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Inhibition of Early Growth Response 1 in the Hippocampus Alleviates Neuropathology and Improves Cognition in an Alzheimer Model with Plaques and Tangles

Xike Qin,^{*} Yunling Wang,^{*} and Hemant K. Paudel^{*†}

From the Lady Davis Institute for Medical Research,^{*} Jewish General Hospital, Montreal, Quebec; and the Department of Neurology and Neurosurgery,[†] McGill University, Montréal, Quebec, Canada

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Address correspondence to
Hemant K. Paudel, Ph.D., Lady
Davis Institute for Medical
Research, Jewish General Hos-
pital, 3755 Cote Ste Catherine,
Montréal, QC, Canada H3T
1E2. E-mail: hemant.paudel@mcgill.ca

A sporadic form of Alzheimer disease (AD) and vascular dementia share many risk factors, and their pathogenic mechanisms are suggested to be related. Transcription factor early growth response 1 (Egr-1) regulates various vascular pathologies and is up-regulated in both AD brains and AD mouse models; however, its role in AD pathogenesis is unclear. Herein, we report that silencing of Egr-1 in the hippocampus by shRNA reduces tau phosphorylation, lowers amyloid- β (A β) pathology, and improves cognition in the 3xTg-AD mouse model. Egr-1 silencing does not affect levels of cyclin-dependent protein kinase 5 (Cdk5), glycogen synthase kinase 3 β , protein phosphatase 1, or protein phosphatase 2A, but reduces p35 subunit of Cdk5. Egr-1 silencing also reduces levels of β -secretase 1 (BACE-1) and BACE-1—cleaved amyloid precursor protein (APP) metabolites (secreted APP β , C99, A β 40, and A β 42) but has no effect on presenilin 1 and presenilin 2. In hippocampal primary neurons, Egr-1 binds to BACE-1 and p35 promoters, enhances tau phosphorylation, activates Cdk5 and BACE-1, and accelerates amyloidogenic APP processing. Blocking Cdk5 action blocks Egr-1—induced tau phosphorylation but has no effect on BACE-1 activation and amyloidogenic APP processing. Blocking BACE-1 action, on the other hand, blocks Egr-1—induced amyloidogenic APP processing but does not affect tau phosphorylation. Egr-1 regulates tau phosphorylation and A β synthesis in the brain by respectively controlling activities of Cdk5 and BACE-1, suggesting that Egr-1 is a potential therapeutic candidate for the treatment of AD. (*Am J Pathol* 2017, 187: 1828–1847; <http://dx.doi.org/10.1016/j.ajpath.2017.04.018>)

Alzheimer disease (AD) is the major cause of dementia in humans. Progressive depositions of senile plaques and neurofibrillary tangles are the neuropathological features of AD.¹ Plaques are composed mainly of amyloid- β (A β) peptide derived from amyloid precursor protein (APP). APP is cleaved by β -secretase 1 (BACE-1) to generate a 99-amino acid membrane-bound protein C99 and soluble sAPP β . C99 is further cleaved by γ -secretase to produce the A β peptides. The BACE-1 cleavage of APP initiates and is the rate-limiting step in A β production. Genetic and biochemical studies have implicated accumulation of A β in the brain as central to AD pathogenesis.² It has been suggested that accumulation of A β triggers toxic events leading to neurodegeneration and perhaps dementia in AD patients.³

Inhibition of A β production by targeting BACE-1 or γ -secretase has been one of the major strategies in the development of therapy against AD.³ However, BACE-1 has a relatively large active site.⁴ Hence, development of BACE-1 inhibitors that are permeable to the blood-brain barrier and neural membrane has been difficult.⁵ In addition, a direct pharmacological inhibition of BACE-1 impairs synaptic plasticity and cognitive functions in the AD mouse model owing to the involvement of BACE-1 in synaptic physiology.^{6,7} The γ -secretase, on the other hand, is

