

Molecular Mechanisms of Environmental Enrichment: Impairments in Akt/GSK3 β , Neurotrophin-3 and CREB Signaling

Yuan-Shih Hu, Nancy Long, Gustavo Pigino, Scott T. Brady, Orly Lazarov*

Department of Anatomy and Cell Biology, University of Illinois at Chicago, Chicago, Illinois, United States of America

Abstract

Experience of mice in a complex environment enhances neurogenesis and synaptic plasticity in the hippocampus of wild type and transgenic mice harboring familial Alzheimer's disease (FAD)-linked APP^{swe}/PS1 Δ E9. In FAD mice, this experience also reduces levels of tau hyperphosphorylation and oligomeric β -amyloid. Although environmental enrichment has significant effects on brain plasticity and neuropathology, the molecular mechanisms underlying these effects are unknown. Here we show that environmental enrichment upregulates the Akt pathway, leading to the downregulation of glycogen synthase kinase 3 β (GSK3 β), in wild type but not FAD mice. Several neurotrophic signaling pathways are activated in the hippocampus of both wild type and FAD mice, including brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF), and this increase is accompanied by the upregulation of the BDNF receptor, tyrosine kinase B (TrkB). Interestingly, neurotrophin-3 (NT-3) is upregulated in the brains of wild type mice but not FAD mice, while insulin growth factor-1 (IGF-1) is upregulated exclusively in the brains of FAD mice. Upregulation of neurotrophins is accompanied by the increase of N-Methyl-D-aspartic acid (NMDA) receptors in the hippocampus following environmental enrichment. Most importantly, we observed a significant increase in levels of cAMP response element-binding (CREB) transcripts in the hippocampus of wild type and FAD mice following environmental enrichment. However, CREB phosphorylation, a critical step for the initiation of learning and memory-required gene transcription, takes place in the hippocampus of wild type but not of FAD mice. These results suggest that experience of wild type mice in a complex environment upregulates critical signaling that play a major role in learning and memory in the hippocampus. However, in FAD mice, some of these pathways are impaired and cannot be rescued by environmental enrichment.

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* E-mail: olazarov@uic.edu

Introduction

Environmental factors and lifestyle are well established as crucial contributing factors for the development of Alzheimer's disease (AD) [1]. We and others have shown that experience of transgenic mice expressing familial Alzheimer's disease (FAD)-linked mutant amyloid precursor protein (APP) and/or presenilin-1 (PS1) in environmental enrichment (EE) rescues impaired neurogenesis, enhances hippocampal long-term potentiation (LTP) and upregulates gene expression of molecular targets associated with learning and memory, synaptic plasticity and neuronal survival [2–4]. This suggests an overall increase of brain plasticity and synaptic function following experience of FAD mice in EE. In addition, environmental enrichment reduces pathological hallmarks in the brains of FAD mice. Specifically, level of soluble oligomeric A β , the neurotoxic precursor of amyloid plaques, is significantly reduced in the brains of FAD mice following EE [3–6]. Moreover, experience in EE decreased the level of hyperphosphorylated tau, the precursor of neurofibrillary tangles in brains of these mice [3,7]. Interestingly, concomitant to reduced levels of hyperphosphorylated tau, we observed an upregulation of the

main anterograde motor protein, kinesin-1, in the brain of enriched mice, suggesting that EE may enhance axonal transport [3].

In spite of the extensive use of EE and the high potential therapeutic value of this experimental paradigm for neurodegenerative diseases and aging, the molecular mechanisms underlying its effects are not fully elucidated. Several studies have reported that physical exercise activates phosphatidylinositol-3-kinase (PI3K)/Akt pathway in the skeletal muscles and in the hippocampus of wild type mice [8–10]. Akt, also known as Protein kinase B, is a major upstream modulator of glycogen synthase kinase 3 beta (GSK3 β) in neurotrophin-dependent signaling pathways. Protein kinase B directly regulates GSK3 β by phosphorylation of GSK3 β at amino acid serine 9, thereby inactivating its kinase activity. Both GSK3 β and cyclin-dependent kinase 5 (CDK5) are key kinases that phosphorylate tau *in vitro* and *in vivo* and their misregulation is implicated in the formation of neurofibrillary tangles [11–16]. Interestingly, both GSK3 β and CDK5 are also key regulators of kinesin-based anterograde axonal transport [17,18].

The predominant neurotrophic factor implicated in AD is brain derived neurotrophic factor (BDNF). Its activation of the tyrosine

kinase B (TrkB) receptor modulates neuronal differentiation, neuronal survival and synaptic plasticity via multiple signaling pathways, i.e. extracellular signal regulated kinases (ERK) implicated in cell differentiation, PI3K/Akt implicated in cell survival, and phospholipase C γ /protein kinase C (PLC γ /PKC) signaling pathways implicated in synaptic plasticity, respectively [19]. Brain derived neurotrophic factor-mediated TrkB activation can promote neuronal synaptic activity through activation of the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding (CREB), a critical signal for the formation of long-term learning and memory [20]. Increased BDNF levels are also associated with enhanced activity of CREB, [21], which in turn drives the expression and activation of intracellular signaling pathways through the actions of two types of glutamate-gated ion channels; α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors.

Upregulation of BDNF protein levels by physical exercise and environmental enrichment was shown previously by numerous studies, including ours [2,22–25]. Alterations in BDNF levels are observed in the cortex and hippocampus of AD patients [26–29], and BDNF immunoreactivity is associated with senile plaques [30,31]. Surprisingly, the nature of BDNF alteration in AD is controversial, with some reports suggesting that BDNF increases [32], and others suggesting it decreases in the hippocampus of AD patients [33,34]. Interestingly, BDNF induces rapid dephosphorylation of tau protein via the PI3K/Akt signaling pathways [35]. Previously we demonstrated that BDNF gene expression is upregulated in APP^{swe}/PS1 Δ E9 mice following EE [2]. Nevertheless, several important questions remain unanswered. These include questions about the effect of EE on BDNF metabolism, the regulation of other neurotrophins and the activation of the pathway(s) downstream of TrkB activation that are directly mediated by EE.

The purpose of this study was to determine the signaling pathways underlying the effects of EE on the brains of nontransgenic and APP^{swe}/PS1 Δ E9 mice. Here we show that expression of the inactive form of GSK3 β is upregulated in the brains of nontransgenic, but not in APP^{swe}/PS1 Δ E9 mice following EE. Similarly, levels of phosphorylated Akt are increased in the brains of nontransgenic, but not in APP^{swe}/PS1 Δ E9 mice following EE. In addition, the levels of several neurotrophic factors, such as BDNF and nerve growth factor (NGF) are also upregulated following EE, both of which are capable of activating Akt/GSK3 β cascade. Experience in EE also increases the levels of neurotrophin-3 (NT-3) in the brains of nontransgenic but not APP^{swe}/PS1 Δ E9 mice, while insulin growth factor-1 (IGF-1) is only upregulated in the hippocampus of APP^{swe}/PS1 Δ E9 mice. Furthermore, we show that NMDA receptor 1 (NMDAR1), but not glutamate receptor-1 (GluR1), is upregulated following EE in the hippocampus of both nontransgenic and APP^{swe}/PS1 Δ E9 mice. Finally, we show that CREB transcription is upregulated following EE in the hippocampus of both nontransgenic and APP^{swe}/PS1 Δ E9 mice. However, CREB phosphorylation is upregulated only in the hippocampus of wild type mice, but not in the hippocampus of APP^{swe}/PS1 Δ E9 mice. This suggests that a critical step in learning and in the formation of long-term memories is defective in APP^{swe}/PS1 Δ E9, and cannot be rescued by EE. This study provides novel insights on a network of cascades altered by EE in the brains of wild type and APP^{swe}/PS1 Δ E9 mice. These findings provide new information about the inhibitory effect of APP^{swe}/PS1 Δ E9 on stimulus-induced upregulation of the Akt/GSK3 β and NT-3 pathways, which may underlie or contribute to defective CREB signaling. Defective EE-induced

CREB phosphorylation APP^{swe}/PS1 Δ E9 mice may suggest that additional intervention will be required for a complete rescue of learning and memory impairments in AD.

Materials and Methods

Transgenic animals

Ethics Statement: All animal procedures were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee (IACUC). FAD-linked APP^{swe}/PS1 Δ E9 transgenic mice coexpressing human PS1 encoding Δ E9 mutation, and mouse APP containing humanized A β and the Swedish mutation (K595N, M596L) were generated as previously described [36]. Transgenic mice and nontransgenic littermates were maintained in standard laboratory conditions (14/10 hr light-dark cycle) and with full access to food and water *ad libitum*. For brain tissue collections, animals were euthanized with isoflurane followed by cervical dislocation. Brain tissues were quickly dissected into different regions (e.g. cortex, hippocampus etc.) and frozen immediately in liquid nitrogen. All samples were stored in -80°C until analyzed.

Environmental enrichment

Twenty one day-old male APP^{swe}/PS1 Δ E9 mice (N = 14, N = 7 for protein analysis, N = 7 for mRNA analysis) and their nontransgenic littermates (N = 14, N = 7 for protein analysis, N = 7 for mRNA analysis) either experienced environmental enrichment for a period of 1 month or were maintained in standard housing (SH) conditions as described previously [3]. Mice were maintained in groups of 3–5 males/cage. The enriched environment was composed of running wheels, color tunnels, visually stimulating toys, and free access to food and water in the enlarged cages (approximately 24 \times 17 \times 11 inches in dimensions). Objects in the cage were changed and repositioned for novel stimulation every day. Mice were exposed to environmental enrichment for 3 hours everyday and returned to the standard housing cage (approximately 11 \times 6 \times 8 inches in dimensions) for the rest of the day. Control groups of APP^{swe}/PS1 Δ E9 mice (N = 14, N = 7 for protein analysis, N = 7 for mRNA analysis) and their nontransgenic littermates (N = 14, N = 7 for protein analysis, N = 7 for mRNA analysis) were singly housed in standard laboratory conditions for 1 month. For the study comparing the effects of young versus old mice, male APP^{swe}/PS1 Δ E9 mice (N = 5) and their nontransgenic littermates (N = 5) were housed in standard laboratory condition for either 2 or 6 months. The number of animals used for each experiment is indicated in the result sections and in each individual figure legends.

