Molecular Mechanisms of Environmental Enrichment: Impairments in Akt/GSK3β, Neurotrophin-3 and CREB Signaling

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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 APPswe/PS1ΔE9. 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 3β (GSK3β), 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 environmental 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.

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 Aβ, 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 (GSK3β) in neurotrophin-dependent signaling pathways. Protein kinase B directly regulates GSK3β by phosphorylation of GSK3β at amino acid serine 9, thereby inactivating its kinase activity. Both GSK3β 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 GSK3β 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

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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 Cγ/protein kinase C (PLCγ/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], in which turn drives the expression and activation of intracellular signaling pathways through the actions of two types of glutamate-gated ion channels; z-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 [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 APPswE/PS1ΔE9 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 APPswE/PS1ΔE9 mice. Here we show that expression of the inactive form of GSK3β is upregulated in the brains of nontransgenic, but not in APPswE/PS1ΔE9 mice following EE. Similarly, levels of phosphorylated Akt are increased in the brains of nontransgenic, but not in APPswE/PS1ΔE9 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/GSK3β cascade. Experience in EE also increases the levels of neurotrophin-3 (NT-3) in the brains of nontransgenic but not APPswE/PS1ΔE9 mice, while insulin growth factor-1 (IGF-1) is only upregulated in the hippocampus of APPswE/PS1ΔE9 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 APPswE/PS1ΔE9 mice. Finally, we show that CREB transcription is upregulated following EE, both of which are capable of activating CREB signaling. Defective EE-induced CREB phosphorylation APPswE/PS1ΔE9 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 APPswE/PS1ΔE9 transgenic mice coexpressing human PS1 encoding ΔE9 mutation, and mouse APP containing humanized Aβ and the Swedish mutation (K595N, M596L) were generated as previously described [36]. Transgenic mice and nontransgenic littersmates 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 APPswE/PS1ΔE9 mice (N = 14, N = 7 for protein analysis, N = 7 for mRNA analysis) and their nontransgenic littersmates (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 24×17×11 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 11×6×8 inches in dimensions) for the rest of the day. Control groups of APPswE/PS1ΔE9 mice (N = 14, N = 7 for protein analysis, N = 7 for mRNA analysis) and their nontransgenic littersmates (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 APPswE/PS1ΔE9 mice (N = 5) and their nontransgenic littersmates (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 nitricellulose 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 ECL™ Plus chemiluminescent substrate (GE Healthcare) and protein expression levels were quantified by densitometric analysis using ImageJ1.40 software (National Institutes of Health, Bethesda, MD, USA). Primary antibodies...
used in this study were polyclonal rabbit anti-phosphorylated GSK3β ser 9 (1:2000, Cell Signaling), monoclonal mouse anti-GSK3β (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 µL 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 µL) 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 µL/well). The colorimetric reaction product was measured at 450 nm using a microplate reader.

**Rice extraction**

Total RNA was isolated from brain tissue using RNAeasy Mini Kit (Qiagen), according to manufacturer’s instruction. Briefly, about 20 mg of tissue were manually homogenized in 300 µL of buffer RLT extraction buffer. The homogenate 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 µg 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 iycyser 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−ΔΔCt 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 ΔΔCt value. Relative fold change was calculated as 2−ΔΔCt.

**Statistical Analysis**

Data are presented as mean ± 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 GSK3β activity plays a critical role in the pathogenesis of AD. One possibility is that GSK3β activity is compromised while its level of expression is unaltered. To evaluate this, we used an antibody that recognizes phosphorylated GSK3β at serine 9 (pGSK3β ser 9), which corresponds to the inactive form of GSK3β [48]. To determine whether GSK3β signaling pathway is compromised in FAD, we compared the ratio of pGSK3β ser 9 to total GSK3β expression levels in APPswe/PS1ΔE9 and wild type littermates maintained in standard housing conditions by Western blot analysis. Although the trend was an increased ratio of inactive GSK3β to total GSK3β, the differences were not statistically significant in cortex and hippocampus of 2-month-old wild type mice (N = 4) compared to APPswe/PS1ΔE9 (N = 4) (Figure 1A), suggesting that in young APPswe/PS1ΔE9 mice, GSK3β levels and activity are not altered. To test whether changes in GSK3β levels were age-dependent, we examined the expression and activity levels of GSK3β in hippocampal protein extracts of APPswe/PS1ΔE9 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 pGSK3β ser 9 to total GSK3β levels in 6 month-old mice, suggesting an increased activity of GSK3β at 6 months compared to 2 months of age (Figure 1B).