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involved in Notch signaling, which is essential for development and differentiation.⁸ Inhibition of γ -secretase, therefore, is associated with potential severe adverse effects.⁹ Moreover, pharmacological inhibitors of γ -secretase that block APP cleavage without any adverse effects on Notch reduced cerebrospinal fluid and plasma levels of A β in healthy individuals,¹⁰ but failed to show any clinical effect in mild to moderate AD patients in phase II clinical trials.¹¹

Neurofibrillary tangles are formed by the aggregation of hyperphosphorylated tau protein. In normal brain, tau binds and stabilizes the microtubule cytoskeleton. In AD brain, tau hyperphosphorylation is implicated to cause tau aggregation, microtubule instability, loss of axonal transport, and perhaps neurodegeneration.¹² Tau hyperphosphorylation is regarded as one of the most critical pathogenic stages of AD.¹³ Tau-based research has focused primarily on compounds that inhibit tau kinases or reduce tau aggregation. Cyclin-dependent protein kinase 5 (Cdk5) and glycogen synthase kinase 3 β (GSK3 β) are among the major brain tau kinases.¹⁴ Cdk5 knockout mice die perinatally,^{15,16} and a number of GSK3 β inhibitors have failed to show any efficacy in clinical trials.^{17–19} Thus, despite substantial efforts in drug development and increased understanding of the pathology, currently no disease-modifying treatment is available for AD.¹

Understanding the mechanism of the disease is the basis for the development of effective therapies. Although mutations that cause the rare familial form of AD have been identified,² the etiology of the sporadic form, which represents approximately 95% of all AD cases, is unknown. However, old age, atherosclerosis, hypertension, hyperlipidemia, and the E4 allele of the apolipoprotein E gene are some of the major risk factors for sporadic AD. These are also risk factors for vascular dementia.^{20,21} Pathological studies have revealed the presence of ischemic lesions in patients with AD, and among nondemented individuals, those with coronary heart disease had greater plaque burden in the brain than those without heart disease.²² Hypoxia and ischemia resulting from vascular insufficiency promote cleavage of APP and increase A β level in human, mouse, and rat brain.²³ Likewise, focal cerebral ischemia promotes tau phosphorylation in mice,²⁴ and hypertension increases neurofibrillary tangles in the hippocampus of nondemented elderly individuals.²⁵ Although increasing numbers of epidemiological, neuroimaging, pathological, pharmacotherapeutics, and clinical studies suggest that pathogenic mechanisms of vascular dementia and AD are somehow related,^{20,21} the molecular link between the two pathologies is yet to be found.

Early growth response 1 (Egr-1) is a zinc finger transcription factor that regulates a number of cell functions, including cell proliferation, apoptosis, cell growth, and signal transduction.^{26,27} In the central nervous system, Egr-1 is induced during ischemic stroke, hypoxia, brain injury, and inflammation and plays an important role in the

induction and maintenance of various vascular pathologies.^{26–29} A recent study analyzed a microarray data set of 31 samples corresponding to hippocampal gene expression from 22 AD subjects of varying degree of severity and 9 controls.³⁰ Using modern bioinformatics methods, this study uncovered 1372 probe gene expression signatures that represented a high degree of consensus with established pathological markers of AD progression. More important, the authors found that Egr-1 is significantly up-regulated in AD brain, correlating with progression of the disease.³⁰ Consistent with this report, a number of subsequent studies have reported up-regulation of Egr-1 in human AD brains.^{31–34} Recently, Egr-1 level was reported to be elevated in the brains of APP,³¹ APP/presenilin 1 (PS1),³⁵ and 3xTg-AD³⁶ mouse models of AD. In addition, genetic deletion of Egr-1 reduces tau phosphorylation in mouse brain, and overexpression of Egr-1 promotes tau phosphorylation in rat primary neurons in culture and in rat hippocampus.³² The pathological significance of these observations is unclear.

