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Enrichment improves cognition in AD mice by amyloid-related and unrelated mechanisms

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Abstract

Lifelong cognitive stimulation is associated with a lower risk of Alzheimer's disease (AD), but causality is difficult to prove. We therefore sought to investigate the preventative potential of environmental enrichment (EE) using mice expressing both human mutant presenilin-1 and the amyloid precursor protein (PS1/PDAPP). At weaning, mice were placed into either an enriched or standard housing environment. Behavioral testing at 4.5–6 months showed that environmentally enriched PS1/PDAPP mice outperformed mice in standard housing, and were behaviorally indistinguishable from non-transgenic mice across multiple cognitive domains. PS1/PDAPP mice exposed to both environmental enrichment and behavioral testing, but not to EE alone, showed 50% less brain β-amyloid without improved dendritic morphology. Microarray analysis revealed large enrichment-induced changes in hippocampal expression of genes/proteins related to Aβ sequestration and synaptic plasticity. These results indicate that EE protects against cognitive impairment in AD transgenic mice through a dual mechanism, including both amyloid dependent and independent mechanisms.

Keywords: Alzheimer's disease; Environmental enrichment; Amyloid; Microarray

1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorder characterized by parenchymal β-amyloid deposition, neurofibrillary tangle formation, neuronal loss, and cognitive decline. A lifelong pattern of high mental activity [68] and educational attainment [56] correlates with lower risk of AD and may be protective. Furthermore, high levels of linguistic ability early in life are associated with a reduced risk of the disease [47,53]. These studies suggest that extra "cognitive reserve" developed throughout life may help buffer against the development of later dementia. However, the extent to which cognitive stimulation (i.e. environmental enrichment, EE) protects against AD remains difficult to assess in humans because: (1) retrospective studies cannot unequivocally isolate environmental enrichment from other factors affecting cognition over a lifetime, and (2) although short-term cognitive stimulation of AD patients has been shown to be beneficial [36], long-term intervention in humans is impractical. Furthermore, epidemiological human studies give no insight about the potential mechanisms by which EE may protect against AD.

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It is known from previous studies that non-transgenic rodents subjected to cognitive stimulation (i.e. EE) perform better in water mazes, exhibit increased dendritic branching and dendritic spine formation [11,22,61], increased synaptogenesis [45], and increased neuronal plasticity-related gene expression [43], while exhibiting decreased levels of apoptotic cell death [70]. The key question about whether EE intervention can protect against AD pathology, or its associated mental decline, was addressed in the present study using a transgenic mouse model of the disease. The mice chosen express both the human mutant amyloid precursor protein ($hAPP^{V717F}$) and mutant presenilin 1 ($hPS1^{M146L}$) genes, which result in moderate brain β-amyloid plaque deposition and significant behavioral impairment by 5–6 months of age. At weaning, mice were placed into either standard housing or a continuous enriched environment. Half of the animals in each housing condition were behaviorally tested between 4.5 and 6 months of age, while the other half remained in their respective housing to control for effects of behavioral testing on neuropathology and gene expression.

Our previous experiments determined that an EE paradigm used therapeutically in aged APPsw transgenic mice with moderate Aβ plaque deposition provided cognitive benefits without affecting the amyloid plaque burden [2]. The results in that study suggest that consistent, intensive cognitive stimulation could provide considerable benefit to mild-moderate stage AD patients. Here we sought to determine the extent to which pre-emptive EE protects memory, impacts Aβ deposition, and affects dendritic morphology. To characterize these changes at the molecular level, hippocampal gene expression was also analyzed using a whole mouse genome microarray. We found that PS1/PDAPP mice raised in EE outperformed those raised in standard housing (SH) across a variety of cognitive tests, in which they were statistically indistinguishable from non-transgenic (NT) mice. Interestingly, brain Aβ deposition in Tg mice was not affected by EE alone. However, EE in combination with an extensive behavioral testing paradigm (an additional enriching experience), resulted in large reductions in brain Aβ deposition. Although the extent of dendritic branching in cortex/hippocampus was unchanged by enrichment, Tg mice given EE did exhibit increased expression of synaptic plasticity-related genes as well as increased expression of transthyretin (a known Aβ sequestering molecule).

In addition to our current and previous environmental enrichment experiments, two other groups have reported on the neuropathological and/or behavioral effects of EE in Alzheimer's transgenic mice. Jankowsky et al. (2003) reported that raising female APP + PS1 mice in a potentially stress provoking EE (i.e., involving frequent addition/removal of different-aged animals) actually increased brain Aβ deposition [30]. In 2005, the same group confirmed their initial findings of EE-induced increases in brain Aβ deposition for female APP + PS1 mice, while surprisingly also reporting associated protection against cognitive impairment [29]. Recently, Lazarov et al. (2005) reported that discontinuous enrichment sessions in male APP + PS1 mice induced a decrease in brain Aβ deposition and favorable changes in gene expression associated with learning, memory, and neurotrophic actions. These opposite effects of enrichment/novelty on brain Aβ deposition have been attributed to the fact that one study involved males while the other utilized female mice. The different results could also be explained by Jankowsky et al. employing continuous EE while Lazarov et al. utilized discontinuous EE sessions. Furthermore, different APP + PS1 transgenic lines were utilized in the two studies. The present study circumvents these confounding issues by placing equal numbers of male and female mice in each housing condition, and by utilizing continuous housing conditions which more appropriately mimic humans living a lifestyle of consistent cognitive enrichment. Moreover, the gene microarray analysis of the present study was done in animals with already moderate amyloid burdens. This would appear to be more relevant to aging humans who have lived a cognitively-stimulating lifestyle than the microarray analysis done by Lazarov et al., which was conducted in young APP transgenic mice after a considerably shorter period of novelty sessions and well before overt Aβ deposition. Our results show a profound protective effect of EE in both male and female mice, and favorable changes in gene expression related to cognitive function. Reductions in Aβ burden also occurred, but only after an intensive 6-week period of behavioral testing was added to 4.5 months of EE.

2. Materials and methods

2.1. Construction of transgenic mice

All procedures involving experimentation on animal subjects were done in accord with the guidelines set forth by the University of South Florida's Institutional Animal Care and Use Committee. Heterozygous PDGF-hAPP(V717F) mice [Swiss-Webster × C57BL/6] were crossed with PDGF-hPS1(M146L) heterozygotes [Swiss-Webster × C57BL/6] to generate mice with an $APP^{+/-}$, $PS1^{+/-}$ genotype. All offspring were screened by PCR to identity the PDGF-hAPP [19] and the PDGF-hPS1 gene [16]. Mice were also screened for the retinal degeneration (rd) mutation (which causes blindness) and found to be negative for this mutation.