SDS-PAGE and Western blot Analysis

Hippocampal and cortical protein extraction were prepared in ROLB buffer as described before [37]. Briefly, 30 μg of detergent soluble protein samples were separated on 7.5% acrylamide gels and transferred onto 0.45 μm nitrocellulose membrane (Bio-Rad) for 2 hours at 100 mV in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3). Membrane blots were blocked for 2 hours in blocking solution (1% BSA in TBS) at room temperature, followed by incubation of primary antibodies diluted in blocking solution overnight at 4°C . On the next day, the membranes were washed three times with TBST (0.1% Tween-20), and incubated in secondary antibody diluted in TBST for 1 hour. Membranes were visualized with ECLTM Plus chemiluminescent substrate (GE Healthcare) and protein expression levels were quantified by densitometric analysis using ImageJ1.41o software (National Institutes of Health, Bethesda, MD, USA). Primary antibodies

used in this study were polyclonal rabbit anti-phosphorylated GSK3 β ser 9 (1:2000, Cell Signaling), monoclonal mouse anti-GSK3 β (1:2500, BD transduction), polyclonal rabbit anti-Akt (1:2000, Cell Signaling), polyclonal rabbit anti-phosphorylated Akt Ser 437 (1:2000, Cell Signaling), polyclonal rabbit anti-ERK C-16 (1:1000, Santa Cruz), monoclonal mouse anti-phosphorylated ERK E-4 (1:1000, Santa Cruz), monoclonal mouse anti-CREB (1:1000, Cell Signaling), monoclonal mouse anti-phosphorylated CREB ser133 (1:1000, Cell Signaling), and monoclonal mouse anti-actin (1:2500, Millipore). Secondary horse peroxidase antibodies used in this study were rabbit anti-mouse HRP (1:5000, Pierce) and donkey anti-rabbit HRP (1:20,000, Promega).

BDNF ELISA Assay

The expression level of BDNF was measured using an ELISA kit, BDNF E_{max} ImmunoAssay System (Promega, WI, USA), according to manufacturer's instructions. Brain tissues were homogenized in modified protein extraction buffer as described [38], followed by BCA quantification assay to determine protein concentration. To measure BDNF levels, 96-well immunoplates were coated with 100 μ L per well of monoclonal anti-mouse-BDNF antibody (1:2000). After an overnight incubation at 4°C, plates were washed three times with wash buffer and the protein samples (100 μ L) were incubated in coated wells for 2 hours at room temperature. Immobilized antigen was incubated with an anti-human BDNF antibody for 2 hours at room temperature. The plates were then incubated with an anti-IgY HRP for 1 hour at room temperature followed by TMB/peroxidase substrate solution and 1 M HCl (100 μ L/well). The colorimetric reaction product was measured at 450 nm using a microplate reader. BDNF concentration was determined based on linear regression of the BDNF standards (range = 7.8–500 pg/mL purified mouse BDNF) that were incubated under similar conditions in each assay. The sensitivity of the assay is about 15 pg/g of BDNF, and cross-reactivity with other related neurotrophic factors (Nerve growth factor, Neurotrophin-3 and Neurotrophin-4) is less than 3%. All samples were assayed in duplicate.

RNA extraction

Total RNA was isolated from brain tissue using RNeasy Mini Kit (Qiagen), according to manufacturer's instruction. Briefly, about 20 mg of tissue were manually homogenized in 500 μ L of buffer RLT extraction buffer. The homogenate was centrifuged for 3 minutes at full speed, and the supernatant was transferred to a new tube. A second centrifugation was performed to remove any remaining cellular debris, followed by adding 95% ethanol for RNA precipitation and binding onto the column. The column was centrifuged and washed several times, and the RNA was eluted from the column using RNase-free water. Total RNA concentration was determined using NanoDrop[®] spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 nm/280 nm, and RNA integrity was determined by running a 1% denaturing agarose gel electrophoresis. Total RNA samples were stored at –80°C until further analysis.

Reverse Transcription and quantitative Polymerase Chain Reaction (qPCR)

Complementary DNA (cDNA) synthesis was performed with SuperScript[®]III First-strand synthesis SuperMix (Invitrogen), using 1 μ g of total RNA and oligo dT primers. cDNA was further diluted in deionized water and stored at –20°C. The lists of primer sequences and references are listed in Table S1. Primer efficiency was tested using conventional PCR. Cycling conditions

were: 10 min at 95°C, followed by 40 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Samples were analyzed in triplicate and a melting curve analysis was performed in each sample at the end of qPCR reaction. Expression level of each gene was determined by BioRad iQ5 icycler real time PCR system employing iQ SYBR Green Supermix (BioRad). Expression levels of 18S ribosomal RNA (18S rRNA) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls. Relative gene expression was determined by $2^{-\Delta\Delta Ct}$ method [39]. The threshold cycle (Ct) value was determined for target genes and the endogenous internal controls in each sample. The difference between target gene Ct and internal control Ct was determined for each sample, resulting in the ΔCt value. The ΔCt of a calibrator sample was subtracted from each sample ΔCt to yield the $\Delta\Delta Ct$ value. Relative fold change was calculated as $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Data are presented as mean \pm SE. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). All biochemical analyses (ELISA, densitometry, RT-PCR data) were analyzed using Student's t test or one-way ANOVA, followed by Tukey's post hoc test. All results were considered statistically significant when $P < 0.05$.

Results

Environmental enrichment downregulates kinase activities involved in pathological tau phosphorylation

Activities of tau and kinesin are both tightly regulated by the level of phosphorylation. Intrigued by our previous observation that EE attenuates tau phosphorylation and upregulates kinesin-1 levels, we examined the role of kinase activities implicated in the regulation of tau and kinesin phosphorylation. Numerous studies in FAD mouse models [16,40–42] and in humans [43–47] suggest that misregulated GSK3 β activity plays a critical role in the pathogenesis of AD. One possibility is that GSK3 β activity is compromised while its level of expression is unaltered. To evaluate this, we used an antibody that recognizes phosphorylated GSK3 β at serine 9 (pGSK3 β ser 9), which corresponds to the inactive form of GSK3 β [48]. To determine whether GSK3 β signaling pathway is compromised in FAD, we compared the ratio of pGSK3 β ser 9 to total GSK3 β expression levels in APP^{swE}/PS1 $\Delta E9$ and wild type littermates maintained in standard housing conditions by Western blot analysis. Although the trend was an increased ratio of inactive GSK3 β to total GSK3 β , the differences were not statistically significant in cortex and hippocampus of 2 month-old wild type mice (N = 4) compared to APP^{swE}/PS1 $\Delta E9$ (N = 4) (Figure 1A), suggesting that in young APP^{swE}/PS1 $\Delta E9$ mice, GSK3 β levels and activity are not altered. To test whether changes in GSK3 β levels were age-dependent, we examined the expression and activity levels of GSK3 β in hippocampal protein extracts of APP^{swE}/PS1 $\Delta E9$ mice at 2 and 6 months of age, corresponding to pre- and post-onset of amyloid deposition, respectively (N = 4 for 2 month-old, N = 4 for 6 month-old). The result showed a significant decrease in pGSK3 β ser 9/total GSK3 β levels in 6 month-old mice, suggesting an increased activity of GSK3 β at 6 months compared to 2 months of age (Figure 1B).

To examine whether EE regulates the level of pGSK3 β ser 9, and hence GSK3 β kinase activity, we examined the protein expression levels of pGSK3 β ser 9 and total GSK3 β in the cortex and hippocampus of nontransgenic and APP^{swE}/PS1 $\Delta E9$ mice following experience in an enriched environment by Western blot