Transgenic mice expressing mutated human APP or mutated both APP and PS1 are often used animal models for AD research. Unlike AD patients, these mice do not display neurofibrillary tangles.¹ 3xTg-AD mice develop age-dependent plaques, tangles, and cognitive decline, recapitulating many salient feature of AD.³⁷ Therefore, to determine the role of Egr-1 in AD pathogenesis, we have administered lentivirus-expressing shRNA against Egr-1 into the hippocampus of 10-month-old 3xTg-AD mice. We show that targeted knockdown of Egr-1 in the hippocampus inhibits activities of Cdk5 and BACE-1, alleviates levels of both phosphorylated tau and A β , and improves cognition in this AD mouse model. Our data suggest that Egr-1 is a potential therapeutic target for the treatment of AD.

Materials and Methods

Animals

All experiments involving animals were performed according to the guidelines of Canadian Council of Animal Care and Lady Davis Institute for Medical Research (Montréal, QC, Canada). Both 3xTg-AD and nontransgenic (nTg) mice in hybrid C57BL/6J129 background were gifts from Dr. Hyman Schipper (Lady Davis Institute for Medical Research). 3xTg-AD mice express human APP with Swedish mutation (KM 670/671NL), human tau with P301L mutation, and human PS1M146V.³⁷ All mice were genotyped by PCR.

Antibodies and Chemicals

Polyclonal antibodies against green fluorescent protein (GFP), Egr-1, Myc, PP1, Thr³²⁰-phosphorylated PP1, PP2A, Tyr-phosphorylated PP2A, tau, and site-specific phosphorylated tau were described previously.³² Antineprilysin,

anti-insulin-degrading enzyme, anti-PS1, anti-presenilin 2 (PS2) were obtained from Millipore (Oakville, ON, Canada). Monoclonal antibodies against Tyr-tubulin and Ac-tubulin were described previously.³⁸ Human and mouse anti-APP-C-terminal and anti-sAPP β were from Millipore. Roscovitine was obtained from Sigma-Aldrich (Oakville, ON, Canada).

Lentiviral Vector and Intracerebral Injection

Detail of construction of lentiviral vector expressing siRNA against mouse Egr-1 is described previously.³⁹ Briefly, four unique siRNA cassettes were obtained through GeneCopoeia (MSH032227; Rockville, MD). Each of these constructs, in a lentivirus GFP vector, expressed a 19 mer, either a non-effective scrambled control (shRNA-Ctl) sequence (5'-CAAAGCCAAGCAAACCAAT-3') or one of the three Egr-1 targeting sequences (Sh-1, 5'-CGCTCTGAATAATGAAG-3'; Sh-2, 5'-ACTGGTCTTTCAGACATGA-3'; or Sh-3, 5'-AGCGCTAGACCATCAAGTT-3') driven by histone H1 promoter. These shRNAs were validated by transfection into the mouse cell line, 3T3L1, followed by Western blot analysis for Egr-1 expression. Although all three shRNAs reduced endogenous Egr-1 by >50%, shRNA-Ctl had no effect. Sh3 was most effective and down-regulated Egr-1 expression by 82% in 3T3L1 cells and 85% in mouse hippocampal primary neurons in culture³⁹ and was selected for *in vivo* experiments. Lentiviruses expressing shRNA-Egr-1 and shRNA-Ctl were produced as per manufacturer's protocol. Briefly, the DNA was transfected along with a Lenti-X HTX Packaging Mix (Clontech, Mountain View, CA) into the Lenti-X 293T cells. The virus was purified from the supernatant 48 and 72 hours after transfection.^{32,39} The titer of purified virus was determined by flow cytometry, and the virus was stored frozen at -80°C .

A total of 20 3xTg-AD and 20 nTg received viral injection. For each group, a total of 10 mice received shRNA-Ctl and the other 10 received shRNA-Egr-1. One 3xTg-AD mouse receiving shRNA-Egr-1 died after 2 weeks. Viral injection was performed as described previously.³² Briefly, 3xTg-AD and nTg control mice (10 months old each) were anesthetized with isoflurane and placed into a Kopf stereotaxic apparatus, where they received bilateral injections for the lentiviral preparations (1.8×10^9 multiplicity of infection) into the hippocampus (coordinates, from bregma: medial/lateral, 1.94; anterior/posterior, 1.25; dorsal/ventral, 1.50). Injections were delivered via a 5 μL Hamilton microsyringe connected to a PhD Ultra Pump Syringe (Harvard Apparatus, Saint-Laurent, QC, Canada) with a 27 gauge needle. Injection (2 μL per side) was delivered at a rate of 0.25 $\mu\text{L}/\text{minute}$. The needle was left for an additional 5 minutes after the injection to allow diffusion of virus into the tissue.