2.2. Environmental enrichment

At weaning, mice were place into two groups that were exposed to standard single housing (PS1/PDAPP $n = 32$; NT = 23) or environmentally enriched housing (PS1/PDAPP $n = 27$; NT $n = 19$) for 4.5–5.5 months. All standard housed (SH) animals were housed in shoe box cages (6.5″ side, 10.5″ long, and 5.5″ high) with static microisolator tops under climate-controlled conditions on a 12 h light/12 h dark cycle, fed Harlan Teklad Global Diet #2018 and provided with tap water ad libitum. Although some enrichment studies in

normal rodents have involved socially-housed animals as the standard housing control, we have found that both single- and socially-housed double transgenic mice are equally impaired across all of our cognitive-based tasks versus NT mice, and have elected to utilize standard single housing as our housing control for this study. Enriched mice had the same diet and light cycle as SH mice, but were socially housed by sex (6–8 to a cage) in a continuous complex environment, which consisted of a 110 l Sterilite container (19″ wide, 32″ long, 13.5″ high) containing an inner "CritterTrailTWO" rodent house which had a running wheel, tunnels, enclosed rooms, and platforms. Within the space surrounding the rodent house were additional running wheels, toys, and huts; these items were changed weekly for subtle novelty in an otherwise continuous enriched environment [2]. In addition to their enriched housing, EE mice were taken out of their cages three times weekly and allowed to explore one of a number of different complex environments (e.g., open field box with toys, Y-maze, large tubes, etc.) for several hours over the entire enrichment period. Approximately equal numbers of male and female mice were included in each of the SH and EE groups. Within any given group, there were never any sex-dependent differences in performance thus allowing combination of data from both sexes for statistical analysis.

2.3. Behavioral testing

Beginning at 4.5–6 months of age, while continuing in either EE or SH housing, approximately half of the mice ($n = 57$) were tested in five cognitive-based tasks as previously described in detail [4]: Y-maze, Morris water maze, circular platform, platform recognition, and radial arm water maze (RAWM) in that order. All testing was conducted during the light phase. Because this report presents results from Morris water maze, platform recognition, and RAWM, the methodology of those tasks is described in detail.

The standard Morris water maze task measures spatial learning and reference memory. For this task, the floor of a 100 cm circular pool was divided into four quadrants, with a 9 cm (dia.) platform submerged in the goal quadrant (quadrant 2). The pool was surrounded by various two- and three-dimensional visual cues, which are necessary for animals to perform well in both this spatial task and the ensuing RAWM spatial task. Reference learning (acquisition) was evaluated over a 9-day period, with four successive trials per day. For each trial, the animals started from a different quadrant, with the same quadrant start pattern taking place on each day of testing. Latency to find the platform (up to 60 s) was recorded for each trial, with daily averages used for statistical comparisons. If an animal failed to discover the platform location in 60 s, it was guided to the platform and allowed a 30 s stay. On days 4, 7, and 10 (prior to acquisition testing for days 4 and 7), each mouse was given a memory retention (probe) trial by allowing the animal to swim in the pool for 60 s after the platform had been removed. Only one trial was performed, with the mouse being placed in the quadrant immediately opposite the former platform quadrant. Retention trials were video recorded, and the amount of time spent in each quadrant was later determined.

The platform recognition task measures the ability to search for and identify/recognize a variably-placed elevated platform. Because it was executed immediately following the Morris water maze spatial task and in the same pool, the platform recognition task requires animals to ignore the spatial cues present around the pool and switch from a spatial to an identification/recognition strategy. It is not a task of visual acuity alone in our paradigm, as underscored by platform recognition performance being directly correlated to levels of brain Aβ deposition, as well as to performance in other cognitive-based tasks [28]. Mice were given four successive trials/day over a 4-day period. Latencies to find an elevated platform (9 cm dia.), bearing a prominent cone-shaped Styrofoam ensign on a wire pole, were determined. For each trial (60 s max.), animals were placed into the pool at the same location and the platform was moved to a different one of four possible locations. Mice that did not find the platform within 60 s were guided to the platform by the experimenter. For statistical analysis, escape latencies for all four daily trials were averaged.

For the RAWM task of working memory, an aluminum insert was placed into the above pool to create six radially-distributed swim arms emanating from a central circular swim area. The latency to locate which one of the six swim arms contained a submerged escape platform (9 cm dia.) was determined for 5 trials/day over 9 days of testing. There was a 30 min time delay between the fourth trial (T4; final acquisition trial) and fifth trial (T5; memory retention trial). The platform location was changed daily to a different arm, with different start arms for each of the five trials semi-randomly selected from the remaining five swim arms. During each trial (60 s max.), the mouse was returned to that trial's start arm upon swimming into an incorrect arm. If a mouse failed to make an arm choice within 20 s, it was returned to that trial's start arm and assigned an error. If the mouse did not find the platform within a 60 s trial, it was guided to the platform for the 30 s stay. Escape latencies during trials 4 and 5 are both considered indices of working memory.

For all of the above tasks, statistical analysis involved either one-way or two-way repeated measure ANOVAs, followed by post-hoc planned comparisons between groups using the Fisher LSD test. Discriminant function analysis (DFA) was performed across the eight cognitive measures that loaded together as the primary cognitive factor in factor analysis. Factor analysis involved all 13 cognitive measures evaluated across all five tasks. The eight cognitive measures in factor 1 that were used for DFA were all from Morris maze (MM), platform recognition (PR), and RAWM. They were: MM overall latency, MM latency during last block, PR overall latency, PR final day latency, RAWM overall T4 latency, RAWM overall T5 latency, RAWM final block T4 latency, and RAWM final block T5 latency. DFA was performed using the DISCRIM subroutine of the Systat software

package. Following completion of the behavioral testing at 6–7.5 months of age, mice were then anesthetized with Nembutal (0.1 mg/g). The animals were intracardially perfused with 0.9% NaCl (25 ml), and their brains were removed.

2.4. Immunohistochemical procedures

Brains were immersion fixed for 24 h in 4% paraformaldehyde in 1× Sorenson's phosphate buffer, followed by cryoprotection via three sequential overnight sucrose immersions, ending in 30% sucrose. Twenty five-micrometer sections were cut using a sliding microtome (Spencer Lens Co.) and a freezing stage (Physitemp). The sections were mounted and processed through antigen retrieval in prewarmed 25 mM citrate buffer (pH 7.3) at +82 °C for 5 min and further processed as previously described [40]. The sections were incubated with primary antibodies against Aβ (6E10, dil. 1:5000) overnight at +4 °C. Secondary antibody was anti-mouse IgG developed with NovaRED substrate kit (Vector). Thioflavine S staining was accomplished using a 5 min incubation in 1% thioflavine S followed by 5 min differentiation in 70% ethanol.

2.5. Image analysis

Data were collected from three equally spaced coronal tissue sections from both dorsal hippocampus and overlying parietal cortex (Bregma −1.30 to −2.30 mm) for each mouse. The sections were examined with a Nikon Eclipse E1000 microscope using either 4× or 10× Plan Fluor objective lenses. A Retiga 1300 CCD (QImaging) with a QImaging RGB LCD-slider was used to capture images. For thioflavine S, a Nikon BV-2B fluorescence filter cube was utilized. Customized software written in Visual Basic 6.0 (Microsoft) utilizing Auto-Pro function calls (Image Pro Plus, Media Cybernetics) was used to segment and quantify images. Aβ deposition was calculated as percent area of interest (=area $\text{stained}_{\text{tot}}$/$\text{area measured}_{\text{tot}}$). Results were analyzed using a two-tailed, unpaired student's *t*-test with Welch's correction.

