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Short-term environmental enrichment enhances synaptic plasticity in hippocampal slices from aged rats

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Abstract

Age-associated changes in cognition are mirrored by impairments in cellular models of memory and learning, such as long-term potentiation (LTP) and long-term depression (LTD). In young rodents, environmental enrichment (EE) can enhance memory, alter LTP and LTD, as well as reverse cognitive deficits induced by aging. Whether short-term EE can benefit cognition and synaptic plasticity in aged rodents is unclear. Here, we tested if short-term EE could overcome age-associated impairments in induction of LTP and LTD. LTP and LTD could not be induced in the CA1 region of hippocampal slices in control, aged rats using standard stimuli that are highly effective in young rats. However, exposure of aged littermates to EE for three weeks enabled successful induction of LTP and LTD. EE-facilitated LTP was dependent upon N-methyl-D-aspartate receptors (NMDARs). These alterations in synaptic plasticity occurred with elevated levels of phosphorylated cAMP response element-binding protein and vascular endothelial growth factor, but in the absence of changes in several other synaptic and cellular markers. Importantly, our study suggests that even a relatively short period of EE is sufficient to alter synaptic plasticity and molecular markers linked to cognitive function in aged animals.

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Keywords

Environmental enrichment; Long-term potentiation; Long-term depression; N-methyl-D-aspartate receptor; Aging

INTRODUCTION

Memory deficits are widely recognized to occur in dementing diseases such as Alzheimer's disease. However, it is also known that memory deficits occur in the elderly in the absence of disease. Among individuals aged 71 and older, 22.2% have cognitive impairment without dementia (Plassman et al., 2008), while 13.9% have dementia (Plassman et al., 2007).

Alarming, cognitive decline is already evident in humans by 45–49 years of age (Singh-Manoux et al., 2012), and progressively increases with age (Plassman et al., 2007, Plassman et al., 2008, Borson, 2010, Daffner, 2010, Salhouse, 2010, Artegiani and Calegari, 2012, Singh-Manoux et al., 2012). Cognitive decline is also seen in aging primates (Aizawa et al., 2009) and rodents (Rapp and Gallagher, 1996, Chen et al., 2004, von Bohlen und Halbach et al., 2006, Villeda et al., 2011, Freret et al., 2012, Seib et al., 2013).

Memory deficit commonly experienced by the elderly in the absence of dementia is termed age-associated memory impairment (AAMI) or cognitive decline (AACD) (Levy, 1994). Even mild AAMI/AACD produces sufficient cognitive deficits to provide a substantial burden for those affected and their families (Langa and Levine, 2014). AAMI/AACD is an increasing burden on society as individuals over the age of 60 are the fastest growing age group (Daffner, 2010). Globally, the proportion of individuals over the age of 60 will increase from 10% in 2000 to 22% in 2050 and to 32% in 2100 (Lutz et al., 2008). This shift will increase the median age of the world's population from 26.6 years in 2000 to 37.3 years in 2050 and to 45.6 years in 2100. Given the prevalence of cognitive decline and its burden both to the individual and to society, it is critical that we seek to understand how to prevent or reverse age-related declines in cognition, and thus improve quality of life for the elderly.

The hippocampus is particularly vulnerable to aging (Mora et al., 2007). Indeed, age-related changes in synaptic plasticity have been reported for all hippocampal subregions (Foster, 2012). Two major forms of synaptic plasticity closely correlated with and critically involved in learning and memory processes, are long-term potentiation (LTP) and long-term depression (LTD) (Sale et al., 2014). Aging impairs the induction, magnitude, and maintenance of LTP that can be evoked by weak stimulation (Landfield et al., 1978, Barnes, 1979, Burke and Barnes, 2006, Freret et al., 2012, Haxaire et al., 2012). In contrast to its effect on LTP, aging has increased susceptibility to induction of LTD (Norris et al., 1998, Foster and Kumar, 2007, Kumar and Foster, 2007).

Environmental enrichment (EE) is an experimental setting in which animals are put in surroundings designed to enhance social interactions, sensory and motor stimulation, and learning and memory. Cages are typically large and contain tunnels, platforms, toys, and running wheels (Mora et al., 2007). Impressively, EE prevents or ameliorates deficits in both hippocampal-dependent and -independent forms of learning and memory in multiple species of aged mammals (Escorihuela et al., 1995, Soffie et al., 1999, Duffy et al., 2001, Leggio et

al., 2005, Bennett et al., 2006, Sale et al., 2014). Moreover, in young rodents, LTP in the hippocampal CA1 region are strengthened after EE (Duffy et al., 2001, Artola et al., 2006, Huang et al., 2006). While one notable study found that 10–12 weeks of EE reversed age-related changes in LTD and LTP (Kumar et al., 2012), the effect of EE on synaptic plasticity in the context of aging requires further examination.