Morris Water Maze Testing

After 4 months of viral injection, mice were tested for spatial learning/memory performance in the Morris water

maze by an observer blind to the experiment and the genotype of mice, as described.⁴⁰ The maze consisted of a circular pool (1.2 m in diameter) filled to a depth of 55 cm with water rendered opaque by addition of white gouache, and maintained at 23°C to 24°C . A square plexiglass platform (10 $\times$ 10 cm), 0.5 cm below water level, was left in the same location (southwest quadrant, approximately 0.5 m in from the side) for the duration of each experiment. Latency, distance, and swimming path to find the platform were recorded with a video camera connected to a computerized tracking system (HVS Image, Buckingham, UK). Each mouse was given a block of four trials/day for 5 consecutive days. Three cardinal points (northeast, northwest, and southeast) were used as release points, with the restriction that no one release point was used more than twice in 1 day. Mice were placed in the pool and allowed to swim for a maximal time of 60 seconds. If a mouse did not find the platform within this time, it was placed on it for 15 seconds. The intertrial intervals ranged between 10 and 15 minutes.

Four hours after the last training trial on day 5, mice were given a probe trial (60 seconds) with the platform removed. Latency and distance traveled in each quadrant were measured. The quadrant in which the platform was previously located (southwest) was designated as the target quadrant. During the probe trial, latencies to former location of the platform were measured and the number of times the mouse crossed the former platform location was recorded.

Immunohistochemistry

After behavior tests, mice were subjected to neuropathological examinations. Mice were anesthetized and transcardially perfused with cold phosphate-buffered saline. The right hemibrain was post-fixed in 4% paraformaldehyde (pH 7.4) at 4°C for 48 hours and divided into sections on a vibratome. Coronal sections (50 μm thick) corresponding to the hippocampus and cortex were used for histology. The right hemisphere was dissected into hippocampal and cortical samples and either frozen at -80°C or used freshly for measurement of proteins. The procedure for immunohistochemistry using immunostainer Discovery XT from Ventana (Tucson, AZ) and quantification of images was described previously.³⁹ Briefly, sections were labeled with mouse anti-Egr-1 or rabbit anti-GFP (1:100; Cell Signaling, Danvers, MA), followed by Alexa-red or fluorescein isothiocyanate-blue conjugated secondary antibodies. For plaque detection, sections were labeled with rabbit anti-human A β 42 (1:200; Cell Signaling), followed by Alexa Fluoro conjugated secondary antibody. For quantification, slides were scanned at $\times 400$ magnification [resolution of 0.25 $\mu\text{m}/\text{pixel}$ (100,000 pixels/inch)] using an Aperio ScanScope AT Turbo (Leica Biosystems, Wetziar, Germany). The background illumination levels were calibrated using a prescan procedure. Acquired digital images representing whole

tissue sections were analyzed applying the Spectrum Analysis algorithm package and ImageScope analysis software version 11.2 (Aperio Technologies, Wetzlar, Germany). A rectangular region of interest, 25 μm^2 in size, was defined. The immunoreactivity value for each region of interest was obtained. The immunoreactivity within each region of interest in every section was averaged to generate a mean immunoreactive value for each animal. Data were averaged from six mice in each group, with four regions ($n = 24$) for each genotype, and are expressed as the fold of nTg.