2.6. Golgi staining

Coronal brain slices 2–3 mm thick were stained en bloc using the Rapid Golgi modification of Valverde [63]. Tissue blocks were initially immersed in a mixture of osmium tetroxide and potassium dichromate for 5–6 days, then rinsed, blotted, and subsequently immersed in a silver nitrate solution for 36–48 h. Blocks were then dehydrated and embedded in nitrocellulose. The stained blocks were then cut at 120 μm on a sliding microtome, cleared in alpha-terpineol, rinsed in xylene, and coverslipped under Permount. All slides were coded for subsequent quantitative analyses. For dendritic branching analysis, randomly selected layer V pyramidal neurons of the parietal cortex (six neurons per brain) had camera lucida drawings prepared of their basilar dendritic arbors. These were subsequently quantified for the amount and distribution of their dendritic domains using the Sholl analysis (Method of Concentric Circles; [50]). Pair-wise statistical comparisons of the Sholl profiles utilized the repeated measures ANOVA with the post-hoc tuckey test.

2.7. ELISA analysis of Aβ levels

Hippocampal protein extracts ($n = 8$) were used for ELISA and western blot analysis. ELISA was performed as previously described [15], with the modification that PBS rather than TBS was used to extract soluble Aβ.

2.8. Microarray

RNA from mice in both EE ($n = 5$) and SH ($n = 4$) was harvested after the period of enrichment and without any behavioral testing. Immediately following saline perfusion, brains were micro-dissected and the hippocampus isolated and frozen in liquid nitrogen. Total RNA was isolated from each hippocampus using an RNeasy kit (Qiagen). The microarray used, Affymetrix GeneChip mouse genome 430 2.0 array, contains over 45,000 probe sets designed from GenBank, dbEST, and RefSeq sequences clustered based on build 107 of the UniGene database. The clusters were further refined by comparison to the publicly available draft assembly of the mouse genome. An estimated 39,000 distinct transcripts are detected including over 34,000 well-substantiated mouse genes. Five micrograms of total RNA from each brain sample served as the mRNA source for microarray analysis. The poly(A) RNA was specifically converted to cDNA and then amplified and labeled with biotin following a previously described procedure [64]. Hybridization with the biotin labeled cRNA, staining, and scanning of the chips followed the proscribed procedure outlined in the Affymetrix technical manual and has been previously described [67]. Scanned output files were visually inspected for hybridization artifacts and then analyzed. Raw Signal intensities for each group, EE and SH, were used for two-tailed *t*-test analysis. Probe sets that yielded a changed *P*-value less than 0.05 were identified as changed (increased or decreased). Furthermore, a signal ratio (SR) (raw probeset signal EE/raw probeset signal SH) of +/−1.40 was used as a lower limit detection boundary. An additional analysis using Affymetrix GCOS 1.2 software (MAS5) generated a second list of changed transcripts, while a third list was generated using Significance Analysis of Microarrays (SAM) (http://www-stat.stanford.edu/~tibs/SAM). Each gene list was compared to a list of over 130 genes known to be related to AD (www.alzforum.org). Furthermore, each gene was manually queried in PubMed (www.pubmed.gov) in combination with the following individual keywords: "memory", "plasticity", "neurogenesis", and "synaptogenesis," the results of which were recorded and cross-referenced to search results from other genes. All genes which had both significantly changed expression levels on at least two of the three microarray analysis methods and an established relevance to memory or AD, as indicated by the above described queries, are reported.

2.9. Rolipram administration

Twelve-month-old PS1/PDAPP/APOE$^{+/-}$ mice with moderate amyloid load were subcutaneously injected daily with either 0.03 mg/kg 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (Rolipram) (5 μg Rolipram/ml vehicle) ($n = 5$) or vehicle only (1 μl DMSO/ml 0.9% Saline) ($n = 4$) for 2 weeks. After 1 day without treatment, all mice were behaviorally tested for 6 days in the radial arm water maze.

3. Results

3.1. Behavioral testing

Cognitive performance for both standard-housed (SH) and environmentally-enriched (EE) mice was determined between 4.5 and 6 months of age through five cognitive-based tasks (Y-maze, Morris maze, Circular Platform, Platform Recognition, and Radial Arm Water Maze)—each measuring discreet cognitive domains. There were no transgenic deficits in either Y-maze spontaneous alternation or circular platform spatial learning, as we have previously reported [31]. In Morris maze acquisition testing, over three 3-day blocks, the performance of Tg/SH mice was impaired on both the second and third blocks of testing (Fig. 1A). By contrast, Tg/EE mice performed indistinguishably from both groups of NT mice. During all three probes trials (days 4, 7, and 10), there were no differences between EE and SH groups for either Tg or NT mice. Thus, EE benefited only Tg mice. Ability to improve in the Morris maze was shown by all mice, indicating that the Tg mice did not suffer from visual or motivational deficits.

In the platform recognition task, which requires mice to switch from the Morris maze's spatial strategy to a search/identification strategy that is cognitively-based [28], similar behavioral benefit was observed for Tg mice. Tg/EE mice had significantly lower escape latencies than Tg/SH mice over all 4 days of testing ($P < 0.01$), as well as on individual days 2 through 4 ($P < 0.01$) (Fig. 1B). Furthermore, Tg/EE mice were not statistically significantly different from either NT group in this task.

Mice were also tested in the radial arm water maze (RAWM), a task designed to assay working memory. Over all three blocks of testing, transgenic mice raised in environmental enrichment (Tg/EE) had significantly lower escape latencies than their SH counterparts for the last acquisition trial (T4; $P < 0.01$) and also the memory retention trial (T5; $P < 0.001$) (Fig. 1C). Indeed, the overall working memory performance of Tg/EE mice was identical to that of NT/SH controls. Interestingly, EE also improved the overall RAWM performance of non-transgenic (NT) mice, as evidenced by their significantly lower escape latencies on T4 compared to their SH counterparts ($P < 0.05$). The only other task in which EE improved upon the performance of NT/SH mice was the circular platform task of spatial learning, wherein NT/EE mice had significantly shorter escape latencies overall compared to NT/SH mice (data not shown). Since mice raised in an enriched environment had larger cages and access to mazes and running wheels, their potential athletic ability could have been superior to mice raised in standard housing, possibly explaining the observed decreased latencies. This was not the case, however, since analysis of the number of seconds taken per arm choice revealed no statistical differences in swim speed between SH and EE groups for either NT or Tg mice. Further evidence of athletic ability-independent cognitive protection is elucidated by the fact that Tg/EE mice made significantly fewer RAWM errors than Tg/SH mice, and again were not statistically different from either NT group (data not shown).

In that we have previously shown that PS1/PDAPP mice do not exhibit any generalized deficits in sensorimotor function [31], the impairments of Tg/SH mice in the above three tasks were cognitively-based and eliminated by EE. Since each group in this study was comprised of approximately equal numbers of male and female mice, statistical analyses were performed to determine whether males and females within a given housing environment differed in cognitive performance. For each of the five cognitive-based tasks of this study, there were no sex-related differences in performance within any group (data not shown).

To determine the effects of EE on overall cognitive performance, we performed discriminant function analysis (DFA) across a number of cognitive measures taken from our test battery which represent multiple cognitive areas such as working memory, reference learning/memory, identification/recognition. Specifically, eight cognitive measures were found by a prior factor analysis of all 13 different behavioral measures, to load together as the primary cognitive factor, and were therefore used for the follow-up DFA [5,40]. As shown in Fig. 1D, DFA was easily able to distinguish the impaired overall cognitive performance of Tg/SH mice from the much better performance of both Tg/EE mice and NT/SH controls ($P < 0.001$ for both comparisons). The latter two groups could not be distinguished from one another by DFA, indicating that EE improves the behavior of Tg mice to such a degree they cannot be classified as different from NT mice over multiple cognitive tasks.