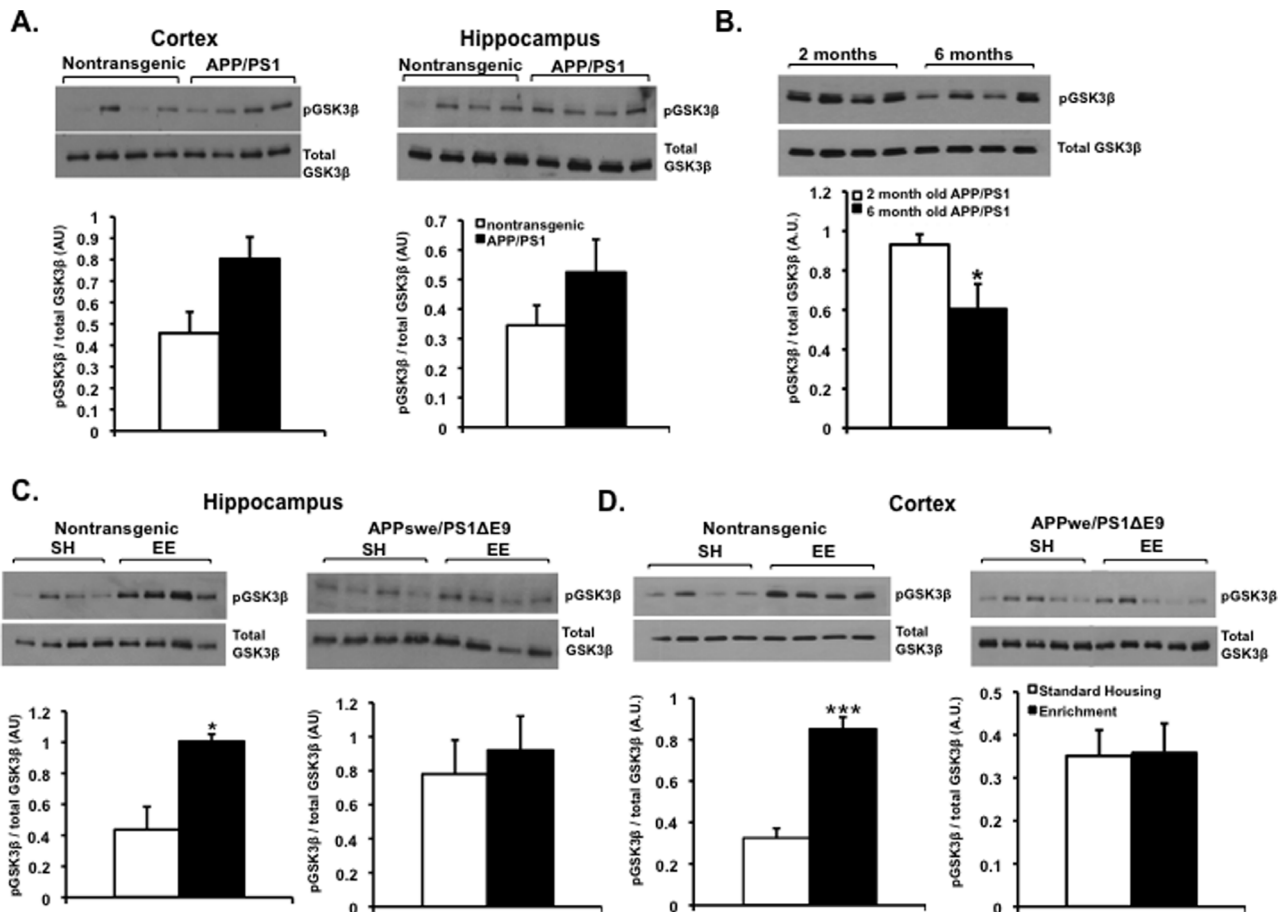


Figure 1. Expression of inactive GSK3 β is upregulated following environmental enrichment in the brains of nontransgenic but not APPsw/PS1 Δ E9 mice. (A) Expression levels of GSK3 β and its inactive form pGSK3 β ser 9 are comparable in the cortex and hippocampus of nontransgenic and APPsw/PS1 Δ E9 mice at 2 months of age as detected by Western blot analysis and densitometric quantification ($P=0.2419$ cortex, $P=0.2161$ hippocampus, $N=4$ for nontransgenic, $N=4$ for APPsw/PS1 Δ E9, Student's t test). (B) Levels of pGSK3 β ser 9 decrease in the hippocampus of 6 month-old APPsw/PS1 Δ E9 ($N=4$) compared to levels at 2 months of age ($N=4$), suggesting an increased activity of GSK3 β at 6 months ($*P<0.05$, Student's t test) as total GSK3 β levels are preserved. (C,D) Levels of pGSK3 β ser 9 increase in the hippocampus (C) and cortex (D) of nontransgenic but not of APPsw/PS1 Δ E9 mice following experience in an enriched environment. Values are means \pm SE (arbitrary units) [$*P<0.05$ nontransgenic hippocampus ($N=4$ for SH, $N=4$ for EE), $P=0.4685$ APPsw/PS1 Δ E9 hippocampus ($N=4$ for SH, $N=4$ for EE), $***P<0.0001$ nontransgenic cortex ($N=4$ for SH, $N=4$ for EE), $P=0.9302$ APPsw/PS1 Δ E9 cortex ($N=5$ for SH, $N=5$ for EE), Student's t test]. doi:10.1371/journal.pone.0064460.g001

analysis. Levels of pGSK3 β ser 9 were significantly upregulated in both cortex and hippocampus of nontransgenic mice following EE [$N=4$ for SH and $N=4$ for EE, (Figure 1C, D left panels)], suggesting a significant reduction in GSK3 β activity in the brains of these mice following EE. However, this effect was not observed in APPsw/PS1 Δ E9 mice following EE [$N=4$ for SH and $N=5$ for EE, (Figure 1C,D right panels)], suggesting that expression of APPsw/PS1 Δ E9 compromises EE-induced downregulation of pGSK3 β activity.

Reduced GSK3 β activity in nontransgenic mice may be induced by the activation of its upstream PI3K/Akt signaling pathway

GSK3 β is one of the major downstream substrates of Akt in the PI3K/Akt-dependent signaling pathway.

Like GSK3 β , the activity of Akt is also tightly regulated by phosphorylation by its upstream regulator, phosphoinositol-3 kinase (PI3K). However, unlike GSK3 β , phosphorylation of Akt at serine 437 by PI3K increases its kinase activity. Thus, we examined the expression levels of phosphorylated Akt using

antibodies that recognize phosphorylation of Akt at Serine 437 (pAkt ser 437) and total Akt in the cortex and hippocampus of these mice. As in the case of GSK3 β , we found no significant difference in the level of total Akt or pAkt ser 437 at 2 months of age in APPsw/PS1 Δ E9 mice ($N=4$) compared to their nontransgenic littermates [$N=4$, (Figure 2A)]. However, we observed a significant decrease of pAkt ser 437 level in 6 month-old mice compared to 2 month-old mice [$N=4$ for 2 month-old, $N=4$ for 6 month-old, (Figure 2B)]. This may suggest that upregulation of GSK3 β activity at 6 months of age in the brains of APPsw/PS1 Δ E9 may directly result from Akt downregulation.

Several studies have reported that physical exercise activates the PI3K/Akt survival-promoting pathway, which in turn regulates GSK3 β activity. This effect is thought to be mediated by increased levels of neurotrophic factors [8,9]. Based on our observation of reduced GSK3 β activity following EE in nontransgenic mice, we analyzed protein expression levels of pAkt Ser 437 in the cortex and hippocampus of standard housing and enriched mice ($N=5$ per group) by Western blot analysis. We observed that pAkt Ser 437 expression level was upregulated in the cortex and

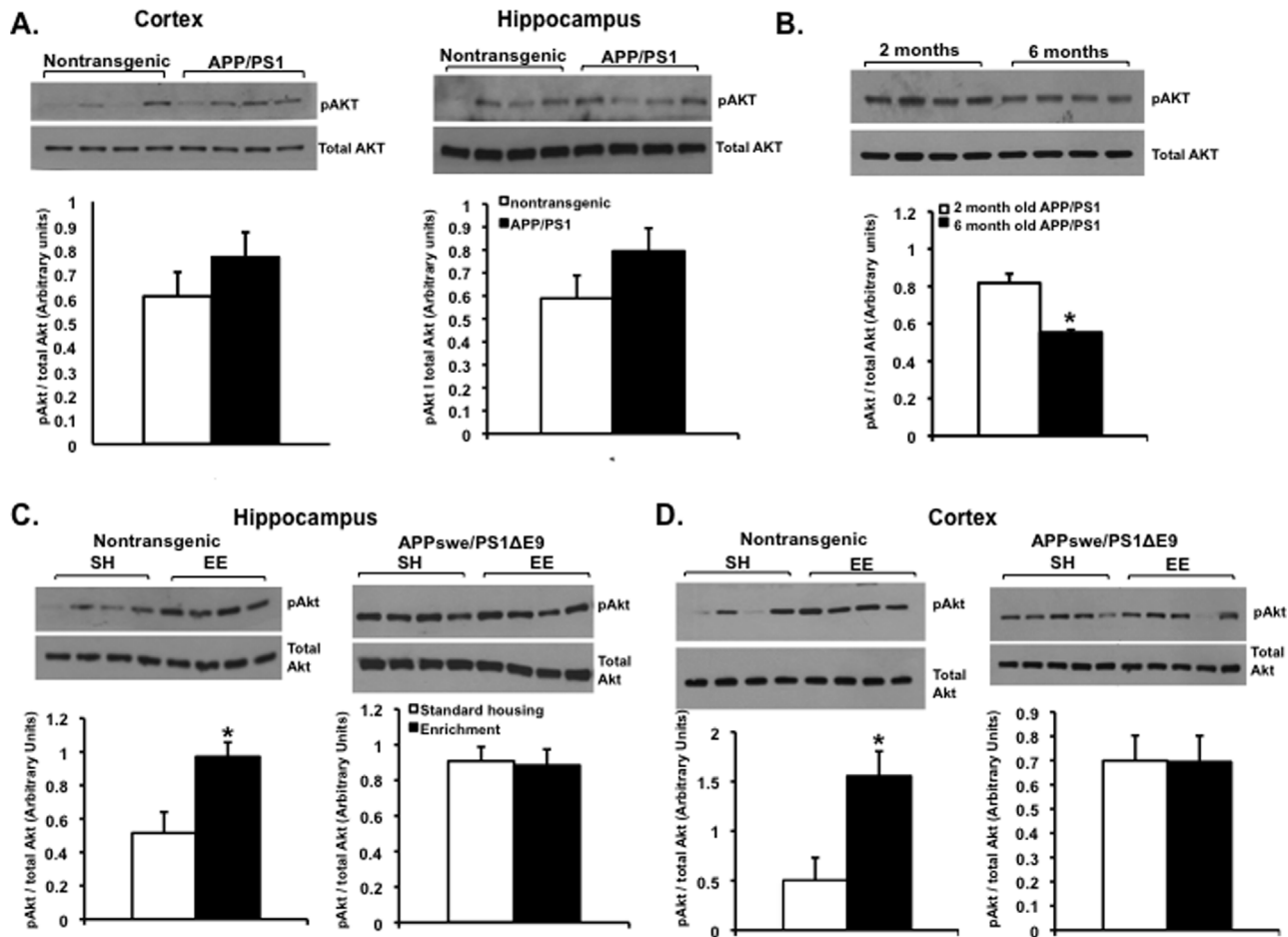


Figure 2. Akt expression and activity are upregulated following environmental enrichment in the brains of nontransgenic but not APPsw/PS1ΔE9 mice. (A) Expression levels of the active form of Akt kinase, pAkt ser 473, is comparable in the cortex and hippocampus of nontransgenic and APPsw/PS1ΔE9 mice at 2 months of age as detected by Western blot analysis and densitometric quantification ($P=0.4331$ cortex, $P=0.4740$ hippocampus, $N=4$ for nontransgenic, $N=4$ for APPsw/PS1ΔE9, Student's *t* test). (B) Levels of pAkt ser 473 decrease in the hippocampus of 6 month-old APPsw/PS1ΔE9 mice ($N=4$) compared to levels at 2 months of age ($N=4$), suggesting decreased activity at 6 months ($*P<0.05$, Student's *t* test). (C,D) Levels of pAkt ser 473 increase in the hippocampus (C) and cortex (D) of nontransgenic but not of APPsw/PS1ΔE9 mice following experience in an enriched environment, again with little or no change in total Akt levels. Values are means \pm SE [(arbitrary units) $*P<0.05$ nontransgenic hippocampus ($N=4$ for SH, $N=4$ for EE), $P=0.9856$ APPsw/PS1ΔE9 hippocampus ($N=4$ for SH, $N=4$ for EE), $*P<0.05$ nontransgenic cortex ($N=4$ for SH, $N=4$ for EE), $P=0.8602$ APPsw/PS1ΔE9 cortex ($N=5$ for SH, $N=5$ for EE), Student's *t* test]. doi:10.1371/journal.pone.0064460.g002

hippocampus of nontransgenic mice that experienced EE (Figure 2C,D left panels), suggesting increased Akt activity following EE. In agreement with our earlier findings on GSK3 β , Akt activity in cortex and hippocampus of APPsw/PS1ΔE9 mice was unaffected by EE (Figure 2C,D left panels). Taken together, this suggests that EE-induced Akt/GSK3 β signaling may be defective in APPsw/PS1ΔE9 mice.