Construction of Mouse shRNA against BACE-1, Lentiviral Production, Cell Culture, and Viral Infection

shRNA against mouse *BACE-1* was constructed as described previously.³⁹ Briefly, 19 mer either *BACE-1* targeting sequence siBACE-1 (5'-GTTCGCTGTCTCACAGTCA-3')⁴¹ or noneffective scrambled control siCtl (5'-CAAAGC-CAAGCAAACCAAT-3') was subcloned into lentiviral vector pLVX-IRWS-ZsGreen1 (Clontech) at the *EcoRI/XbaI* site. Lentivirus was produced as described previously.³⁹ DNA was transfected along with a Lenti-X HTX Packaging Mix into the Lenti-X 293T cells, and the virus was harvested after 48 and 72 hours from the supernatant and purified. The titer of purified virus was determined by flow cytometry, and the virus was stored at -80°C . To validate the shRNA construct, lentivirus was infected into mouse hippocampal primary neurons. After 48 hours of infection, neurons were analyzed by Western blot analysis. Construction and production of lentivirus expressing human Egr-1 with a Myc tag at the C terminus was described previously.³⁹ Hippocampal neurons were prepared from P0 new born rat or mouse pups (Charles River, Senneville, QC, Canada), as described previously.³⁹ Neurons 2 weeks in culture were infected with Ln-vector or Ln-Egr-1 (multiplicity of infection of 20 each). After 72 hours, neurons were either analyzed by immunocytochemistry or homogenized in extraction buffer for Western blot analysis.

Immunocytochemistry, Image Acquisition, and Quantification

Immunocytochemistry was performed as described previously.³⁹ Briefly, Ln-Egr-1- or Ln-vector-infected neurons were fixed and labeled with rabbit anti-BACE-1 (1:200; Cell Signaling), rabbit anti-Myc (1:200; Cell Signaling), or GFP (mouse, 1:1000; Cell Signaling), followed by Alexa Fluor 532 or 488 goat anti-rabbit or goat anti-mouse secondary antibody. Labeled neurons were viewed by using the LSM Pascal (Zeiss, Oberkochen, Germany) confocal microscopy system. At least 15 labeled neurons were randomly chosen from each group for quantification from three to four coverslips. Immunointensity was analyzed along 30 to 40 μm of dendritic length of each neuron by Volocity image analysis software version 6.3 (Improvision,

Coventry, England). Data were averaged from four independent cultures.

BACE-1 Activity Assay, Sandwich Enzyme-Linked Immunosorbent Assay, and Western Blotting for A β 40 and A β 42

BACE-1 activity was measured by using the β -Secretase fluorogenic activity assay kit (catalog number 565785; Calbiochem, Oakville, ON, Canada), following the manufacturer's instruction manual. Briefly, cells were homogenized in the extraction buffer and centrifuged, and the supernatant (50 μL each) was transferred to a fluorescence plate reader. β -Secretase substrate was added to each well, and the plate was incubated for 1 hour at 37°C in the dark. After incubation, fluorescence was measured with excitation at 335 to 355 nm and emission at 495 to 570 nm. Background reading from buffer and the substrate was subtracted. A β 42 in the cell culture medium was measured using mouse A β 42 ELISA Kit (Invitrogen, Carlsbad, CA), following the instruction manual. Culture medium (50 μL) was pipetted into the enzyme-linked immunosorbent assay plate well coated with mouse antibody specific to the N terminal of A β 42. After incubation and washing, rabbit polyclonal antibody specific to A β 42 C terminal was added. Bound antibody was detected by using horseradish peroxidase-labeled secondary antibody and an enzyme-linked immunosorbent assay plate reader at OD₄₅₀ nm. For Western blotting, samples were separated on 10% to 20% Tricine gel (Invitrogen) and probed against either mouse or human anti-A β 40 or anti-A β 42 antibody.