3.2. Alzheimer's disease pathology

To study the effect of environmental enrichment on AD pathology, overall Aβ immunoreactivity and mature β-amyloid (Aβ) deposition were measured. In order to eliminate behavioral testing as an interfering form of cognitive stimulation that could alter brain pathology, we raised two cohorts of mice in both SH and EE conditions through 6 months of age. Aβ immunohistochemistry was measured in both cohorts, but only one cohort underwent behavioral testing between 4.5 and 6 months of age. As was the case for behavioral performance, there were no sex-related dif-

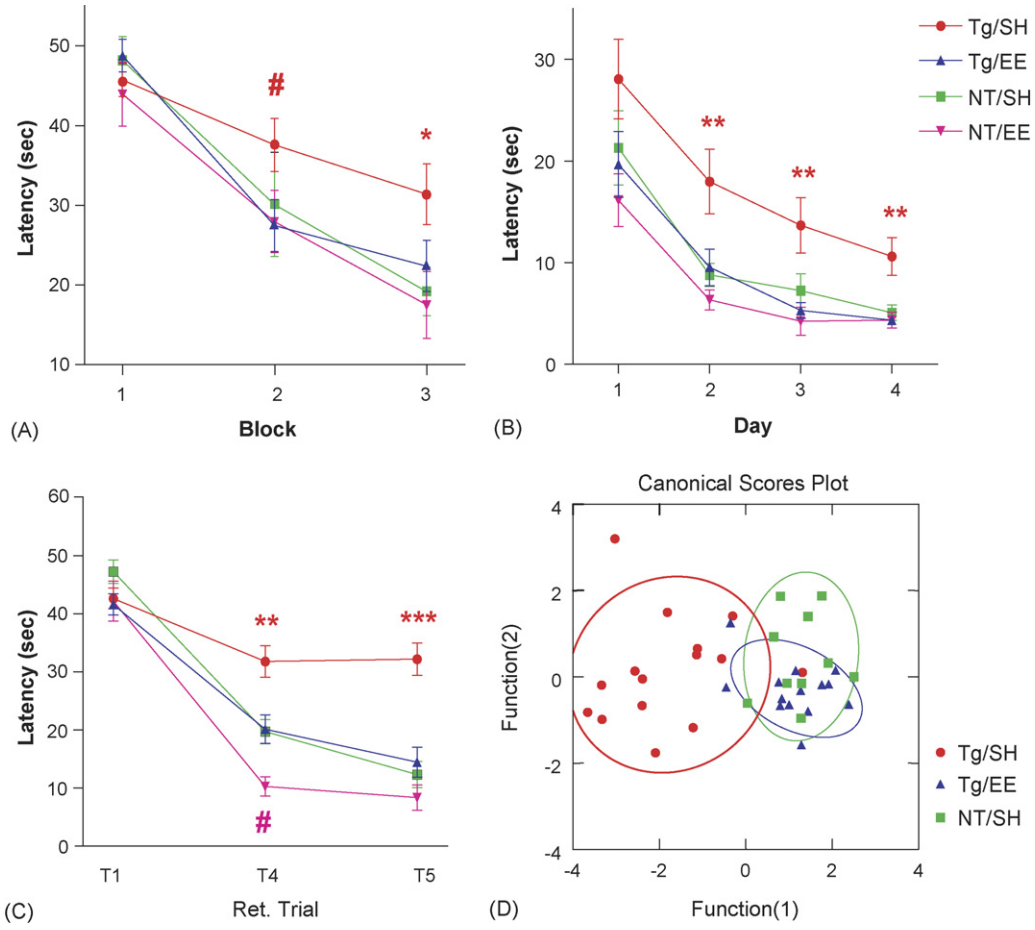

Fig. 1. Environmental enrichment (EE) protects PS1/PDAPP mice against cognitive impairment across multiple behavioral tasks. (A) In Morris water maze acquisitional testing, Tg/SH mice exhibited impaired escape latencies during the second and third blocks of testing, while Tg/EE mice performed identically to both groups of NT mice. $^{*}P<0.05$ for Tg/SH impaired vs. NT/SH and NT/EE; $^{\#}P<0.05$ for Tg/SH impaired vs. Tg/EE. (B) In the platform recognition task of search/identification, the performance of Tg/EE mice over all 4 days of testing was significantly better than impaired Tg/SH controls and identical to NT/SH mice. This effect was evident on individual days 2–4 of testing. $^{**}P<0.01$ for Tg/SH vs. all other groups for that day. (C) In the radial arm water maze task of working memory, Tg mice given standard housing (Tg/SH) showed impaired performance over all days of testing on working memory trials 4 (last acquisition trial) and 5 (retention trial) (T4, T5), while Tg mice raised in EE (Tg/EE) exhibited working memory on trials 4 and 5 that was indistinguishable from non-transgenic standard-housed (NT/SH) mice. On working memory T4, EE also significantly improved performance of non-transgenic mice raised in EE (NT/EE) compared to NT/SH mice. All groups were similar in escape latencies during the semi-randomized initial trial (T1). $^{***}P<0.001$, $^{**}P<0.01$ for Tg/SH vs. both Tg/EE and NT/SH; $^{\#}P<0.05$ for NT/SH vs. NT/EE. In (A) thru (C), means ± S.E.M.s are plotted. (D) Environmental enrichment completely protected PS1/PDAPP mice from overall cognitive impairment across eight behavioral measures evaluated by discriminant function analysis (DFA). This canonical scores plot depicts a linear representation of the two functions resulting from DFA (Wilks' lambda for overall discrimination was $P<0.0001$). The plot graphically shows the poorer overall cognitive performance of individual control Tg/SH mice relative to both enriched Tg/EE mice and control NT/SH mice, which could not be discriminated from one another.

ferences in Aβ deposition for any housing group. Total Aβ immunoreactivity, using the 6E10 monoclonal antibody, revealed that there was no difference in total Aβ load between Tg/EE and Tg/SH mice in either cerebral cortex or hippocampus for the "non-behaviorally tested" group (Fig. 2A and B). Aβ 1-42 enzyme linked immunosorbent assay (ELISA) further verified that the Aβ levels (either soluble or insoluble) were not statistically significantly different between non-behaviorally tested SH versus EE transgenic groups (data not shown). Some pathological chaperones, such as apolipoprotein E, exert their contributory effect on AD pathology by promoting compact plaque formation rather than overall Aβ levels [12]. Taking these prior findings into account, it is not unreasonable to expect EE to elicit changes in only mature (compact) Aβ deposition. We find however, that mature Aβ plaque loads (as measured by thioflavine S staining) were also not significantly different between non-behaviorally tested EE and SH transgenic groups in either cerebral cortex or hippocampus (Fig. 2C and D).