Despite these encouraging findings, critical details regarding the implementation of EE and its downstream molecular mechanisms remain uncertain. In attempts to understand how EE improves cognitive function, existing studies have identified a plethora of cellular and molecular changes associated with the beneficial effects of EE. For example, rodents housed in enriched conditions show increased brain weight and size, dendritic branching, synapse formation, number of astrocytes, and neurogenesis in several areas of the brain such as the hippocampus, cerebral cortex, and basal ganglia (Comery et al., 1996, Soffie et al., 1999, van Praag et al., 2000, Kolb et al., 2003, Leggio et al., 2005, Mora et al., 2007, Diniz et al., 2010). However, we do not know the age of exposure to EE or duration of EE required to evoke these changes since existing studies have largely used chronic protocols with widely differing ages of EE onset and durations of EE exposure. Given these knowledge gaps, we have investigated the effects of short-term EE instated in old age on synaptic plasticity and a panel of hippocampal molecular markers. We hypothesized that short-term EE instated in old age would ameliorate aging-related decays in LTP and LTD in hippocampal slices.

EXPERIMENTAL PROCEDURES

Animals

All animal procedures were approved by the Washington University Animal Studies Committee, Division of Comparative Medicine, Washington University School of Medicine, St. Louis, MO, and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23). All efforts were made to minimize the number of animals used and their suffering. Four to five rats per group were used in each experiment.

Environmental Enrichment (EE)

Male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN) at 21 months of age. Upon arrival, rats were single-housed for 7 to 10 days before being randomly divided into two groups: standard housing (control) or environmental enrichment (EE) training. All rats remained single-housed; however, rats in the EE group were transferred in groups of three to a large (L 15" × W 30" × D 30"), two-story cage, for three hours a day (from 5:00 pm to 8:00 pm), seven days a week, for three weeks. The cage contained toys and various objects whose locations were rearranged every day. Both groups had food available *ad libitum*.

Hippocampal Slice Preparation

Rats were moved to the dissection room the night prior to sacrifice. Dissections were performed between 10:30 am and 12:30 pm to account for possible effects of transportation and the light/dark cycle, respectively. Rats were deeply anesthetized with halothane or

isoflurane in a chemical fume hood and decapitated via guillotine. The brain was quickly removed and hippocampi were rapidly dissected and placed in gassed (95% O₂–5% CO₂), 30°C standard artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 5 KCl, 2 CaCl₂, 2 MgSO₄, 1.25 NaH₂PO₄, 22 NaHCO₃, and 10 D-glucose. Transverse slices (500 µm thick) of the dorsal hippocampus were cut with a custom-made rotary slicer (Tokuda et al., 2010). Slices were then maintained in an incubation chamber for 1 hour at 30°C in ACSF. Individual slices were transferred to a submersion recording chamber where they were constantly perfused with standard solution (2 ml/min) at 30°C.

Electrophysiology

Extracellular recordings in gassed ACSF were obtained from the dendritic layer of the CA1 region with the use of 5- to 10 MΩ glass electrodes filled with 2 M NaCl. A bipolar electrode was placed in stratum radiatum to stimulate the Schaffer collateral/commissural pathway. The stimulus intensity was set to evoke 40–50% of the maximal amplitude of field excitatory postsynaptic potentials (fEPSPs). Different types of afferent stimulation were performed at the same relative intensity in individual slices. LTP was induced by delivering 100 Hz × 1 sec high frequency stimulation (HFS) to the Schaffer collateral pathway. LTD was induced by delivering low frequency stimulation (LFS) consisting of 900 pulses at 1 Hz to the Schaffer collateral pathway. These stimulus parameters were chosen because they produce robust and reliable LTP and LTD, respectively, in hippocampal slices from young (30 day old) rats (Izumi and Zorumski, 1995).

fEPSPs were monitored and analyzed with an IBM computer-based data acquisition system. The magnitude of potentiation was expressed as the percent change in the maximal slope of fEPSPs. Changes of the EPSP slope by more than 20%, 60 minutes after conditioning stimulation was considered to represent successful induction and maintenance of LTP or LTD.