Expression levels of BDNF are comparable in APPsw/PS1ΔE9 and nontransgenic littermates at 2 months of age

The binding of brain derived neurotrophic factor (BDNF) to its receptor activates the PI3K/Akt signaling pathway [49]. Changes in BDNF expression were observed in AD patients [26] and BDNF immunoreactivity was closely associated with senile plaques [30,31]. However, whether the levels of BDNF and its receptors are increased [32,50], or decreased is still controversial in both human post-mortem Alzheimer's brains and in many AD mouse models. [33,34,51]. In fact, depending on the regions studied, the

levels of different neurotrophins in AD patients may vary significantly [29,34].

To address this, we first asked whether mRNA levels of BDNF are altered early in life in APPsw/PS1ΔE9 mice. For this purpose, levels of BDNF mRNA were examined in the hippocampus of nontransgenic and APPsw/PS1ΔE9 mice at 2 months of age, before the onset of AD pathology. While many studies have shown altered expression levels of BDNF in AD patients and AD mouse models at older ages, we did not observe any significant difference in the mRNA expression level of BDNF at 2 months of age, raising the possibility that alterations in BDNF mRNA levels during progression of AD pathology may be through indirect mechanisms rather than by the direct effects of APPsw/PS1ΔE9 mutant expression, or that alterations in BDNF expression are pronounced at a later stage (Figure 3A).

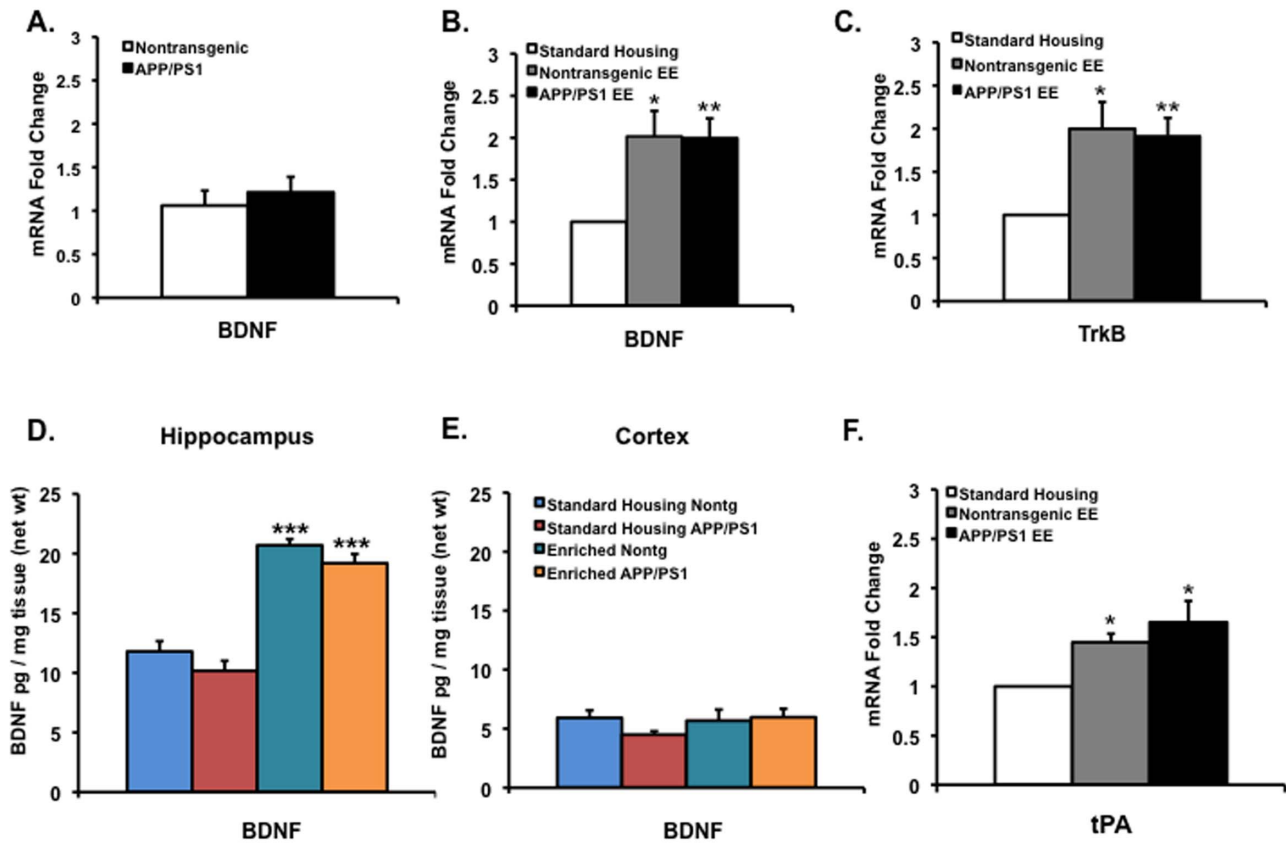


Figure 3. Hippocampus-specific upregulation of BDNF following environmental enrichment in nontransgenic and APPswe/PS1ΔE9 is accompanied by increased levels of TrkB receptors and tPA. (A) Real time RT-PCR of RNA extract of the hippocampus of nontransgenic and APPswe/PS1ΔE9 mice revealed comparable levels of BDNF in the hippocampus of nontransgenic and APPswe/PS1ΔE9 at 2 months of age ($P = 0.5517$, $N = 5$ for nontransgenic SH, $N = 5$ for APPswe/PS1ΔE9 SH, Student's *t* test). (B) BDNF levels increase in the hippocampus of nontransgenic and APPswe/PS1ΔE9 mice following environmental enrichment as detected by real time RT-PCR [$*P < 0.05$ for nontransgenic SH ($N = 5$) vs. nontransgenic EE ($N = 5$), $**P < 0.01$ for APPswe/PS1ΔE9 SH ($N = 5$) vs. APPswe/PS1ΔE9 EE ($N = 5$), Student's *t* test]. (C) Upregulation of TrkB receptors was also observed in the hippocampus of nontransgenic and APPswe/PS1ΔE9 mice following environmental enrichment as detected by real time RT-PCR [$*P < 0.05$ for nontransgenic SH ($N = 5$) vs. nontransgenic EE ($N = 5$), $**P < 0.01$, for APPswe/PS1ΔE9 SH ($N = 5$) vs. APPswe/PS1ΔE9 EE ($N = 7$), Student's *t* test]. (D,E) BDNF levels increase in the hippocampus [$***P < 0.001$ for nontransgenic SH ($N = 6$) vs. nontransgenic EE ($N = 6$), $***P < 0.001$ for APPswe/PS1ΔE9 SH ($N = 6$) vs. APPswe/PS1ΔE9 EE ($N = 6$), one-way ANOVA], but not in the cortex [$P = 0.8356$ for nontransgenic SH ($N = 6$) vs. nontransgenic EE ($N = 6$), $P = 0.0842$ for APPswe/PS1ΔE9 SH ($N = 6$) vs. APPswe/PS1ΔE9 EE ($N = 6$), one-way ANOVA] of nontransgenic and APPswe/PS1ΔE9 following environmental enrichment as determined by ELISA. (F) Upregulation of tPA mRNA level in the hippocampus of nontransgenic and APPswe/PS1ΔE9 following environmental enrichment as determined by real time RT-PCR [$*P < 0.05$ for nontransgenic SH ($N = 6$) vs. nontransgenic EE ($N = 6$), $*P < 0.05$, for APPswe/PS1ΔE9 SH ($N = 6$) vs. APPswe/PS1ΔE9 EE ($N = 7$)]. doi:10.1371/journal.pone.0064460.g003

Environmental enrichment upregulates BDNF and TrkB receptor expression exclusively in the hippocampus of nontransgenic and APPswe/PS1ΔE9 mice

To determine whether environmental enrichment upregulates BDNF at 2 months of age, mRNA expression levels of BDNF were examined in the hippocampus of nontransgenic and APPswe/PS1ΔE9 mice following EE by real time RT-PCR. We observed a two-fold increase in BDNF mRNA expression level in the hippocampus of enriched mice compared to their standard housing littermates in both nontransgenic and APPswe/PS1ΔE9 mice (Figure 3B), suggesting that APPswe/PS1ΔE9 mice retain the ability to upregulate BDNF to the same extent as nontransgenic mice. To gain more insight into the downstream effectors of BDNF modulated by EE, we sought to examine whether EE-induced upregulation of BDNF mRNA is accompanied by upregulation of its receptor TrkB. Here we show that TrkB mRNA level was significantly upregulated following EE in both nontransgenic and APPswe/PS1ΔE9 mice (Figure 3C).

To confirm the upregulation of BDNF protein in the hippocampus of nontransgenic and APPswe/PS1ΔE9 mice following EE, and to examine whether this upregulation is confined to the hippocampus, we examined protein expression levels of BDNF in cortex and hippocampus of nontransgenic and APPswe/PS1ΔE9 mice by ELISA. We found a marked increase in BDNF in the hippocampus of both nontransgenic and APPswe/PS1ΔE9 mice following EE (Figure 3D). However, there was no change in BDNF levels in the cortex of these mice (Figure 3E).