Importantly, we did observe that animals that received both behavioral testing and lived in an enriched environment had significantly lower Aβ deposition than those mice that underwent only EE. This was consistent for both total and compact Aβ deposition and for both cortex and hippocampus (Fig. 2A–D). For behaviorally-tested Tg/EE mice, their 57.6% and 69.0% decreases in compact Aβ deposition in

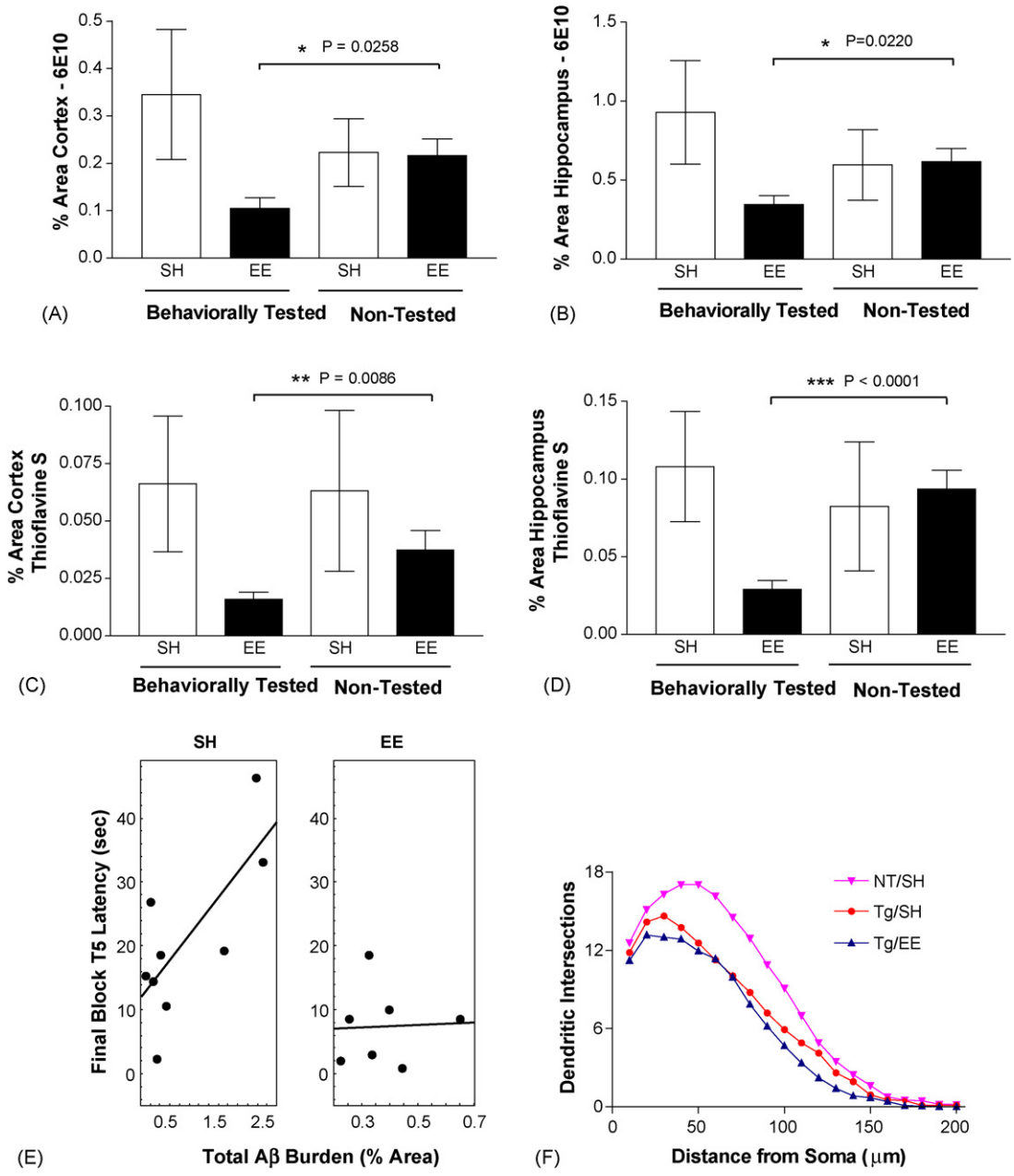

Fig. 2. Total Aβ and compact amyloid plaque levels are greatly reduced in Tg mice both environmentally enriched and behaviorally tested despite dendritic deficits. (A) Immunohistochemical analysis, using the monoclonal antibody 6E10, reveals a 51.5% reduction in area immunostained in cerebral cortex ($P < 0.05$) in those mice behaviorally tested and raised in EE ($n = 8$) vs. mice only raised in EE ($n = 11$). (B) Similarly, in hippocampus, mice behaviorally tested and raised in EE had a 44.1% ($P < 0.05$) reduction in immunostaining vs. those only raised in EE. There was no statistically significant difference in immunostaining in either cortex or hippocampus between EE and SH mice in either tested or non-tested cohorts. (C) Compact plaque deposition, as measured by thioflavine S staining, shows, in cerebral cortex, a 57.6% reduction in mature plaque deposition ($P < 0.01$) and (D) a 69.0% reduction ($P < 0.0001$) in hippocampal deposition in mice behaviorally tested and raised in EE vs. those only raised in EE. As with 6E10 immunostaining, there was no statistically significant difference in thioflavine S staining in either cortex or hippocampus between EE and SH mice in either tested or non-tested cohorts. Data in A–D are means ± S.E.M. (E) Among behaviorally-tested mice, increased Aβ deposition correlated with RAWM working memory impairment in standard-housed mice, but not in enrichment-housed mice. (F) EE does not rescue the transgene-induced dendritic arborization deficits, as shown by Golgi staining ($P < 0.0001$) (Sholl method).

cortex and hippocampus, respectively, as compared to non-behaviorally tested Tg/EE mice, are particularly noteworthy. These reductions in Aβ deposition between behaviorally-tested and non-behaviorally tested animals were seen for both male and female Tg/EE mice alike, analyzed separately or together. Our Aβ deposition results from both cohorts thus indicate that neither EE nor behavioral testing alone is sufficient to alter Aβ pathology. However, when they are combined there is a large statistically significant effect provided by both of these forms of cognitive stimulation.

In behaviorally-tested mice, correlation analyses performed between hippocampal Aβ burdens and cognitive

measures in individual Tg mice revealed a clear dependency on housing condition. Strong positive correlations between Aβ burdens and cognitive impairment were present in standard-housed Tg mice, as exemplified by the correlation between hippocampal 6E10 staining and working memory errors during the final block of RAWM testing (Fig. 2E). Thus, increased Aβ deposition was associated with poorer working memory performance, as we have seen in prior studies [3,24,34,40]. By contrast, no such correlations existed between Aβ deposition and cognitive performance in EE-housed Tg mice (Fig. 2E), indicating that EE-induced cognitive improvement seen in Tg mice is at least partially independent of any reduction in Aβ pathology.

The extent and distribution of dendritic arborization in layer V cortical neurons was measured in behaviorally tested mice via Golgi staining. Using the Sholl method of quantification, there was no observed difference in dendritic branching between transgenic EE and SH groups (Fig. 2F). However, both Tg groups had significantly reduced dendritic arborization compared to non-transgenic standard-housed (NT/SH) mice ($P<0.001$), indicating that EE could not increase dendritic arborization in Tg mice to the level of NT controls. Therefore, despite having reduced dendritic branching, Tg/EE mice performed identically to NT/SH controls. Evidentially, EE must induce changes in the brains of PS1/PDAPP Tg mice that allow them to compensate for any cognitive damage that may be imparted by the dendritic branching deficits and the Aβ pathology typical of these transgenic mouse lines.