Immunohistochemistry

The right hippocampus was drop-fixed in 4% paraformaldehyde overnight, equilibrated sequentially in 15% and 30% sucrose overnight, frozen, and stored at –80°C until sectioning. 30 µm coronal sections in a 1 in 8 series were made by cryostat and stored at –30°C in cryoprotectant until use. Tissue sections were incubated with 3% H₂O₂ for 15 minutes to remove endogenous peroxidase activity. Blocking/permeabilization solution contained 10% normal goat serum, 1% BSA, and 0.3% Triton-X in PBS. Tissue sections were blocked/permeabilized for 45 to 60 minutes prior to 24 or 48 h of incubation with primary antibodies in 5% normal goat serum and 0.1% Triton-X in PBS at 4°C at the following concentrations: BDNF (1:100, Santa Cruz, sc-546, rabbit), Iba1 (1:500; Wako, #019-19741, rabbit), Gfap (1:1000; Millipore, MAB360, mouse), Map2 (1:500, Sigma, M9942, mouse), Neuropeptide Y (NPY, 1:1000, GeneTex, GTX10980, rabbit), phospho-Creb (Ser133, 1:200, Millipore, 05-807, mouse), Synaptophysin (1:1000, Sigma, S5768, mouse), Vegf (1:50, Santa Cruz, sc-65617, mouse), VGlut1 (1:1000, Synaptic Systems, 135 304, guinea pig). Alexa488 (1:200), Alexa488 (1:200), or Cy3 (1:400) conjugated-secondary antibodies (Jackson ImmunoResearch) diluted in 2% normal goat serum, 1% BSA, and 0.1% Triton-X in PBS were added for 2 h at room temperature. Detection of BDNF was

performed using the TSA-Plus kit (PerkinElmer, Boston, MA). Nuclei were stained with 4,6-diamidino-2-phenylindole (Sigma) for 10 minutes at room temperature.

High-magnification (20x, 0.8DICII) microscopic imaging was performed using a Zeiss Axioimager.Z1 or an Olympus NanoZoomer 2.0-HT. Images were taken in z-stacks of 1 μm steps through the range of tissue section immunoreactivity. ImageJ was used to 3D render z-stacks and quantify signal immunoreactivity. Specificity of signal immunoreactivity was verified by substituting primary antibodies with same species IgG. Five sections across the dorsal hippocampus from the aforementioned 1 in 8 series were quantified per mouse. For quantification of BDNF, P-Creb, and Vegf, background immunoreactivity was subtracted from signal immunoreactivity. This was done by selecting a region outside of the tissue, measuring the immunoreactivity in this region, and subtracting this immunoreactivity from the immunopositive reactivity measured in each image. For quantification of GFAP, Iba1 or NPY, cells were considered immunoreactive only if cell soma and processes were clearly discernible to avoid oversampling.

Western Blotting

Protein was isolated and analyzed as previously described (Stein et al., 2014). Briefly, protein extracts (15–50 μg) were prepared from acutely isolated rat cortices flash frozen in liquid nitrogen, and stored at -80°C until use. Membranes were incubated with primary antibodies in Tris-buffered saline containing 0.1% Tween 20 (TBST) overnight at 4°C . Primary antibodies used included: BDNF (1:500, Abcam, ab72439, rabbit), Gapdh (1:1000; Millipore, CB1001, 6C5), Map2 (1:1000, Sigma, M9942, mouse), Synaptophysin (1:2000, Sigma, S5768, mouse).

Statistical Analyses

Numerical data are presented as mean $\pm$ standard error of the mean (S.E.M.). Statistical analyses were performed using Excel, RStudio, SigmaPlot 5.01 and 9.0, or SigmaStat 3.1 (Systat Software Inc., Richmond, CA). Statistical significance was determined by Student's *t*-tests or Mann-Whitney U-tests for instances when normal distributions can or cannot be assumed, respectively. *P*-values less than 0.050 were considered significant. Sample sizes are stated in the figure legends and refer to individual rats.

RESULTS

Short-term EE results in weight loss

Since EE has been found to provide cognitive benefits for hippocampal-dependent and -independent learning and memory in multiple species of aged mammals (Escorihuela et al., 1995, Soffie et al., 1999, Duffy et al., 2001, Leggio et al., 2005, Bennett et al., 2006, Sale et al., 2014), we hypothesized that EE could enhance synaptic plasticity in hippocampal slices from aged rats. Successful EE regimens have used durations of EE spanning much of life, such as the entirety from weaning to old age. However, such chronic treatments cannot distinguish whether the tested outcomes are prevented or reversed. We chose to test whether changes in synaptic plasticity were reversed. To do this, we split a cohort of 21–22 month old rat littermates into two groups: one group (control) was kept under standard conditions

whereas the other group (EE) was transferred in groups of three to a large, two-story cage containing various toys and objects for three hours a day, for three weeks (Figure 1). The toys and objects were rearranged every day. During this three week period, rats exposed to EE lost a significant amount of body weight relative to control rats (two way repeated measures ANOVA: Time $\times$ Treatment interaction: $F(2,27) = 4.2$, $p = 0.025$; Treatment effect: $F(1,27) = 59.1$, $p < 0.0001$). While control rats gained 13.4 ± 11.1 g ($n=5$ rats) during the three week period, EE rats lost 52.2 ± 9.6 g ($n=6$ rats; $p<0.01$, Student's t -test).

