (Hullinger et al., 2015) and this report on aged rats. In our study, we found that 1-month EE was sufficient to increase memory, synaptic plasticity, and biochemical signaling in the aging brain. In our previous studies using young F344 rats, we found that 1-month EE did not discriminate between the effects of EE and SE in the NOR and MWM tasks. On the other hand, 4 months did show differences in behavioral performance (Hullinger et al., 2015). In this study, we selected a 1-month time frame for enrichment based on several studies that have provided evidence that the effects of EE differ between the young and aged rodent based on the duration of enrichment exposure. Young rodents showed to benefit more than aged rats from an extended exposure to enrichment (3–4 months) when tested for performance on learning and memory behavioral tasks, as well as assessment of synaptic plasticity (Harburger et al., 2007; Hullinger et al., 2015; Mora-Gallegos et al., 2015). In contrast, this study and those of others have shown that aged animals displayed increased learning ability, as well as enhanced synaptic plasticity following a short exposure to enrichment (3–5 weeks) (Buschler and Manahan-Vaughan, 2012; Fuchs et al., 2016; Harburger et al., 2007; Kobayashi et al., 2002; Mora-Gallegos et al., 2015; Morse et al., 2015; Stein et al., 2016). Changes in gene expression following EE have been shown to occur after brief periods of enrichment (3 hours, 6 hours, 2 days, and 14 days), suggesting that changes in cellular function may precede detectable changes in learning and memory behavior in young rats (Rampon et al., 2000; Sato et al., 2013). Another explanation could be that young animals perform well in the tasks used and there is not much room for improvement and/or that more sensitive tasks should be used to detect subtle differences in performance in young rats at earlier time points. The combination of both aging and stress converges to negatively impact hippocampal function and cognition (Cortese and Burger, 2017; Meaney et al., 1992; Stranahan et al., 2008). EE has been shown to reduce stress-like behaviors such as anxiety (as measured by increased exploratory behavior and open-arm entries in the elevated plus maze), decreased corticosterone levels, and decreased defecation and freezing; therefore, aged rats may benefit more than young rats from decreased stress in the early stages of enrichment exposure, reflecting on enhanced behavioral performance (Chapillon et al., 1999; Fernandez-Teruel et al., 1997; Fox et al., 2006; Sanchez et al., 2001; Sztainberg et al., 2010). Finally, EE exposure to aged rats may be more beneficial compared with young rats because of their ability to adapt to aging-related changes in the nervous system (Ash et al., 2016).

Here, we show that sensitive behavioral tests may be necessary for identifying subtle behavioral changes to differentiate the benefits of EE and SE independently. In this study, we sought to examine specific changes associated with EE and SE, in an effort to truly dissect the benefits of environmental changes. In doing so, we did not find any differences in behavioral ability between EE and SE rats using the MWM and NOR tasks. However, when tested in the RAWM task, we observed significant differences in behavioral performance (number of errors) between EE and SE animals on days 1 and 2 of reference memory training. This may be due in part to the fact that the RAWM is a more sensitive behavioral task that incorporates features of the land-based radial-arm maze with the elements of the MWM to detect subtle behavioral changes in rodents (Alamed et al., 2006; Gallagher et al., 1993). Specifically, in the RAWM task, rats cannot use a nonspatial egocentric strategy (i.e., swimming at a certain distance of the pool wall to learn the location of the platform in the MWM task) (Burger et al., 2007; Day and Schallert, 1996; Hyde et al., 1998; McDonald and White, 1994; Shukitt-Hale et al., 2004; Whishaw et al., 1987). Instead, rats have to enter an arm and therefore make a correct (or incorrect) choice that can be quantified. Indeed, our behavior data indicate that rats can be segregated into EE and SE using the RAWM task. In addition, at this 1-month time point, we found significant differences between SE and EE animals when measuring synaptic efficacy, as well as the biochemical signaling cascades triggered by activation of mGluR5. Thus, the sensitivity of

the RAWM may best highlight and represent specific differences we see physiologically and biochemically between SE and EE aged rats.