Environmental enrichment-induced BDNF increases may be due to either increased production of BDNF or increased processing of immature proBDNF into mature BDNF. Interestingly, studies have suggested that physical exercise affects hippocampal plasticity by promoting proBDNF proteolytic cleavage, thereby increasing the levels of mature BDNF [52,53]. To address the possibility that EE-induced increases in BDNF were due to its enhanced proteolytic cleavage, we examined the expression of tissue-type plasminogen activator (tPA), the enzyme that converts plasminogen into plasmin, which in turn cleaves

proBDNF to yield mature BDNF. Interestingly, we observed a significant upregulation of tPA mRNA expression following EE in both nontransgenic and APP^{swe}/PS1 Δ E9 mice, suggesting that proteolytic processing of proBDNF to mature BDNF may be enhanced following EE (Figure 3F).

In addition to the PI3K/Akt pathway, BDNF-TrkB signaling modulates neuronal synaptic plasticity via extracellular signal regulated kinases (ERK), and the PLC γ /PKC signaling pathways. To address whether experience of wild type and transgenic mice in EE upregulates these pathways, we examined levels of total ERK and phosphorylated ERK (pERK) in the cortex of enriched nontransgenic and APP^{swe}/PS1 Δ E9 mice by Western blot analysis. While in wild type mice there was a trend of increased levels of pERK following EE, we observed no statistically significant changes in levels of either total ERK or pERK (Figure 4A,B). Gene expression level of PKC γ mRNA by real time RT-PCR revealed no change in PKC γ expression following enrichment in either genotype (Figure 4C).

EE upregulates NGF expression and differentially modulates levels of NT-3 and IGF-1 in the hippocampus of nontransgenic and APP^{swe}/PS1 Δ E9 mice

To determine whether other neurotrophic factors may be upregulated in the hippocampus of nontransgenic and APP^{swe}/PS1 Δ E9 mice, we examined levels of the nerve growth factor (NGF), neurotrophin-3 (NT-3) and insulin growth factor-1 (IGF-1), that were previously reported to be upregulated in the brains of adult rats following EE [24,54–58]. These neurotrophins have been shown to be differentially regulated in the brains of the APP23 transgenic mouse model [4]. Using real time RT-PCR we observed that none of the neurotrophic factors were deficient in the hippocampus of 2 month-old APP^{swe}/PS1 Δ E9 mice, when compared to their nontransgenic littermates (Figure 5A, C, E). However, NGF levels were upregulated in the hippocampi of both nontransgenic and APP^{swe}/PS1 Δ E9 mice following EE (Figure 5B). Interestingly, we observed a significant increase in NT-3 mRNA expression in the hippocampus of nontransgenic mice that experienced EE, but not in enriched APP^{swe}/PS1 Δ E9 mice (Figure 5D). Conversely, IGF-1 mRNA expression was upregulated in the hippocampus of enriched APP^{swe}/PS1 Δ E9

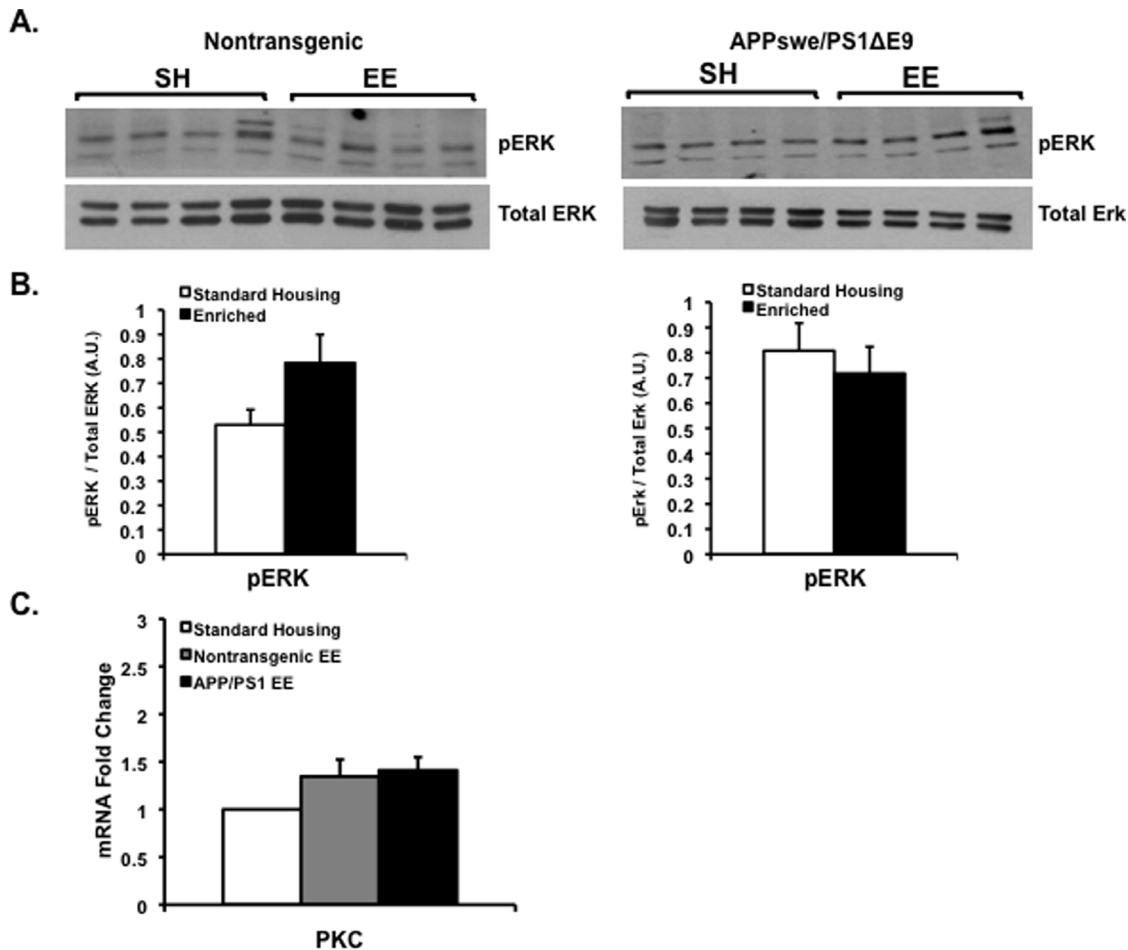


Figure 4. No changes in levels of Akt-induced ERK or PKC signaling following environmental enrichment. (A,B) Protein expression levels of pERK were comparable in the cortex of nontransgenic and APP^{swe}/PS1 Δ E9 mice following experience in an enriched environment as detected by Western blot analysis (A) and densitometric quantification (B), [$P=0.5874$ for nontransgenic ($N=4$ per group), $P=0.1031$ for APP^{swe}/PS1 Δ E9 ($N=4$ per group), Student's t test]. (C) No statistically significant change in the level of PKC following environmental enrichment as detected by real-time RT-PCR [$P=0.6504$ for nontransgenic SH ($N=6$) vs. nontransgenic EE ($N=6$), $P=0.1364$ for APP^{swe}/PS1 Δ E9 SH ($N=6$) vs. APP^{swe}/PS1 Δ E9 EE ($N=6$), Student's t test].

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mice, but not in the hippocampus of enriched nontransgenic mice (Figure 5F). This suggests that EE exhibits differential effects in the hippocampus of nontransgenic and APP^{swe}/PS1 Δ E9 mice. Another possibility is that expression of mutant APP^{swe}/PS1 Δ E9 induces a blockage of EE-induced NT-3, while the upregulation of IGF-1 is a compensatory mechanism for neurotrophin blockage. Nevertheless, further experiments are warranted in order to unravel the mechanism underlying these differences.

Learning and memory-linked CREB phosphorylation is enhanced in wild type but not APP^{swe}/PS1 Δ E9 mice following EE

Expression of cAMP response element-binding (CREB) is critical for formation of long-term memory and learning [20]. Brain derived neurotrophic factor-mediated TrkB activation has been shown to promote neuronal synaptic activity through activation of the transcription factor, CREB, which drives the expression and activation of intracellular signaling pathways