Short-term EE alters synaptic plasticity in the hippocampus

Typically, it is difficult to induce LTP in hippocampal slices from aged rats via conventional methods: a single $100 \text{ Hz} \times 1 \text{ s}$ HFS in the presence of 2.0 mM calcium and 2.0 mM magnesium (Izumi and Zorumski, 1995, Burke and Barnes, 2006, Foster, 2012, Freret et al., 2012, Haxaire et al., 2012). To confirm the effect of aging on LTP, we delivered HFS using conventional methods to the CA1 region of hippocampal slices from control rats. As anticipated, conventional HFS failed to induce LTP in slices from aged rats housed under standard conditions (Figure 2A; $p=0.172$, paired t -test before and after HFS).

To test the idea that EE reverses age-related changes in synaptic plasticity (Kumar et al., 2012, Sale et al., 2014), we delivered HFS under conventional conditions to the CA1 region of hippocampal slices from aged rats exposed to EE. In stark contrast to their littermates housed under standard conditions (fEPSP slope 60 minutes after HFS: $106 \pm 6\%$ of baseline, $n=9$ rats, open circles in Figure 2A), we could successfully induce LTP with conventional methods in the aged rats exposed to EE ($159 \pm 16\%$ of baseline, $p<0.01$, Student's t -test relative to aged rats housed under standard conditions; $p=0.002$, Wilcoxon Signed Rank Test before and after HFS; $n=10$, closed circles in Figure 2A).

Like LTP, we could not induce LTD in slices from control rats using a conventional $1 \text{ Hz} \times 900$ pulse LFS in the presence of 2.0 mM calcium and 2.0 mM magnesium (fEPSP slope 60 minutes after LFS: $92 \pm 5\%$; $p=0.822$, paired t -test before and after LFS; $n=5$, closed circles, Figure 2B). In contrast, robust LTD was evoked in slices from EE rats ($68 \pm 3\%$, open circles, $p<0.01$, Student's t -test relative to aged rats housed under standard conditions; $p=0.004$, paired t -test before and after LFS; $n=5$, Figure 2B).

Short-term EE enhances NMDAR-dependent LTP

Differing stimuli can induce different forms of LTP. For example, induction of LTP via conventional methods requires activation of N-methyl-D-aspartate receptors (NMDARs). In contrast, induction of LTP with higher frequency stimulation ($200\text{--}250 \text{ Hz}$) or in the presence of elevated extracellular calcium (Izumi and Zorumski, 1998) involves activation of voltage-dependent calcium channels (VDCCs) (Teyler et al., 1995). Since we were able to induce LTP with conventional methods in EE rats, we hypothesized that the LTP enhanced by EE was NMDAR-dependent. To test if LTP induction after EE training was mediated by NMDARs or voltage gated calcium channels, we attempted to induce conventional LTP in the presence of the NMDAR antagonist D,L-APV ($100 \mu\text{M}$) or the calcium channel inhibitor nifedipine ($20 \mu\text{M}$). As expected, EE-mediated LTP was blocked by D,L-APV (fEPSP slope: $100 \pm 7\%$, $p<0.01$, Student's t -test, $n=5$, open squares in Figure 3), but not by nifedipine

($128 \pm 5\%$, $p=0.218$, Student's t -test, $n=5$, open triangles in Figure 3). Thus, EE-mediated LTP is primarily NMDAR-dependent.

Short-term EE does not affect the immunoreactivity of several synaptic or cellular markers

Having found that aging impaired induction of LTP and LTD in rats housed under standard, but not EE, conditions, we hypothesized that there would be accompanying histological manifestations of these electrophysiological differences. First, we evaluated basic morphology by visually comparing hematoxylin and eosin (H&E) staining on cryosectioned hippocampal slices. By eye, we could not distinguish differences in gross morphology between control and EE rats (Figure 4A).

Rodents housed in enriched conditions have been shown to have increased glial populations in several areas of the brain, including the hippocampus (Mora et al., 2007). To test if short-term EE also increased the glial population, we checked the immunoreactivity of the astrocytic marker Gfap and the microglial marker Iba1. However, numbers of Gfap+ and Iba1+ cells were unchanged in the EE rats relative to the control rats (Figure 4B,C,D).

Rodents housed in enriched conditions have also been shown to have new synapse formation and enhanced dendritic branching in the hippocampus (Mora et al., 2007). However, when we immunostained for the synaptic marker synaptophysin (Figure 4E) and quantified synaptophysin signal intensity in the *stratum radiatum* (Figure 4F) or *stratum oriens* (Figure 4G) after correcting for background signal, no significant differences were found. Similarly, immunoblotting for synaptophysin in cortical extracts failed to reveal differences between the control and EE rats (Figure 4H). To assess GABAergic integrity and the balance between glutamatergic and GABAergic neurons, we also immunostained for Neuropeptide Y (NPY), an interneuron subtype, in control and EE rats. However, we only detected a trend towards an increase in the number of NPY+ cells in CA1 (Figure 4I). We also did not observe differences between control and EE rats upon immunostaining for a dendritic marker, Map2 (Figure 4J) (Matesic and Lin, 1994, Folkerts et al., 1998, Hoskison et al., 2007), or a marker for glutamatergic synaptic transmission, Vglut1 (Figure 4J) (Santos et al., 2009). Immunoblotting for Map2 in cortical extracts confirmed the lack of differences between control and EE rats (Figure 4K). Thus, short-term EE in old age does not alter astrocytic, microglial, dendritic, or synaptic markers examined in this study.