To examine whether EE regulates the level of pGSK3β ser 9, and hence GSK3β kinase activity, we examined the protein expression levels of pGSK3β ser 9 and total GSK3β in the cortex and hippocampus of nontransgenic and APPswe/PS1ΔE9 mice following experience in an enriched environment by Western blot.
Levels of \(\text{pGSK3}_\beta\) ser 9 were significantly upregulated in both cortex and hippocampus of nontransgenic mice following EE (\(N = 4\) for SH and \(N = 5\) for EE, Figure 1C, D left panels), suggesting a significant reduction in \(\text{GSK3}_\beta\) activity in the brains of these mice following EE. This effect was not observed in APP\(\text{swe}/\text{PS1}_\Delta\text{E9}\) mice following EE (\(N = 4\) for SH and \(N = 4\) for EE, Figure 1C, D right panels), suggesting that expression of \(\text{APPswe}/\text{PS1}_\Delta\text{E9}\) compromises EE-induced downregulation of \(\text{GSK3}_\beta\) activity.

Reduced \(\text{GSK3}_\beta\) activity in nontransgenic mice may be induced by the activation of its upstream PI3K/Akt signaling pathway

\(\text{GSK3}_\beta\) is one of the major downstream substrates of Akt in the PI3K/Akt-dependent signaling pathway.

Like \(\text{GSK3}_\beta\), the activity of Akt is also tightly regulated by phosphorylation by its upstream regulator, phosphoinositol-3 kinase (PI3K). However, unlike \(\text{GSK3}_\beta\), 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 (\(\text{pAkt ser 437}\)) and total Akt in the cortex and hippocampus of these mice. As in the case of \(\text{GSK3}_\beta\), we found no significant difference in the level of total Akt or \(\text{pAkt ser 437}\) at 2 months of age in APP\(\text{swe}/\text{PS1}_\Delta\text{E9}\) mice (\(N = 4\)) compared to their nontransgenic littermates (\(N = 4\), Figure 2A). However, we observed a significant decrease of \(\text{pAkt ser 437}\) level in 6 month-old mice compared to 2 month-old mice (\(N = 4\) for 2 month-old, \(N = 5\) for 6 month-old, Figure 2B). This may suggest that upregulation of \(\text{GSK3}_\beta\) activity at 6 months of age in the brains of APP\(\text{swe}/\text{PS1}_\Delta\text{E9}\) may directly result from Akt downregulation.

Several studies have reported that physical exercise activates the PI3K/Akt survival-promoting pathway, which in turn regulates \(\text{GSK3}_\beta\) activity. This effect is thought to be mediated by increased levels of neurotrophic factors [8,9]. Based on our observation of reduced \(\text{GSK3}_\beta\) activity following EE in nontransgenic mice, we analyzed protein expression levels of \(\text{pAkt Ser 437}\) in the cortex and hippocampus of standard housing and enriched mice (\(N = 5\) per group) by Western blot analysis. We observed that \(\text{pAkt Ser 437}\) expression level was upregulated in the cortex and
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 GSK3β, Akt activity in cortex and hippocampus of APPswe/PS1ΔE9 mice was unaffected by EE (Figure 2C,D left panels). Taken together, this suggests that EE-induced Akt/GSK3β signaling may be defective in APPswe/PS1ΔE9 mice.

Expression levels of BDNF are comparable in APPswe/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 APPswe/PS1ΔE9 mice. For this purpose, levels of BDNF mRNA were examined in the hippocampus of nontransgenic and APPswe/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 expression are pronounced at a later stage (Figure 3A).
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).