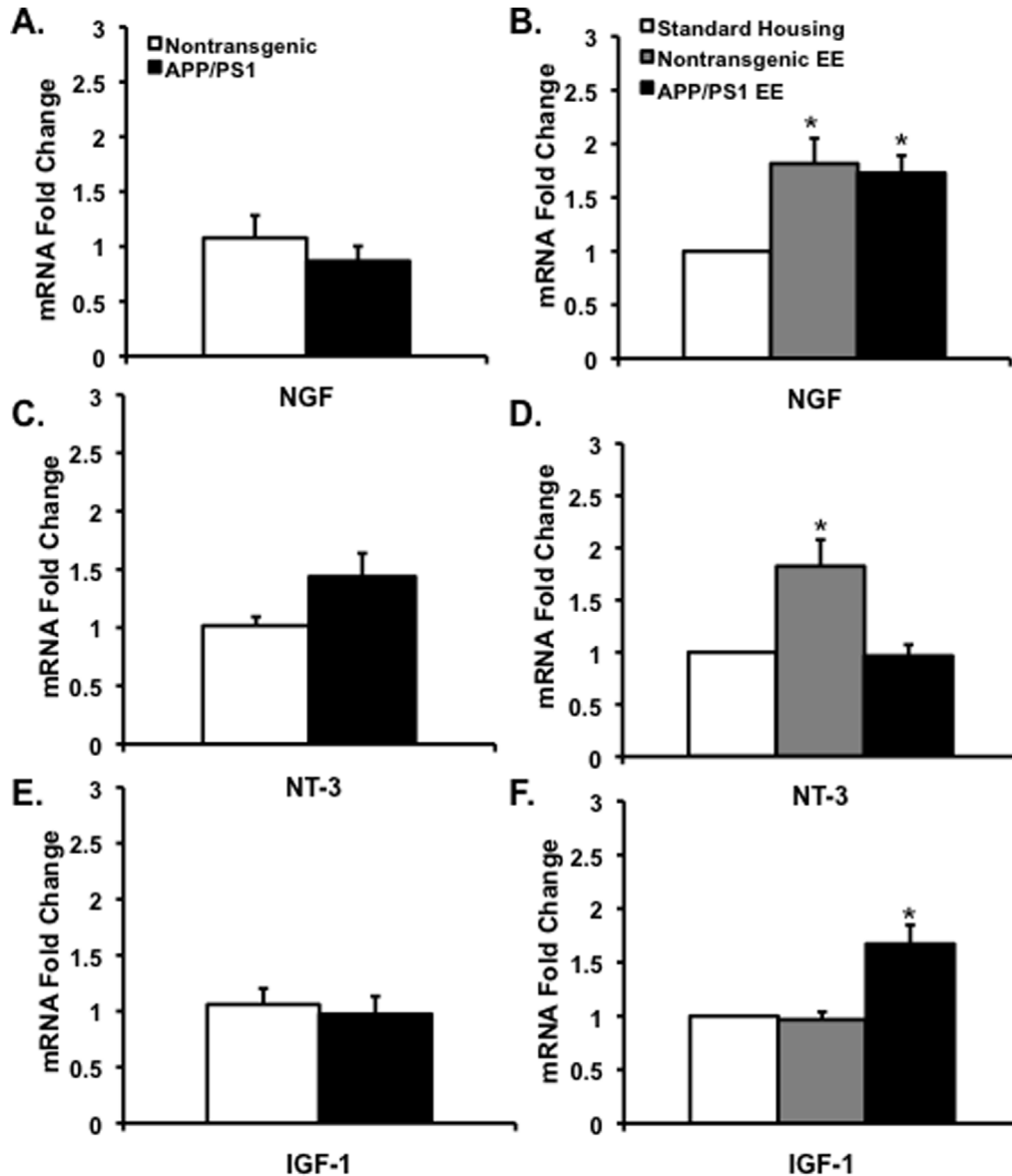


Figure 5. Differential regulation of neurotrophins following environmental enrichment in the brains of nontransgenic and APP^{swe}/PS1 Δ E9 mice. (A,C,E) mRNA levels of NGF (A), NT-3 (B) and IGF-1 (C) are comparable in the hippocampus of nontransgenic and APP^{swe}/PS1 Δ E9 mice at 2 months of age, as determined by real time-RT-PCR (NGF, $P=0.3945$; NT-3, $P=0.0914$ and IGF-1, $P=0.6949$, $N=6$ for nontransgenic, $N=6$ for APP^{swe}/PS1 Δ E9, Student's t test). (B) NGF levels increased in the hippocampus of nontransgenic and APP^{swe}/PS1 Δ E9 mice following environmental enrichment [$*P<0.05$ for nontransgenic SH ($N=6$) vs. nontransgenic EE ($N=6$), $*P<0.05$ for APP^{swe}/PS1 Δ E9 SH ($N=6$) vs. APP^{swe}/PS1 Δ E9 EE ($N=6$), Student's t test]. (D) Levels of NT-3 were upregulated in the hippocampus of nontransgenic but not APP^{swe}/PS1 Δ E9 following environmental enrichment [$*P<0.05$ for nontransgenic SH ($N=6$) vs. nontransgenic EE ($N=6$), $P=0.0640$ for APP^{swe}/PS1 Δ E9 SH ($N=6$) vs. APP^{swe}/PS1 Δ E9 EE ($N=7$), Student's t test]. (E) Levels of IGF-1 increased following environmental enrichment in the hippocampus of APP^{swe}/PS1 Δ E9 but not nontransgenic mice [$P=0.4077$ for nontransgenic SH ($N=7$) vs. nontransgenic EE ($N=7$) $*P<0.05$ for APP^{swe}/PS1 Δ E9 SH ($N=6$) vs. APP^{swe}/PS1 Δ E9 EE ($N=6$), Student's t test].

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through the action of two types of glutamate-gated ion channels; AMPA and NMDA receptors [20,59]. To test the hypothesis that EE upregulates learning and memory-dependent signals, we examined gene expression of CREB following EE. We show that EE upregulated mRNA expression level of CREB by 3.5 fold (Figure 6A), possibly through the activation of neurotrophins and growth factors.

Calcium-calmodulin-dependent protein kinase IV subunit (CaMKIV) has emerged as the most important Ca^{2+} -activated CREB kinase *in vivo* [60,61]. Therefore we examined the expression of CaMKIV in the hippocampus of EE mice. CaMKIV acts as a kinase for the CREB binding protein (CBP). CREB binding protein is known as a transcriptional co-activator that interacts with CREB and proteins in the basal transcriptional complex. Interestingly, CBP is required for EE-induced neurogenesis and cognitive enhancement [62]. While we observed a trend of increased levels of CaMKIV mRNA following EE, this trend did not reach statistical significance (Figure 6B). Another important mediator of learning and memory is the calcium-calmodulin-dependent protein kinase II (CaMKII) subunit. It regulates ion channel properties and synaptic trafficking of AMPA receptors during hippocampal LTP [63–65]. However, gene

expression levels assayed by real time RT-PCR did not show any significant changes in CaMKII mRNA level (Figure 6C). Likewise, we did not observe upregulation of the postsynaptic density protein 95 (PSD-95), a well-characterized postsynaptic marker for plasticity, following experience in EE (Figure 6D).

Previously, we showed that EE upregulates hippocampal LTP [3], suggesting a role for EE in regulating synaptic plasticity. BDNF has been shown to increase mRNA expression level of members of the AMPA receptor family, glutamate receptor-1 (GluR1) and glutamate receptor-2 (GluR2) in hippocampal neurons, as well as mRNA and protein levels of ionotropic glutamate receptor, NMDA receptor subunits, NR1, NR2A and NR2B [66,67]. By increasing the number of NMDA receptors, BDNF upregulates receptor activity and promotes LTP formation. To examine whether EE can induce expression of NMDA and AMPA receptors in APPSwe/PS1 Δ E9 mice, we examined gene expression of NMDAR1 and GluR1 receptors following EE. We show that mRNA expression of NMDAR1 (Figure 6E), but not the AMPA receptor GluR1 (Figure 6F), was significantly induced following EE.

To examine the functional manifestation of increased CREB transcript following EE, we examined levels of total CREB and