Short-term EE increases the expression of P-Creb and Vegf, but not BDNF

Several factors have been identified as mediators of the effects of EE on the brain. However, these factors were identified in young rodents subjected to chronic EE. Thus, we next asked if these factors were similarly altered in old rodents subjected to short-term EE. One of these crucial mediators of the effects of chronic EE on brain development is brain-derived neurotrophic factor (BDNF). An increase in BDNF levels and activity in the hippocampus and cortex occurs following physical exercise (Berchtold et al., 2002) and EE (Young et al., 1999, Hu et al., 2013) and is thought to contribute to the beneficial effects of both interventions (Vaynman et al., 2004, Mora et al., 2007, Eckert and Abraham, 2013, Sale et al., 2014). To test if changes in BDNF also mediate the effects of short-term EE, we

immunostained hippocampal slices for BDNF. Yet, we only detected a trend towards increased BDNF immunoreactivity in the EE rats due to high individual variability (Figure 5A,B). To verify these findings, we immunoblotted for BDNF in cortical extracts. However, we failed to detect a change in cortical levels of BDNF (Old: 0.070 ± 0.015 Bdnf/Gapdh, $n=5$; EE: 0.072 ± 0.011 Bdnf/Gapdh, $n=4$; $p=0.92$, Student's *t*-test, Figure 5C). To investigate if these trends resulted in an increase in BDNF activity, we also immunostained for phosphorylated cAMP response element-binding protein (P-Creb) (Figure 5A,D). Creb is a key target gene of BDNF signaling (Sale et al., 2014). EE is also known to upregulate Creb phosphorylation in the hippocampus (Young et al., 1999, Hu et al., 2013) and increased phosphorylation of Creb mimicked the effects of EE on visual acuity maturation (Sale et al., 2014). We observed nuclear P-Creb immunostaining, as reported (Lu and Hawkins, 2002, Hattiangady et al., 2005). The intensity of this staining was 1.71 fold higher in EE rats relative to control rats ($p=0.030$, Student's *t*-test), supporting the notion that EE increases P-Creb signaling.

A second factor known to be increased in the hippocampus by daily exercise (Latimer et al., 2011, Speisman et al., 2013) and decreased with age (Shetty et al., 2005) in rodents is vascular endothelial growth factor (Vegf). Thus, we compared Vegf immunoreactivity in the control and EE rats (Figure 5E). As reported, EE also increased Vegf immunoreactivity 1.56 fold relative to control rats ($p=0.009$, Student's *t*-test). Our findings that P-Creb and Vegf are changed as previously reported suggest that EE activates the same pathways in youth and old age, and that these changes have taken place by three weeks of treatment.

DISCUSSION

Here we confirmed that LTP is difficult to induce at Schaffer collateral synapses in aged rats using stimulus conditions that are highly effective in young rodents. We further demonstrated that LTD is also dampened in the aged hippocampus. Importantly, only three weeks of EE for three hours per day was sufficient to enhance both LTP and LTD in aged rats and EE-facilitated LTP was NMDAR-dependent. These alterations in synaptic plasticity were not accompanied by changes in numbers of astrocytes or microglia, dendritic immunostaining by Map2, or excitatory synaptic vesicle immunostaining by VGlut1. However, they did correlate with elevated levels of phosphorylated Creb as well as the growth factor Vegf. Since alterations in LTP and LTD at Schaffer collateral synapses are associated with age-related cognitive decline (Landfield and Lynch, 1977, Deupree et al., 1993, Shankar et al., 1998, Schulz et al., 2002, Tombaugh et al., 2002, Lee et al., 2005, Burke and Barnes, 2006, Boric et al., 2008), our findings highlight the importance of social, physical, and/or intellectual activity with age. Our findings also support studies in humans that older age is not too late for enriching activities to improve brain function (Hertzog et al., 2008).