3.3. Microarray

We used microarray analysis to examine gene expression changes in the hippocampus that might contribute to the aforementioned memory enhancement. At 6 months of age, non-behaviorally tested EE ($n=5$) and SH ($n=4$) transgenic mice were analyzed using an Affymetrix GeneChip designed for the mouse genome. Twenty genes had both significantly changed expression levels on at least two of three analysis algorithms and an established relevance to one or more aspects of memory or AD (Table 1). Significantly, 17 of the 20 genes, which were reported in an unbiased manner, were changed in a direction that would be expected to confer a beneficial effect for AD according to the available literature ($P<0.002$, Sign test). For example, transthyretin (TTR), an Aβ binding protein shown to sequester Aβ and inhibit amyloid formation, was up-regulated in EE mice (signal ratio range from 4.91 to 3.02; $P<0.01$). Another group of transcripts, all related to nuclear factor-kappaB (NF-KB) inhibition, were up-regulated due to EE. Inhibiting NF-KB has been shown to be neuroprotective in multiple studies [9,72]. The levels of a number of transcripts related to presynaptic vesicle binding and neurotransmitter release were also positively affected by EE, indicating a likely altering of synaptic plasticity that could be involved in the observed changes in cognitive performance. Interestingly, three immediate early genes (IEGs), genes often implicated in mediating changes in neuronal plasticity – activity regulated cytoskeletal-associated protein (Arc), immediate early response 2 (Ier2), and early growth response 1 (Egr1) – were slightly decreased due to EE (SR −1.53, −1.21, −1.18, respectively, $P<0.05$) contradicting a recent report by Lazarov et al. [33]. The fact that the microarray analysis was performed using RNA from mice sacrificed immediately after EE and before a significant reduction in amyloid deposition had been induced (by the additional experience of behavioral testing), suggests that the observed cognitive benefits were likely related to favorable changes in gene expression and were partially independent of amyloid load. A number of genes implicated by the microarray analysis were further validated by real-time PCR (specifically TTR, SNAP23, Col8α1, Cldn3, Nfkbiα, and EGR1), all of which exhibited changes which were in full accord with the microarray results (Table 2).

A primary objective in the analysis of our microarray results was to elucidate targets for pharmacological intervention. Based on the observed synaptic transcript changes, inhibition of NF-KB, and our preliminary microarray experiments (not shown), we chose to target phosphodiesterase 4 (PDE4). PDE4 is known to localize in presynaptic terminals [42] and to mediate both memory [71] and long-term potentiation [39]. It has also been shown to be involved in NF-KB-related induction of inflammation [57]. The PDE4 inhibitor, Rolipram, has been found to alleviate memory deficits in rodent models, including transgenic mice [6,7,23]. We therefore injected PS1/PDAPP mice with Rolipram over a 2-week period, and then subjected them to 6 days of RAWM testing. The Rolipram-treated mice had significantly lower escape latencies than those mice receiving vehicle alone for the last acquisition trial (T4; $P<0.05$) and also the memory retention trial (T5; $P<0.05$), indicating that Rolipram treatment effectively accomplishes the same benefits in cognition as environmental enrichment (Fig. 3). Indeed, a comparison of the results shown in Figs. 1 and 3 reveals that only 2 weeks of Rolipram treatment is able to restore normal function to severely impaired AD mice.

4. Discussion

Our previous studies, and studies from others, have shown that mice that overexpress a mutant human APP gene linked to AD are significantly impaired in a number of behavioral tasks [3,5,31,40,41]. Here we show that doubly transgenic PS1/PDAPP Alzheimer's mice raised in a mentally-stimulating environment were "protected" from otherwise certain cognitive impairment, as evidenced by their superior performance in a variety of behavioral tasks compared to transgenic mice raised in standard housing conditions. In fact, long-term EE produced transgenic mice whose performance across multiple cognitive measures was indistinguishable from non-transgenic mice. This cognitive protection afforded by EE was apparent in both male and female trans-

Table 1
Comparative microarray analysis of hippocampal gene expression between environmentally enriched and standard housed PS1/PDAPP mice

Gene	Symbol	Accesssion number	Affymetrix ID	*t*-Test	SR	Mas5	SAM
Transthyretin	Ttr	NM_013697	1451580_a_at	0.002	4.91	2.14	3.92
			1455913_x_at	0.001	4.24	1.73	
			1459737_s_at	0.001	3.49	1.57	
			1454608_x_at	0.003	3.02	1.37	2.41
Synaptosomal-associated protein 23	Snap23	NM_009222	1420896_at	0.002	1.77		1.41
			1420897_at			0.245	
Synaptogyrin 2	Syngr2	NM_009304	1417081_a_at	0.035	1.52	0.225	
Calcium/calmodulin-dependent protein kinase II alpha	Camk2a	NM_009792	1442707_at	0.049	1.50	0.365	
Neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	NM_008745	1420838_at	0.012	1.44	0.205	1.16
			1435196_at				1.21
Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Nfkbia	NM_010907	1449731_s_at	0.009	1.74		
			1448306_at	0.005	1.73	0.41	1.39
			1438157_s_at			0.39	
Inhibitor of kappaB kinase beta	Ikbkb	AF026524	1426333_a_at	0.012	1.54		1.23
Inhibitor of kappaB kinase gamma	Ikbkg	NM_010547	1421209_s_at	0.013	1.49		1.19
Procollagen, type VIII, alpha 1	Col8a1	NM_007739	1418440_at	0.008	6.03	1.905	4.83
			1418441_at	0.044	3.94	1.29	
			1455627_at			2.065	
Procollagen, type IX, alpha 3	Col9a3	NM_009936	1460693_a_at	0.049	4.50	1.53	
			1460734_at			1.975	
Procollagen, type IV, alpha 6	Col4a6	NM_053185	1421006_at	0.002	2.09		1.67
Occludin	Ocln	NM_008756	1448873_at	0.023	2.00	0.36	1.60
Claudin 1	Cldn1	NM_016674	1437932_a_at	0.042	5.12	1.77	
			1450014_at			0.19	
Claudin 2	Cldn2	NM_016675	1417232_at	0.029	2.69	0.815	
			1417231_at			2.93	
Caspase 6	Casp6	NM_009811	1415995_at	0.015	1.50		1.20
Caspase 8	Casp8	BC006737	1424552_at	0.001	1.99		1.59
Amyloid beta (A4) precursor-like protein 2	Aplp2	NM_009691	1421888_x_at	0.043	1.43		
			1432344_a_at			0.27	
Nicastrin	Ncstn	NM_021607	1418570_at	0.023	1.60		
			1418570_at			0.3	
Protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	Ppp2r5e	NM_012024	1428462_at			0.23	1.18
Transforming growth factor, beta 2	Tgfb2	NM_009367	1423250_a_at	0.031	1.64	0.32	
			1450922_a_at			0.37	
			1438303_at			0.35	

SR: signal ratio (EE/SH); Mas5: average affymetrix signal log ratio; SAM: significance analysis of microarrays fold change.