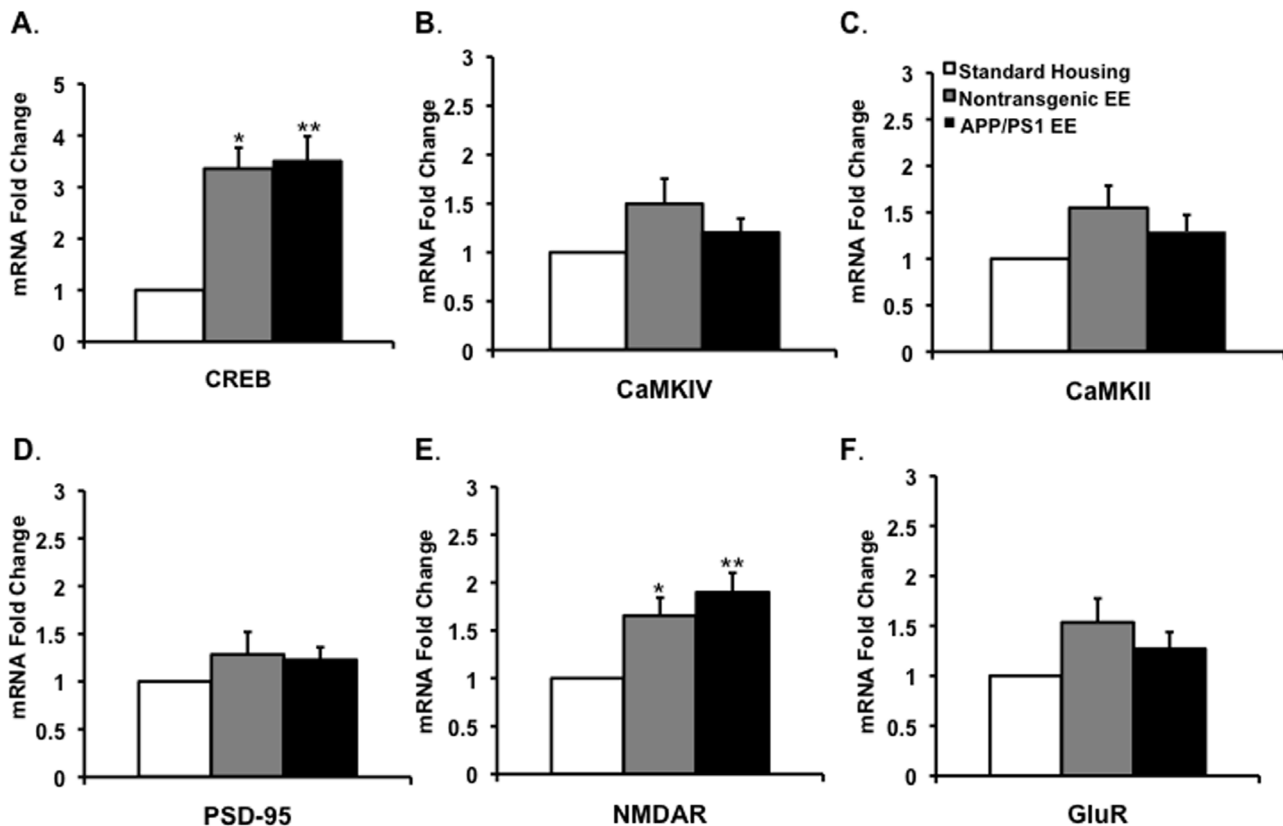


Figure 6. Upregulation of CREB and NMDAR transcription in the hippocampus of nontransgenic and APPSwe/PS1 Δ E9 mice following environmental enrichment. Real time RT-PCR suggests that levels of (A) CREB were significantly upregulated in the hippocampus of nontransgenic and APPSwe/PS1 Δ E9 mice following environmental enrichment [$*P < 0.05$ for nontransgenic SH (N = 7) vs. nontransgenic EE (N = 7), $**P < 0.01$ for APPSwe/PS1 Δ E9 SH (N = 6) vs. APPSwe/PS1 Δ E9 EE (N = 6), Student's t test], but not levels of (B) CaMKIV [$P = 0.2326$ for nontransgenic SH (N = 6) vs. nontransgenic EE (N = 6), $P = 0.5772$ for APPSwe/PS1 Δ E9 SH (N = 6) vs. APPSwe/PS1 Δ E9 EE (N = 7)], (C) CaMKII [$P = 0.0642$ for nontransgenic SH (N = 7) vs. nontransgenic EE (N = 7), $P = 0.3524$ for APPSwe/PS1 Δ E9 SH (N = 6) vs. APPSwe/PS1 Δ E9 EE (N = 7)] or (D) PSD-95 [$P = 0.4337$ for nontransgenic SH (N = 7) vs. nontransgenic EE (N = 7), $P = 0.4236$ for APPSwe/PS1 Δ E9 SH (N = 7) vs. APPSwe/PS1 Δ E9 EE (N = 7)]. (E,F) Levels of NMDAR1 [$*P < 0.05$ for nontransgenic SH (N = 6) vs. nontransgenic EE (N = 6), $**P < 0.01$ for APPSwe/PS1 Δ E9 SH (N = 6) vs. APPSwe/PS1 Δ E9 EE (N = 6)], but not GluR [$P = 0.1256$ for nontransgenic SH (N = 6) vs. nontransgenic EE (N = 6), $P = 0.3937$ for APPSwe/PS1 Δ E9 SH (N = 6) vs. APPSwe/PS1 Δ E9 EE (N = 6)], are upregulated in the hippocampus following environmental enrichment. doi:10.1371/journal.pone.0064460.g006

phosphorylated CREB at serine 133 (pCREB). Following neuronal stimulation, phosphorylation of CREB at serine 133 induces gene transcription that plays a crucial role in the initiation of learning and memory [68]. Western blot analysis shows that levels of total CREB are comparable in mice that experience either standard housing or EE (Figure 7A–C). Interestingly, there was an increase in levels of pCREB in the hippocampus of wild type mice following EE (Figure 7A,B), but not in the hippocampus of APP^{swe}/PS1 Δ E9 mice (Figure 7A,C), suggesting a defect in the signaling cascade initiating learning and memory. In summary, we propose a scheme that outlines the network of signaling pathways involved in EE-induced hippocampal plasticity as obtained in this study (Figure 8).

Discussion

This study provides several important insights concerning the molecular mechanism underlying EE in wild type and APP^{swe}/PS1 Δ E9 mice. First, we show that EE modulates Akt and GSK3 β activities in wild type mice. In contrast, these changes do not take place in APP^{swe}/PS1 Δ E9 mice, suggesting that APP^{swe}/PS1 Δ E9

mutations impair context-induced activation of Akt signaling and suppression of GSK3 β activity. While it is plausible that EE enhances learning and memory and hippocampal plasticity in APP^{swe}/PS1 Δ E9 mice via one of the many other signaling pathways activated following EE, there appears to be a blockade of the Akt activation/GSK3 β suppression pathway. GSK3 β activity is altered in AD patients and in many AD mouse models [42,69,70], and possibly with aging. Thus, the lack of Akt activation and/or GSK3 β suppression may have progressively detrimental effects on hippocampal plasticity, resulting in abnormal tau phosphorylation and inhibition of fast axonal transport. Further studies are warranted in order to determine whether impairments in the Akt pathway following EE in APP^{swe}/PS1 Δ E9 mice result of lack of activation or constitutive activation of this pathway.

Second, we show that EE upregulates several neurotrophic factors, including BDNF, NGF, IGF and NT-3. While an increase in BDNF has been observed previously in several FAD mouse models following EE, it wasn't clear whether other neurotrophic factors were being modulated as well. Increased levels in NGF

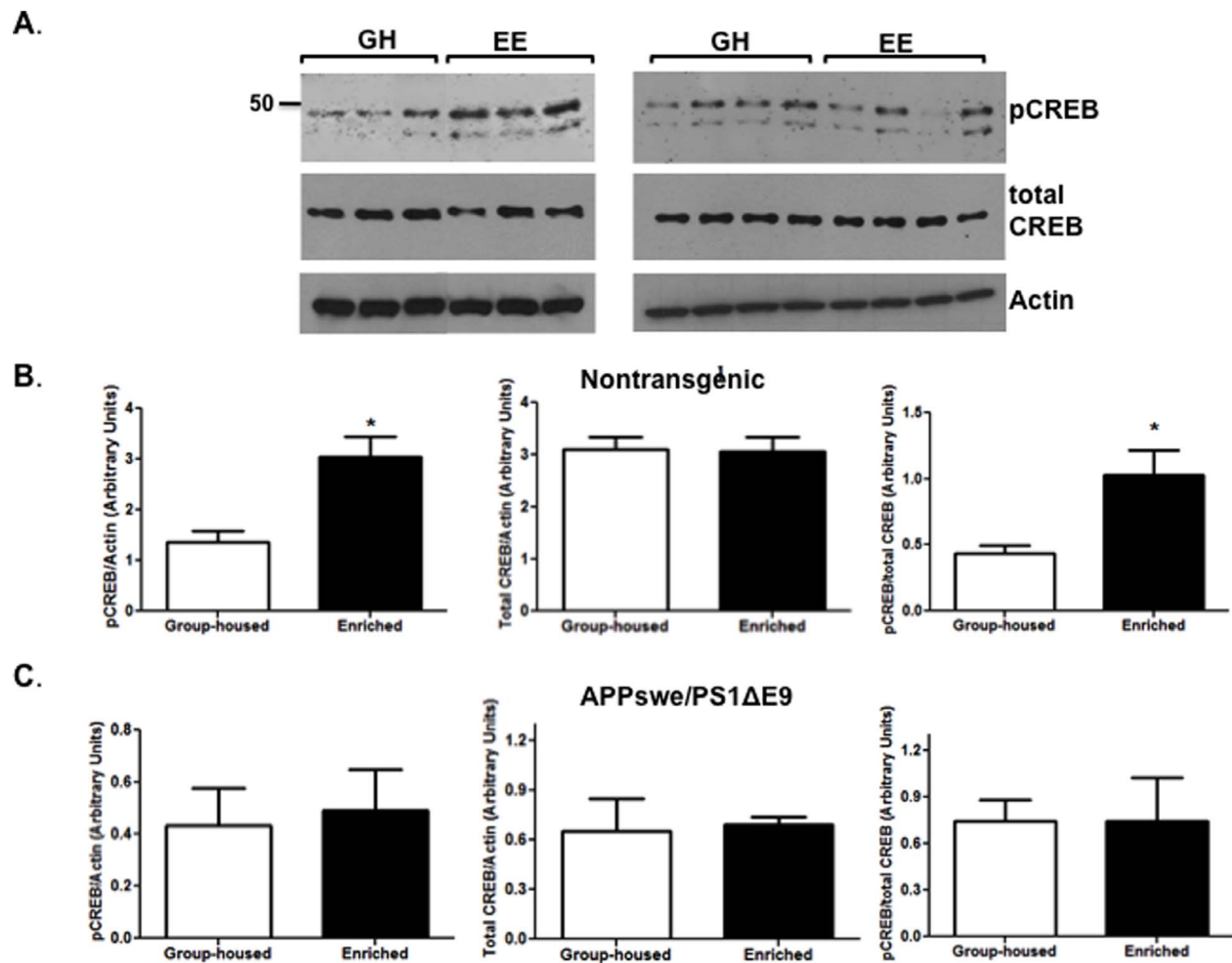


Figure 7. Environmental enrichment upregulates CREB phosphorylation in the hippocampus of wild type but not APP^{swe}/PS1 Δ E9 mice. (A) Western blot analysis of expression level of total CREB and phosphorylated CREB (pCREB) shows an increase in CREB phosphorylation in the hippocampus of wild type but not APP^{swe}/PS1 Δ E9 mice. Levels of total CREB were comparable in mice maintained in standard group-housing (GH) or EE in both genotypes. (B,C) Quantification of total CREB and pCREB levels in the hippocampus of (B) nontransgenic (* P <0.05 pCREB/actin, * P <0.05 pCREB/total CREB, P =0.9161 total CREB/actin, N =3 per group) and (C) APP^{swe}/PS1 Δ E9 mice (P =0.8451 pCREB/actin, P =0.9827 pCREB/total CREB, P =0.8295 total CREB/actin, N =4 per group). doi:10.1371/journal.pone.0064460.g007