A major finding of our study is that we were unable to evoke both LTP and LTD by conventional methods in aged rats single-housed under standard conditions. Using our techniques, induction of LTP and LTD peaks at approximately 30 days of age, becoming more difficult to induce after 120 days of age (Izumi and Zorumski, 1995, 1999). Others have also shown reduction or loss of conventional, NMDAR-dependent LTP with age [(Hsu

et al., 2002, Burke and Barnes, 2006, Boric et al., 2008, Foster, 2012, Freret et al., 2012, Haxaire et al., 2012); but see (Norris et al., 1996)]. In contrast, the magnitude of NMDAR-independent LTP, which generally requires activation of VDCCs, increases with age (Shankar et al., 1998, Tombaugh et al., 2002, Kumar and Foster, 2007). Consistent with this work, the form of LTP we observed after EE was primarily dependent upon NMDARs, but independent of L-type VDCCs. In contrast, previous work has shown that induction of LTD is facilitated by aging (Norris et al., 1996, Hsu et al., 2002, Rosenzweig and Barnes, 2003, Foster and Kumar, 2007). A potential reason for this difference is that these studies characterized different forms of LTD. LTD can be induced by the activation of NMDARs, metabotropic glutamate receptors (Oliet et al., 1997, Palmer et al., 1997), VDCCs (Norris et al., 1996), and intracellular calcium stores (Kumar and Foster, 2005).

EE enhanced both LTP and LTD in aged rats within three weeks. This finding is remarkable considering that most investigations of EE show beneficial results after long treatment durations. For example, working and spatial memory was improved in rats after 22 months of EE (Soffie et al., 1999). Similarly, EE for 6 months prevented memory deficits, reduced anxiety, attenuated age-related impairment of basal glutamatergic neurotransmission in CA1, and reversed the decrease in NMDAR-dependent synaptic potentials in mice (Freret et al., 2012). In contrast, 3 months of EE has yielded mixed results on cognition (Bouet et al., 2011, Kumar et al., 2012). Previous work had shown that EE influences LTP and LTD in young rodents. Two weeks of EE was sufficient to enhance early LTP whereas 5 weeks was necessary to affect LTD (Artola et al., 2006, Buschler and Manahan-Vaughan, 2012). While little was known regarding the effects of EE on CA1 synaptic plasticity in aging rodents, studies had shown short-term EE to be effective in aged animals. For example, in aging rats, three and four weeks of EE, respectively, were able to reactivate ocular dominance plasticity in the visual cortex (Scali et al., 2012) and shift the cortical excitation/inhibition balance (Mainardi et al., 2014). Our study expands this existing knowledge linking EE with enhanced synaptic plasticity to show that these effects hold true in old age.

We also established that LTP enhanced by EE is NMDAR-dependent. This finding is consistent with the fact that induction of LTP by conventional 100 Hz stimulation largely activates NMDARs (Landfield et al., 1978, Barnes, 1979, Foster and Kumar, 2007, Foster, 2012), whereas higher frequency stimulation (200 Hz) induces LTP via VDCCs, not NMDARs (Foster, 2012). Our finding that EE-facilitated LTP is NMDAR-dependent also supports the hypothesis that aging impairs LTP through effects on NMDARs. During aging, hippocampal NMDAR activation and function decrease (Barnes, 1979, Potier et al., 2000, Clayton et al., 2002, Haxaire et al., 2012) whereas calcium from VDCCs and intracellular calcium stores increases (Foster, 2012). Accordingly, there is an age-related shift from NMDAR-dependent LTP to VDCC-dependent LTP (Foster, 2012). Other proposed mechanisms by which aging impairs LTP include neuroinflammatory changes and oxidative stress (Rosenzweig and Barnes, 2003, Foster, 2012). Our work cannot eliminate the possible contributions of neuroinflammatory changes and oxidative stress towards EE-facilitated LTP. Moreover, these mechanisms are not mutually exclusive. For instance, the decline in NMDAR function during aging may be mediated by intracellular redox state (Bodhinathan et al., 2010, Yang et al., 2010). On the other hand, the lack of changes in astrocytes and microglia between control and EE rats suggests that gross neuroinflammatory changes are

not causally involved. Although we did not investigate the form of EE-facilitated LTD, we speculate that it is NMDAR-dependent based on our use of a conventional stimulus paradigm. Like LTP, with age, induction of LTD relies more on metabotropic glutamate receptors (Kumar and Foster, 2007), VDCCs (Norris et al., 1996), and intracellular calcium stores (Kumar and Foster, 2005), while less on NMDARs (Foster, 2012). Given this indirect evidence, future work should focus on delineating how EE modulates NMDAR function.