Environmental enrichment-induced BDNF increases may be due to either increased production of BDNF or increased processing of immature proBDNF into 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 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 following environmental enrichment as determined by ELISA. (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. nontransgenic EE (N = 6), *P<0.05, one-way ANOVA] of nontransgenic and APPswe/PS1ΔE9 following environmental enrichment as determined by real time RT-PCR [*P<0.05 for nontrangenic SH (N = 6) vs. nontransgenic EE (N = 6), **P<0.01, for APPswe/PS1ΔE9 SH (N = 6) vs. APPswe/PS1ΔE9 EE (N = 7), Student’s t test]. (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 nontrangenic SH (N = 5) vs. nontransgenic EE (N = 5), *P<0.05, for APPswe/PS1ΔE9 SH (N = 5) vs. APPswe/PS1ΔE9 EE (N = 5)].

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proBDNF to yield mature BDNF. Interestingly, we observed a significant upregulation of tPA mRNA expression following EE in both nontransgenic and APPswe/PS1ΔE9 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 PLCγ/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 APPswe/PS1ΔE9 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 PKCγ mRNA by real time RT-PCR revealed no change in PKCγ 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 APPswe/PS1ΔE9 mice

To determine whether other neurotrophic factors may be upregulated in the hippocampus of nontransgenic and APPswe/PS1ΔE9 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 APPswe/PS1ΔE9 mice, when compared to their nontransgenic littermates (Figure 5A, C, E). However, NGF levels were upregulated in the hippocampi of both nontransgenic and APPswe/PS1ΔE9 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 APPswe/PS1ΔE9 mice (Figure 5D). Conversely, IGF-1 mRNA expression was upregulated in the hippocampus of enriched APPswe/PS1ΔE9 mice.

**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 APPswe/PS1ΔE9 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 APPswe/PS1ΔE9 (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 APPswe/PS1ΔE9 SH (N = 6) vs. APPswe/PS1ΔE9 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 APPswe/PS1ΔE9 mice. Another possibility is that expression of mutant APPswe/PS1ΔE9 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 APPswe/PS1ΔE9 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.
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/PS1ΔE9 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/PS1ΔE9 mice following environmental enrichment.](image_url)
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 APPswe/PS1ΔE9 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 APPswe/PS1ΔE9 mice. First, we show that EE modulates Akt and GSK3β activities in wild type mice. In contrast, these changes do not take place in APPswe/PS1ΔE9 mice, suggesting that APPswe/PS1ΔE9 mutations impair context-induced activation of Akt signaling and suppression of GSK3β activity. While it is plausible that EE enhances learning and memory and hippocampal plasticity in APPswe/PS1ΔE9 mice via one of the many other signaling pathways activated following EE, there appears to be a blockade of the Akt activation/GSK3β suppression pathway. GSK3β 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 GSK3β 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 APPswe/PS1ΔE9 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...
and NT-3 were observed following EE in wild type rat brain. Expression of both BDNF and NGF is upregulated following EE in both wild type and APPswe/PS1D 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 APPswe/PS1D mutants impair NT-3 signaling. In contrast, gene expression of IGF-1 was upregulated in the APPswe/PS1D, 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 increased expression of 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.

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CREB phosphorylation is impaired following Morris Water Maze (MWM) and has been attributed to elevated levels of Aβ in AD patients. 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 NDMA receptors [21,78]. In support of that mechanism, we show that CREB transcription is upregulated following EE in both wild type and APPswe/PS1E9 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 APPswe/PS1E9 mice. Disregulation 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 Aβ [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 APPswe/PS1E9 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 GSK3β, 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 APPswe/PS1E9 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 APPswe/PS1E9 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]. Levy and colleagues report that EE cannot elicit enhancement in learning and synaptic plasticity in apolipoprotein E (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-APPswe/PS1E9 mice, and suggests that EE induces multiple molecular pathways, some of which are impaired in APPswe/PS1E9 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.

**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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