Table 2
qRT-PCR verification of select transcripts from hippocampal microarray analysis

Gene	Forward primer	Reverse primer	$\Delta\Delta C_T$	Fold change ($2^{-\Delta\Delta C_T}$)
Transthyretin	atggcttcccttcgactctt	gcatccaggactttgaccat	−5.39 ± 1.28	41.98
Synaptosomal-associated protein 23	atggtcagcctcagcaaact	cccatatccagagccatgtt	−0.47 ± 0.26	1.39
Procollagen, type VIII, α 1	tgagatgcctgcgtttactg	ctgtctgccgttgtagagca	−4.72 ± 0.44	26.35
Claudin 3	gcacccaccaagatcctcta	gtagtccttgcggtcgtagg	−2.97 ± 0.71	7.84
NF-kappa light chain gene enhancer in B-cells inhibitor, α	tgcttttgattgaaccacca	aggacacactgggctcactt	−0.29 ± 0.44	1.22
Early growth response 1	gagggacacgctcaccttag	cccccaaaacatcactccta	0.75 ± 0.31	−0.59

Comparative threshold cycle: $\Delta\Delta C_T = \Delta C_T(EE) - \Delta C_T(SH)$. $\Delta C_T = C_T$ (target) $- C_T$ (reference).

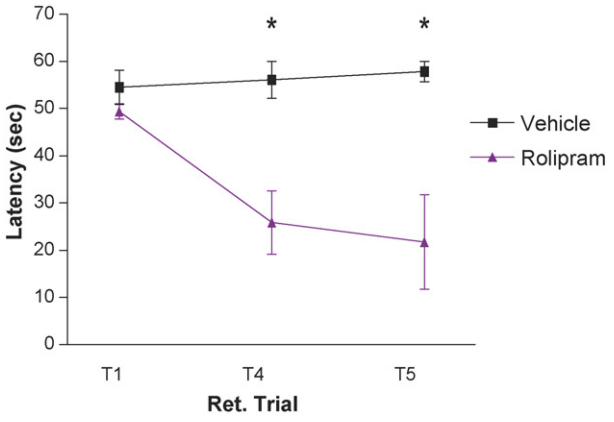

Fig. 3. Administration of Rolipram to Tg mice results in memory improvement which mimics environmental enrichment. (A) In the radial arm water maze task of working memory, Tg mice injected with Rolipram showed significantly improved performance across days 4–6 of testing on working memory trials 4 and 5 (T4, T5), while Tg injected with vehicle only exhibited working memory deficits typical of PS/PDAPP mice. Both groups were similar in escape latencies during the semi-randomized initial trial (T1).

genic mice. Thus, the cognitive benefits of EE were global in nature, affecting multiple cognitive domains and both sexes. However, EE provided only minimal and limited benefit to non-transgenic mice, most likely because of their already superior level of performance even in standard housing conditions. EE also led to reductions in brain Aβ deposition, but only by adding subsequent behavioral-testing, suggesting a possible threshold for the Aβ limiting/sequestering action of cognitive stimulation. Moreover, EE produced beneficial changes in expression of genes involved with Aβ sequestration, memory, and neuronal plasticity. These results not only provide unequivocal evidence that EE can protect against Alzheimer's-like cognitive impairment, but also suggest that both amyloid-independent and amyloid-dependent mechanisms are involved in that protection.

Our previous work has shown that the levels of amyloid deposition in AD (i.e. APP) transgenic mice correlate with the extent of cognitive impairment [5,34,40]. For example, when the Aβ promoting genes Apolipoprotein E (apoE) and/or α-1 antichymotrypsin (ACT) are introduced into a mouse with an APP, apoE-KO, $ACT^{-/-}$ background, the severity of amyloid deposition depends on the presence of these pathology promoters, and, more importantly, directly correlates with the level of cognitive decline [40]. Such correlations between Aβ deposition and cognitive impairment were also evident in the present study for standard-housed Tg mice, but not for EE-housed Tg mice, indicating that EE decreased the relevance of Aβ deposition as a cognitive-impairment factor. More importantly, in the current study, we determined that neither EE nor the 6-week behavioral test period alone was sufficient to affect brain Aβ deposition. However, combining both these forms of cognitive stimulation (i.e. long-term EE and 6 weeks of intense behavioral testing) resulted in dramatic (up to 69%) reductions in both total and mature (compact) Aβ deposition. Since it is unlikely that Aβ deposition during the final 6 weeks of EE would have increased by 2–3 fold in non-tested mice, it is reasonable to conclude that the combination EE experience actually resulted in removal/sequestration of brain Aβ from both diffuse and compact deposits. This EE-induced removal of Aβ resulted not only in lower group means for Aβ deposition, but also much lower variance in Aβ deposition among individual animals within any given EE group. We hypothesize that long-term environmental enrichment primed molecular pathways in the brain that could be further affected by behavioral testing, resulting in the profound reductions in brain Aβ deposition we observed in the behaviorally tested EE animals. It should be noted that the above novel findings could not have been attained without determining EE effects on Aβ deposition in both behaviorally-tested and non-behaviorally tested cohorts of transgenic mice.

In the present study, EE-induced reductions in Aβ deposition were seen in both male and female PS1/PDAPP mice alike. A recent report by Lazarov et al. [33] used a different mouse model (APPsw/PS1dE9) and involved only male mice. Their study involved novelty sessions rather than the continuous EE of the present study. Further confirming our findings, these novelty sessions given by Lazarov et al. were also effective in reducing Aβ deposition. We do not, however, see any changes in the levels of neprilysin gene expression, an Aβ degrading enzyme that these authors attribute to the EE-induced lowering of Aβ, therefore indicating that other sequestration/degradation molecules or mechanisms, possibly the up-regulation of transthyretin, are likely responsible for the changes in pathology that we observed. Furthermore, we do not find an increase in any immediate early genes (data not shown). On the contrary, most IEGs reported to be up-regulated by Lazarov et al. were either slightly down-regulated or not changed at all in our study. Expression of immediate early genes, as a class, may be rapidly (within 15 min) and transiently induced by exposure to a novel environment [27,46], possibly suggesting that: (1) our handling of the animals prior to sacrificing was done in a way as to not induce artifactual changes in IEG expression, or (2) our constant EE conditions (as opposed to short novelty sessions) acclimated the mice to small environmental changes, again preventing a large IEG response immediately prior to sacrifice.

Our results are in partial conflict with two recent studies by Jankowsky et al. [29,30] that focused on Aβ deposition changes in an APPsw/PS1dE9 transgenic mouse model of AD. Their initial study [30] reported that raising female transgenic mice in an unstable enriched environment (i.e. new animals continually added and others removed) resulted in increased brain Aβ deposition. Although this increase could be attributable to the stress of constantly adding new littermates, a later study by the same group, involving stable EE and the same transgenic line again, reported EE-induced elevations in brain Aβ levels [29]. Despite this increased Aβ deposition, this latter study also reported EE-induced cognitive protection in both Morris maze and radial arm water maze tasks. The authors suggest that there may be a

sexually dimorphic Aβ response to enrichment, with EE-induced increases in females and EE-induced reductions in males. The present study provides strong evidence against this premise by showing that both male and female transgenic mice benefit greatly from EE, not only in terms of reduced Aβ deposition, but also in behavioral protection against cognitive impairment. We do show however that, like Jankowsky et al. (2005), behavioral improvement need not rely on decreased Aβ burdens. Based on our research, and that of other authors, we conclude that differences in age, sex, genotype, and EE housing paradigms alter the patterns, either positively or negatively, of Aβ deposition, yet in all cases of EE wherein behavior is measured, cognitive performance improves.