It is interesting that short-term EE enhanced LTD in our aged rats whereas others have found EE to reduce LTD (Shum et al., 2007, Eckert et al., 2010, Kumar et al., 2012). In addition to the duration of EE, there are some key differences among EE paradigms used by previous studies and this study that may explain these differences. For example, studies that conflict with ours assessed a brain region other than the hippocampus (Shum et al., 2007), used mice instead of rats (Shum et al., 2007, Bouet et al., 2011, Freret et al., 2012), changed cage objects twice a week instead of every day (Eckert et al., 2010), and/or group-housed instead of single-housed control mice (Shum et al., 2007, Bouet et al., 2011, Freret et al., 2012, Kumar et al., 2012). Perhaps EE initially changes LTD, but the hippocampal network compensates to reserve these changes. Thus, our three weeks of EE treatment was long enough to induce changes in LTD, but short enough that compensatory adaptations had not occurred. Alternatively, perhaps EE stresses the animals, and a longer duration of time is needed for the animals to adjust to this stress. Indeed, many aspects of our EE treatment could have caused stress, such as the unfamiliar new environment, increased handling, and increased social contact. While the groups of EE animals did not vary from day to day, the daily transfer of EE rats from individual to larger, group cages could represent a chronic social stressor. In fact, the weight loss that we observed in rats subjected to EE could reflect increased physical activity or stress. Older animals are more disposed to stress due to social interactions, novelty, or handling (McEwen, 2001). Supporting this possibility, other mild stresses, such as bright lights or placement on an elevated platform, can augment LTD (Kim et al., 1996, Xu et al., 1997, Artola et al., 2006, Holderbach et al., 2007). And yet, the effects of these stressors were absent in animals that were preconditioned to the stresses for two weeks. Moreover, these same mild stresses blocked induction of LTP, whereas EE facilitated LTP in our paradigm. Thus, if our EE treatment was stressful for the rats, the rats would likely have acclimated to the stress and the effects of stress would have vanished by our time point. And, if they had not acclimated, we should have seen a negative effect on LTP. Importantly, stress-induced increases in LTD are reversed by EE (Yang et al., 2007). Together, these studies and ours may indicate that LTP and LTD are differentially sensitive to EE, the duration of EE, or mild stressors.

A potential caveat to our study is the fact that we single-housed our rats upon receiving them at 21 months of age from their vendor due to space constraints. This single-housing continued for 7–10 days before, as well as during, the three weeks of control or EE treatments. While the EE group was single-housed, their three hours per day of EE was in the presence of other rats. Despite the short duration of this social isolation, it may have negatively affected our control group. Post-weaning social isolation in rats for four or more weeks impaired spatial cognition (Lu et al., 2003, Quan et al., 2010) and reduced NMDAR-dependent LTP in CA1 (Lu et al., 2003; Roberts and Greene, 2003). In mice, social isolation for only 72 hours depressed induction of LTP (Kamal et al., 2014). While, to our knowledge,

the effect of social isolation commencing in old age has not been tested for its effects on LTP, we must consider the possibility that social isolation affected our results. We also could not find compelling evidence for the effect of post-weaning social isolation on LTD. However, neonatal social isolation delayed the age-related decline in the magnitude of LTD (Ku et al., 2008). If social isolation has the same effect in adult rodents, we would expect it to positively influence induction of LTD. However, LTD could not be induced in aged, single-housed rats in our study, suggesting a minimal effect of social isolation on LTD in aged rats.

Our work leaves the mechanism(s) by which EE affects synaptic plasticity unknown. We investigated molecular mediators suggested by previous work, such as BDNF (Young et al., 1999, Mora et al., 2007, Eckert and Abraham, 2013, Hu et al., 2013, Sale et al., 2014), phosphorylated CREB (Young et al., 1999, Hu et al., 2013), and Vegf (Latimer et al., 2011, Speisman et al., 2013). While BDNF was not clearly altered by EE, we detected an increase in CREB phosphorylation and Vegf expression. It may be that alteration in BDNF requires a longer duration or a different type of EE. For example, our EE did not include a running wheel. Also, it may be that CREB phosphorylation is elevated in anticipation of exposure to EE. Accordingly, future work should investigate if changes in CREB phosphorylation and Vegf expression are causal or secondary to changes in synaptic plasticity or a byproduct of EE itself. Our regimen of EE included social, exploratory, and physical components. Because EE and exercise have at least some distinct effects (Kumar et al., 2012), it would be informative to dissect the component(s) of EE responsible for changes in synaptic plasticity and the molecular players involved.

Conclusions

Our study demonstrates that improvement in synaptic plasticity in elderly rats does not require continuous EE commencing in youth, but only three weeks of intermittent EE in old age. While future work is needed to understand the mechanism behind EE-facilitated synaptic plasticity, our work illustrates the importance of lifestyle variables in altering the aging brain.