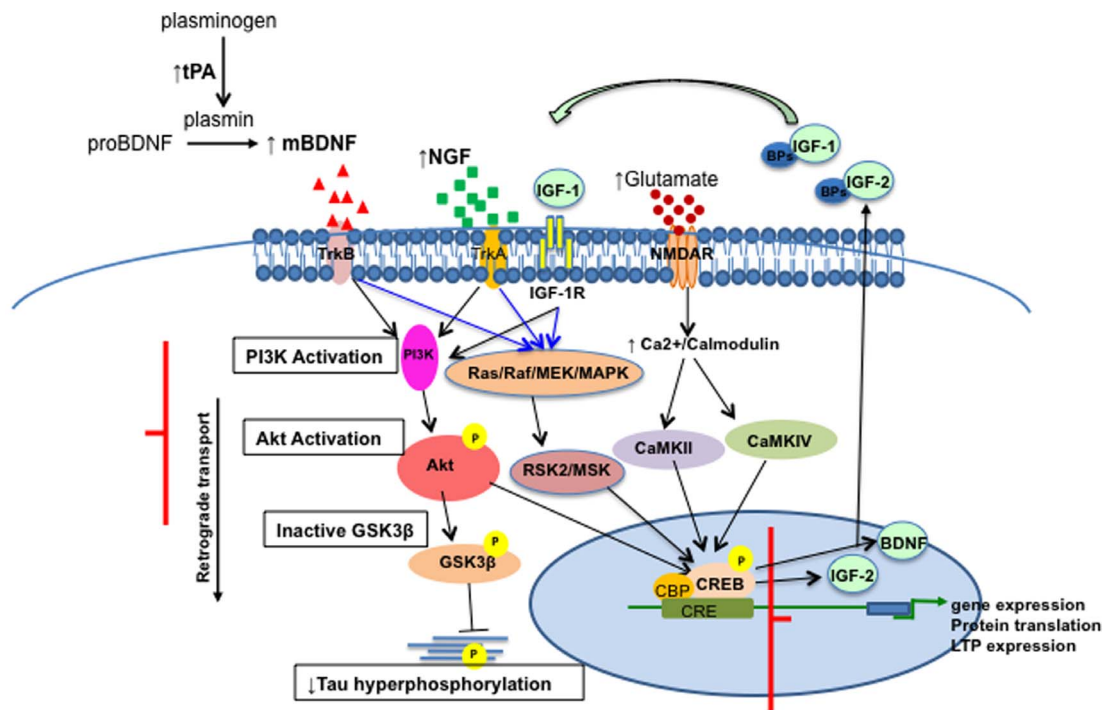


Figure 8. Proposed signaling network upregulated in the hippocampus of wild type and FAD-linked APPsw/PS1ΔE9 transgenic mice following environmental enrichment. Experience in environmental enrichment upregulates the expression of several neurotrophic factors, i.e., brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Upregulation of insulin growth factor 1 (IGF-1) is observed in the hippocampus of APPsw/PS1ΔE9 but not wild type mice, while upregulation of neurotrophin-3 (NT-3) is observed in the hippocampi of wild type but not APPsw/PS1ΔE9 mice. Upregulation of tissue plasminogen activator (tPA) suggests that upregulation of BDNF signaling may be enhanced by the conversion of immature BDNF into the mature form by plasmin. Based on the observed upregulation of the BDNF receptor tyrosine kinase B (TrkB), we hypothesize that neurotrophin binding to their receptors activates intracellular signaling cascades. Intracellularly, several pathways are activated: Protein kinase B (Akt) is phosphorylated, and may downregulate glycogen synthase kinase β (GSK3β) activity that in turn, results in downregulation of tau phosphorylation. This pathway is blocked in the APPsw/PS1ΔE9 mice. Alternatively, a Mitogen-activated protein kinase pathway Ras/Raf/MEK/MAPK, 40S ribosomal protein S6 kinase and mitogen- and stress-activated protein kinase (RSK2/MSK) signaling might be activated following neurotrophin activation. In addition, neurotransmitter (i.e., NMDAR)-induced Ca²⁺/calmodulin-dependent protein kinase cascade is activated. These pathways lead to upregulation of CREB phosphorylation in wild type mice, which in turn, regulates gene expression necessary for the formation of long-term memory, including BDNF and IGF-2.

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[24,54,71,72] and NT-3 [57,58] were observed following EE in wild type rat brain. Expression of both BDNF and NGF is upregulated following EE in both wild type and APPsw/PS1ΔE9 mice, but NT-3 and IGF-1 are differentially regulated by EE in wild type and FAD mice. Interestingly, an increase in NT-3 gene expression level following EE occurs only in wild type mice, suggesting that APPsw/PS1ΔE9 mutants impair NT-3 signaling. In contrast, gene expression of IGF-1 was upregulated in the APPsw/PS1ΔE9, but not in wild type mice. Insulin growth factor-1 is implicated in promoting cell survival signaling pathways following environmental enrichment, and in regulation of neurogenesis through its survival promoting capacity [73,74]. Activation of IGF-1 via its receptors promotes multiple molecular cascades including the PI3K/Akt pathway and the c-Src non-receptor tyrosine kinase, thereby modulating cell proliferation and cellular metabolism. Activity of IGF-1 receptor also appears to play a role in BDNF-mediated effects of physical activity on brain function. For example, IGF-1 signaling via IGF-1 receptor is necessary for exercise-induced upregulation of BDNF [55]. Increased IGF-1 production following exercise training may interact with BDNF to modulate synaptic plasticity, but the nature of functional overlap between the exercise-induced regulation of BDNF and IGF-1 has yet to be determined. Wolf and colleagues (2009) reported a different spectrum of changes in neurotrophin activity, with an

increase in NT-3 and BDNF, but not in NGF or IGF in APP23 mice following EE. Such variations may be due to differences in transgene expression, genetic background, age of animals and duration of EE period [4]. Notably, the studies by Wolf and colleagues were examined in 17 month-old APP23 mice that experienced an EE for 11 months, whereas in our studies young mice were used, and their EE experience was for 1 month.

Third, we show that increased BDNF levels are accompanied by increased mRNA levels of TrkB receptors. It has been shown that expression levels of TrkB are decreased in the frontal cortex and hippocampal formation in AD, while the truncated form of TrkB is increased in association with decreases in BDNF mRNA levels in these brain regions [31,75]. It is also suggested that decreased BDNF and TrkB expression may take place as a function of age or progression of the disease. We observed that BDNF and TrkB expression levels are comparable in wild type and FAD early in life, and that both receptor and ligand levels are upregulated following EE. Importantly, our data suggests that this increase is confined to the hippocampus, as no increase was observed in other cortical regions of these mice. Interestingly, we observed increased expression of tPA in both wild type and FAD hippocampus, suggesting that the increased level of BDNF may result from enhanced production of mature BDNF. Our data further suggest that enhanced BDNF/TrkB signaling following EE does not result

in activation of the downstream signals, ERK and PLC γ /PKC pathways [19], suggesting that these pathways may not play a role in EE under the conditions examined in this study.

Fourth, we show that the expression of NMDA receptors, critical players in learning and memory, are significantly increased in the hippocampus of both wild type and APP^{swe}/PS1 Δ E9 mice following EE. The function of NMDA receptors is significantly reduced in AD patients, possibly contributing to memory deficits [76]. The increase in levels for the NMDA receptor subunits, NR1, NR2A and NR2B may be induced by BDNF [66,67]. Our findings are consistent with Andin and colleagues who showed by *in situ* hybridization that EE upregulates NMDA mRNA expression, while the expression of AMPA mRNA is unchanged in the rat hippocampus [77]. Importantly, BDNF-mediated TrkB activation promotes neuronal synaptic activity via the activation of the CREB transcription factor, which drives expression and activation of intracellular signaling pathways through the action of AMPA and NMDA receptors [21,78]. In support of that mechanism, we show that CREB transcription is upregulated following EE in both wild type and APP^{swe}/PS1 Δ E9 mice. A previous study reported that EE induces hippocampal level of CaMKII and CREB, but not ERK in neurogranin knockout mice [79]. While we show that mRNA levels of CREB are induced following EE, we do not observe an increase in total CREB levels. Nevertheless, we show for the first time that EE upregulates CREB phosphorylation in wild type mice, a critical process in the formation of long-term memory. In addition, we show that CREB phosphorylation following EE is impaired in the APP^{swe}/PS1 Δ E9 mice. Dysregulation of CREB has been implicated in a number of neurodegenerative diseases, including Alzheimer's disease [80]. Several studies suggest dysregulation of CREB, most of which has been attributed to elevated levels of A β [80–84]. Our observations are in agreement with Caccamo and colleagues suggesting that CREB phosphorylation is impaired following Morris Water Maze (MWM) in the 3XTg-AD mice [84]. Previous studies suggest that experience of mice in EE rescues learning and memory deficits in the MWM task in APP^{swe}/PS1 Δ E9 mice [85]. However, it is possible that other aspects of learning and memory not reflected in the MWM, cannot be rescued by EE.

To further elucidate molecular signaling pathways for CREB activation, we examined the expression of CaMKIV, an important Ca²⁺-activated CREB kinase *in vivo* that modifies CREB binding protein (CBP), and CaMKII. The activation via AMPA receptors and autophosphorylation of CaMKII has been shown to be critical for LTP formation [86]. However, we found no significant increase in levels of these kinases following EE. Further experiments are warranted for the understanding of the role of

CREB signaling following EE, its dysfunction in AD and the implications of this dysfunction for learning and memory in these mice.

This study may suggest that an additive therapy aimed at regulating GSK3 β , Akt and pCREB, critical players in multiple cellular processes and in learning and memory, might be necessary in order to reverse deficits that result from dysfunction of these pathways in AD. The functional implications of the lack of induction of CREB phosphorylation following EE in the APP^{swe}/PS1 Δ E9 mice is yet to be determined. In that regard, whether experience of FAD mice in EE fully rescues cognitive deficits is controversial and inconclusive [4,85,87,88]. Several reports suggest that experience of FAD-linked APP^{swe}/PS1 Δ E9 and APP23 mice in EE rescues the performance of mice in the Morris Water Maze and the performance of PS1/PDAPP mice in the platform recognition and radial arm water maze [4,85]. However, other studies report that EE could not rescue deficits in the Barnes maze tests and object recognition tasks, both of which are hippocampus-dependent tasks in TgCRND8 mice [87]. Levi and colleagues report that EE cannot elicit enhancement in learning and synaptic plasticity in apolipoprotein E4 (ApoE4)-expressing mice [88]. Importantly, whether Morris Water Maze and other learning and memory tests used in mice faithfully reflect all aspects of memory and cognition affected in AD is highly questionable. Thus, we claim that while EE has many beneficial effects on wild type and AD mouse brains, there might be aspects that are not adequately or sufficiently rescued.

In summary, this study sheds new light on the complex network of signaling pathways upregulated following EE in the brains of wild type and FAD- APP^{swe}/PS1 Δ E9 mice, and suggests that EE induces multiple molecular pathways, some of which are impaired in APP^{swe}/PS1 Δ E9 mice. This may suggest that translational approaches of EE, such as exercise, may be required but insufficient for the correction of learning and memory impairments in AD.

Supporting Information

Table S1 Primer sequences used for real time RT-PCR. (DOC)

Author Contributions

Conceived and designed the experiments: YSH NL OL. Performed the experiments: YSH NL. Analyzed the data: YSH NL OL. Contributed reagents/materials/analysis tools: GP STB. Wrote the paper: YSH NL GP STB OL.

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