Environmental enrichment was not able to protect against the dendritic branching defects observed in PS1/PDAPP mice despite cognitive benefits observed in the same animals. Analysis of Golgi-stained layer V pyramidal cells in cortex revealed Tg/SH and Tg/EE were nearly identical in their extent of dendritic branching, with both groups having significantly less dendritic branching than NT/SH controls. By contrast, prior studies involving normal rodents have provided evidence for EE-induced changes in dendritic/synaptic morphology. In those studies, EE induced: (1) greater synaptophysin levels (an index of synaptic surface area, but not number of synapses) in hippocampus and neocortex [18], and (2) an increase in Golgi-stained dendritic branches and dendritic spines in neocortex [11,61]. The inability of EE in the present study to increase dendritic branching in Alzheimer's Tg mice suggests that EE's protective effect in those same mice does not primarily involve changes in dendritic morphology. Nonetheless, subtle morphologic changes may have occurred or neuronal populations other than those presently evaluated may have been affected by EE. For example, we see a number of changes in transcripts encoding presynaptic proteins, such as snap23, synaptogyrin 2, etc. Related SNAP and SNARE proteins have often been implicated in synaptic plasticity, and similar genes have been shown to be up-regulated in NT mice that have experienced EE [46]. Furthermore, microarray experiments using human AD brain have indicated that a number of synaptic vesicle genes are down-regulated as a result of the disease process [69], the depression of which is associated with the severity of cognitive deficits and may, in some cases, correlate with disease pathology [25].

To study possible biochemical/genetic changes that might be independent of, or additive to, Aβ reducing/sequestering effects of EE in transgenic mice, we performed microarray analysis using hippocampal RNA from nine non-behaviorally tested transgenic mice (EE $n=5$, SH $n=4$). The resultant expression should reflect the effects of environmental enrichment and help identify genes that are either up- or down-regulated by EE and that may underlie the cognitive protection evident in these transgenic mice. It is important to note that our microarray analysis was performed following 4–5 months of continuous EE and at an age (approximately 6 months old) when PS1/PDAPP mice already have modest Aβ deposition. This design was purposeful in that we wished to mimic the impact that a life-long pattern of enrichment might have on gene expression in humans at an age when brain Aβ deposition is already underway. The microarray analysis reported by Lazarov et al. [33] was completed in young adult Tg mice bearing no Aβ deposits and following only 2 months of three-times-weekly novelty sessions, and therefore did not reflect a continuous cognitively-enriched lifestyle, but rather a series of novelty sessions that does not mimic human lifestyles as closely.

Seventeen of the 20 transcripts which met the specific criteria for inclusion in Table 1 were changed, due to EE, in a direction appropriate to confer a benefit against AD—a striking and statistically significant event. It is particularly interesting that transthyretin was up-regulated in our transgenic mice raised in EE. TTR is a plasma and CSF protein that is known to bind to [59,60] and sequester [48] Aβ, thus inhibiting plaque formation. It is hypothesized that high levels of kidney TTR prevent Aβ plaque formation despite the fact that kidneys have the highest levels of Aβ 1–40 and 1–42 in the body, after the brain [58,60]. In vitro work has shown that TTR co-localizes with amyloid plaques, and in vivo inhibition of TTR increases Aβ deposition [54]. Previous microarray experiments have shown an increase in TTR in an APPsw mouse model of AD as compared to NT mice, and this is hypothesized to be partly responsible for the lack of neurodegeneration typically characteristic of AD mouse models [55]. Similarly, we show an increase in TTR expression due to EE. Perhaps the reduced levels of TTR in human AD patients [49] contribute to their neurodegeneration and may therefore be alleviated by cognitive stimulation. Despite TTR's known Aβ-sequestering activity, the expression increase in our non-behaviorally tested transgenics was unable to limit/decrease their brain amyloid deposition, but may have contributed to the apparent "priming" of the EE animals such that subsequent behavioral testing did lead to reduced amyloid load.

A number of other EE-induced changes in gene expression, all of which could potentially impart a benefit to Alzheimer's disease pathology or phenotype, were observed in this study. For example, memory improvement in EE mice may, at least in part, be attributable to changes in the level of calcium/calmodulin-dependent protein kinase II α (Camk2α), a transcript up-regulated due to EE. Camk2α is required for the induction of long term potentiation in vitro [38], and in vivo [17], and its deletion in animal models results in attenuated synaptic plasticity [20,21] and deficits in both spatial learning tasks [51,52] and long term memory consolidation [35]. In addition, studies of human AD postmortem brain indicate that insufficient activation of Camk2α may underlie disease-related memory impairment [1]. Another transcript up-regulated due to EE, amyloid beta precursor-like protein 2 (Aplp2), is also related to cognitive decline in AD. Mice deficient in Aplp2 have a reduced number of presynaptic vesicles [66], and in vitro experiments show that Aplp2 can stimulate neurite outgrowth [8]. This change is consistent with expected memory- and cognition-related effects con-

ferred by aforementioned increases in presynaptic vesicle gene expression. Learning and memory can additionally be improved by the overexpression of the BDNF receptor, trkB [32], another transcript up-regulated due to EE. Studies in mice by others show a correlation between trkB expression and maze performance [10], and it was recently shown that Aβ can suppress TrkB signaling [13], an event likely thwarted by EE intervention. In all, a number of transcripts were positively changed due to environmental enrichment that may, either individually or in concert, benefit memory and cognition, explaining the observed improvement in behavioral testing performance.

Another group of genes changed due to EE are those related to tight junction integrity. Specifically, Occludin and two members of the Claudin family were shown to be up-regulated, an occurrence linked to alterations in BBB permeability and integrity [14,37]. It has been shown, in the Tg2576 mouse model of AD, that BBB permeability is increased compared to NT controls [62], and that systemic disease-related inflammation may be responsible for these changes due to disruption in tight junction proteins [26]. Therefore an increase in tight junction gene expression may bolster the integrity of the BBB to compensate for inflammation-mediated changes. Additionally, a group of NF-kappaB inhibitory transcripts were up-regulated (Nfkbia, Ikbkb, and Ikbkg), likely reducing inflammation in EE mice [28,65], thereby attenuating downstream events known to promote AD pathology such as IL-1 and ACT activation [44] while preserving BBB function. Another plausible aspect whereby environmental enrichment may be protective against AD may therefore lie either in the reduction of inflammation and/or the regulation of BBB permeability.

Finally, we determined that the combination of two forms of mental stimulation (long-term EE and intense behavioral testing) resulted in dramatic reductions in both total and mature (compact) Aβ deposition occurring over the 6-week period of behavioral testing. Since neither EE nor behavioral testing alone was sufficient to decrease Aβ deposition, yet, by itself, EE reverses the cognitive deficits in AD Tg mice, mechanisms independent of Aβ pathology which ameliorate these symptoms must exist. This conclusion is also supported by our previous study of the effects of EE on aged amyloid-bearing Tg2576 mice in which cognitive decline was reversed but amyloid burden was unaffected. Changes in the expression of neuronal plasticity-related genes, such as those responsible for presynaptic vesicle release, are likely candidates for such an amyloid independent effect.

In sum, we propose that both Aβ-lowering and favorable gene expression pathways are involved in the cognitive protection afforded by EE. Thus, therapeutics designed to impact either Aβ-dependent or -independent pathways may provide cognitive protection against, or viable treatment for, Alzheimer's disease. This prediction has been supported by the finding that pharmacological inhibition of PDE4 by Rolipram yields cognitive benefits similar to that of EE without affecting Aβ burden.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2006.04.009.

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