The faster acquisition of the RAWM by EE rats in early trials (days 1 and 2; Fig. 1D) is consistent with an increase in mGluR5 function. Metabotropic GluR5 is necessary for the acquisition phase of memory formation. For example, mGluR5-knockout mice display deficits in the acquisition of contextual fear conditioning but eventually learn the task with sufficient training (Lu et al., 1997; Xu et al., 2009). In our study, we found that by day 3, all experimental groups had made the same number of errors (Fig. 1D). This was not unexpected because aged rats can reach asymptote levels with enough training, and this training benefits both aged unimpaired and impaired animals equally (Barnes et al., 1980; Gallagher, 1985; Rapp et al., 1987). There is also evidence indicating that mGluR5 is involved in reversal learning that is impaired in animal models of schizophrenia and in extinction of cocaine contextual memory (Gass and Olive, 2009; Gastambide et al., 2012; Liu et al., 2008; Xu et al., 2009). Reversal learning is defined as the ability to reverse an acquired behavior by switching strategies in response to changes to learn a novel one, and it has been shown that aging has a detrimental effect on reversal learning (Guidi et al., 2014; Rapp et al., 1987; Wiescholleck et al., 2014). In our study, all groups made the same amount of perseverative errors in the reversal trials. Yet, EE rats learned the new platform location faster than SC rats by reversal trial 3 (Fig. 1E). Our results suggest that the enhanced mGluR5/Homer1c activity observed in aged rats following EE is responsible for both the improved acquisition of reference memory in RAWM, with both EE and SE improving learning behavior in the MWM and NOR tasks, as well as in reversal learning. The cognitive benefits that result from EE and SE exposure cannot be limited to the hippocampus and hippocampal function, rather the entorhinal and retrosplenial cortices play an equally important role in spatial and object recognition memory (Ash et al., 2016; Parron and Save, 2004).

4.2. EE targets hippocampal processes that regulate plasticity and synaptic-signaling pathways

The synaptic mechanism by which EE may give rise to cognitive benefits is beginning to be elucidated. EE can enhance LTP and LTD in the hippocampus (Artola et al., 2006; Buschler and Manahan-Vaughan, 2017; Hullinger et al., 2015; Kumar et al., 2012; Malik and Chattarji, 2012; Stein et al., 2016). LTP is regulated by changes in synaptic strength that require the activity of ionotropic and metabotropic receptors (Bortolotto et al., 1999; Malinow and Malenka, 2002), specifically the mGluR5 receptor (Anwyl, 2009; Bortolotto et al., 2005; Hullinger et al., 2015; O'Riordan et al., 2014). We and others have shown that EE increases LTP via mechanisms that depend on mGluR5 activity in both young (Buschler and Manahan-Vaughan, 2017; Hullinger et al., 2015) and aged rats (Buschler and Manahan-Vaughan, 2017). The role of mGluR5 activity and signaling in hippocampal benefits following EE has been investigated in young mice (Burrows et al., 2015; Lu et al., 1997) and young rats (Hullinger et al., 2015; Manahan-Vaughan and Braunewell, 2005); however, it is not known if these same pathways are consistent with age. We have identified a potential mechanism through the sustained activity-dependent phosphorylation of the p70S6 kinase, a pathway we previously reported in young rodents following 4-month EE exposure (Hullinger et al., 2015). p70S6K and other synaptic proteins that include Homer1c and mGluR5 are known to be important for synaptic processes associated with cognition (Gerstein et al., 2012; Hullinger et al., 2015; Menard and Quirion, 2012; O'Riordan et al., 2014). Our data now indicate that activity-dependent increased phosphorylation of p70S6K occurs following 1-month EE in hippocampal synaptoneurosomes prepared from aged rats, an increase that is consistent with heightened levels of mGluR5 and Homer1c. Thus, there exists an intriguing link between mGluR5-Homer1c activity and downstream phosphorylation of p70S6K following enrichment in aged rodents. Based on our previous findings in young rats (Hullinger et al., 2015), there seems to be no significant changes in LTP and signaling events both in young and aged EE rats, whereas normal aging is known to reduce LTP and synaptic activity (Reviewed in Burger, 2010 and Rosenzweig and Barnes, 2003). Rather, our data indicate that EE may preserve this mechanism and rescue age-related perturbations and cognitive decline. Furthermore, based on this report there seems to be a significant mechanistic benefit with EE compared with SE. These findings suggest a potential mechanism by which EE benefits overall cognition in the aging brain.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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