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ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
Creb	cAMP response element-binding protein
D,L-APV	2-amino-5-phosphonovarelic acid
EE	Environmental enrichment
fEPSP	Field excitatory postsynaptic potential

H&E	Hematoxylin and eosin
HFS	High frequency stimulation
LFS	Low frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
Map2	Microtubule-associated protein 2
NMDAR	N-methyl-D-aspartate receptor
NPY	Neuropeptide Y
PS	Population spike
Vegf	Vascular endothelial growth factor
VGlut1	Vesicular glutamate transporter 1
VDCC	Voltage-dependent calcium channel

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HIGHLIGHTS

1. Standard stimulus hippocampal LTP and LTD could not be induced in aged rats housed under normal conditions.
2. LTP and LTD could be induced in aged rats after short-term environmental enrichment.
3. The LTP induced in aged rats after short-term environmental enrichment was NMDAR-dependent.
4. Short-term environmental enrichment selectively enhanced phospho-Creb and Vegf expression in aged rats.
5. Short-term environmental enrichment resulted in weight loss in aged rats.

Figure 1. Environmental enrichment (EE) cage

Three aged rats were transferred at a time to this large, two-story cage for three hours a day, for three weeks. The cage contained various toys and objects that were rearranged every day.

Figure 2. Short-term environmental enrichment (EE) enhances synaptic plasticity in the hippocampus

A) High frequency stimulation (HFS, 100 Hz $\times$ 1 sec, arrow) of the Schaffer collateral pathway in the presence of 2.0 mM calcium and 2.0 mM magnesium failed to induce LTP in slices from control rats (closed circles, $n=9$ rats) but successfully induced LTP in the rats exposed to EE (open circles, $n=10$ rats). Traces from EE (upper) and control (bottom) rats are field excitatory postsynaptic potential (fEPSP) waves recorded before (dotted) and 1 hour after HFS (solid traces). Scale: 1 mV, 5 msec. **B)** In slices from control rats, LTD could not be induced by low frequency stimulation (LFS, 1 Hz $\times$ 900 pulses, bar) in the presence of 2.0 mM calcium and 2.0 mM magnesium (closed circles, $n=5$ rats). In contrast, LTD was robust in slices from EE rats (open circles, $n=5$ rats). Traces from EE (upper) and control (bottom) rats are fEPSP waves recorded before (dotted) and 1 hour after HFS (solid traces). Scale: 1 mV, 5 msec. Data are presented as mean $\pm$ S.E.M.

Figure 3. Short-term environmental enrichment (EE) enhances NMDAR-dependent LTP
 EE-mediated LTP is blocked by 100 μ M D,L-APV, an NMDAR antagonist (squares), but is not blocked by 20 μ M nifedipine, which inhibits L-type VDCCs (triangles). Antagonists were administered during the period represented by the black bar (10 min). Traces are from nifedipine (upper) and D,L-APV (bottom) treated slices. fEPSP waves are depicted before (dotted) and 1 hour after HFS (solid traces). $n=5$ rats in each group. Scale: 1 mV, 5 msec. Data are presented as mean $\pm$ S.E.M.

Figure 4. Short-term environmental enrichment (EE) does not affect the immunoreactivity of several synaptic and cellular markers

A) Hematoxylin and eosin (H&E) staining of coronal brain sections. Scale bars represent 500 μ m. **B)** Representative images of immunofluorescence for Dapi (blue), Gfap (green), and Iba1 (red) in CA1. Scale bars represent 50 μ m. **C)** Quantification of the number of Gfap + cells per area (mm^2) in a region of interest spanning the pyramidal cell layer in CA1. **D)** Quantification of the number of Iba1+ cells in CA1 (mm^2) in a region of interest spanning the pyramidal cell layer in CA1. **E)** Representative images of immunofluorescence for Dapi (blue), Synaptophysin (green), and NPY (red) in CA1. Scale bars represent 50 μ m. **F–G)** Quantification of average intensity of synaptophysin immunoreactivity in CA1's *stratum oriens* (**F**) and *stratum radiatum* (**G**). **H)** Quantification of immunoblot of cortical extracts for synaptophysin. **I)** Quantification of the number of NPY+ cells in per area (mm^2) in a region of interest spanning the pyramidal cell layer in CA1. **J)** Representative images of

immunofluorescence for Dapi (blue), VGlut1 (green), and NPY (red) in CA1. Scale bars represent 100 μ M. **K)** Quantification of immunoblot of cortical extracts for Map2. **A–K)** n=4–5 rats in each group. Data are presented as mean $\pm$ S.E.M.

Figure 5. Short-term environmental enrichment (EE) increases the expression of P-Creb and Vegf, but not BDNF

A) Representative images of immunofluorescence for Dapi (blue), BDNF (green and lower panel), and P-Creb (red and middle panel) in CA1. Scale bars represent 50 μ M. **B,D,E)** Quantification of the average intensity of immunoreactivity of the denoted protein using a region of interest spanning the pyramidal cell layer in CA1. **C)** Quantification of immunoblot of cortical extracts for BDNF. **A–E)** $n=4-5$ rats in each group. Data are presented as mean $\pm$ S.E.M. * $P < 0.05$. ** $P < 0.01$.