RNA Extraction and qPCR

Total RNA was purified using RNeasy minikits (Qiagen, Montreal, QC, Canada) from hippocampal primary neurons using the manufacturer's instruction manual and as described previously.³⁹ Total RNA (1 μg) was used for the first-strand synthesis with Super Script Reverse Transcriptase II and oligo (dT) or random primer (Invitrogen). Real-time quantitative PCR (qPCR) was performed using an SYBR Green PCR kit (Qiagen) with gene-specific primers (Table 1) using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Data were analyzed by qPCR software 7500 version 2.0.4 (Applied Biosystems). The relative concentrations of the genes of interest were determined using the comparative threshold cycle (ΔC_T) method after normalization to the endogenous control (glyceraldehyde-3-phosphate dehydrogenase).³⁹

Chromatin Immunoprecipitation Assay

Hippocampal primary neurons infected with Ln-vector or Ln-Egr-1 for 48 hours were subjected to chromatin immunoprecipitation assay using Magna ChIP T/MA/G Millipore Kit (Millipore), following the manufacturer's instructions

Table 1 Primer List

Genes	Primers
qPCR	
Rat <i>BACE-1</i>	Forward: 5'-CCTCCGAAAGTCTGTGTATGTG-3' Reverse: 5'-CAGTTGGAACCATTTGATGAAGA-3'
Rat <i>p35</i>	Forward: 5'-GAAATCTCCTACCCGCTCAA-3' Reverse: 5'-GTCCCAAAAGGCTTCCTTG-3'
Rat <i>GAPDH</i>	Forward: 5'-CGCCTGCTCTCTGTGTT-3' Reverse: 5'-CCATGGTGTCTGAGCGATGT-3'
ChIP assay	
Rat <i>BACE-1</i>	Forward: 5'-AGAGGGCATCCCAGACCCCTTTC-3' Reverse: 5'-AAAGCCAAAGGGTTTTTGTCTTTGGG-3'
Rat <i>p35</i>	Forward: 5'-CGCGCGTTCCCGCGCCCGCGCCG-3' Reverse: 5'-AGCCCGCCGGCTCCGCCCGCCCGCCG-3'
Rat <i>GAPDH</i>	Forward: 5'-GACATGCCGCTGGAGAAAC-3' Reverse: 5'-AGCCCAGGATGCCCTTTAGT-3'

ChIP, chromatin immunoprecipitation; qPCR, real-time quantitative PCR.

and as described previously.³⁹ Neurons were cross-linked, and the DNA was fragmented to 200 to 1000 bp in length. Chromatin solution (100 μ L) was immunoprecipitated using 5 μ g of either monoclonal anti-Egr-1 (MABC 1023; Millipore) or IgG control. Input and immunoprecipitated samples were digested with proteinase K to reverse the cross-linking. The DNA was purified and analyzed by qPCR using primers flanking *BACE-1*,⁴² *P35*,⁴³ or *GAPDH* (control) promoter (Table 1).

Kinase Assay

Activities of GSK3 β and Cdk5 were assayed as described previously.³² To prepare GSK3 β substrate, a synthetic peptide derived from cAMP response element-binding protein (KRREILSRPSYR) was first phosphorylated by protein kinase A, and then the phosphorylated peptide was purified by high-performance liquid chromatography. Cdk5 substrate was KTPKKAKPKTPKKAKKL peptide. Kinase activity assays were performed using any of the above peptides as the substrate. The final concentrations of the various assay components were 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 0.1 mmol/L EGTA, 10 mmol/L MgCl₂, 0.5 mmol/L γ -³²P-ATP, 20 μ mol/L of substrate peptide, and carryover components of cell extract. The reaction was initiated by the addition of 10 μ L of cell extract to 90 μ L of mixture containing the rest of the assay components. After 1 hour at 30°C, aliquots were removed and analyzed by filter paper assay.

Statistical Analysis

All data were analyzed by one-way analysis of variance, followed by Newman-Keuls post hoc test for multigroup comparisons and *t*-test for comparisons between two groups. For water maze data, latencies and the distances swam across the two trials for each mouse every day were averaged. These means were analyzed across the 5 days for testing. A one-way repeated measurement of analysis of variance was used for main effect (ie, group or treatment

comparison) with day as the repeated measure and latency or distance swam as the dependent variable. The Newman-Keuls test was used for post hoc analysis. For probe trials, the means were compared between groups by one-way analysis of variance, followed by Newman-Keuls post hoc test. All data are presented as means $\pm$ SEM, and differences with *P* < 0.05 were considered significant.

Results

Inhibition of Egr-1 in the Hippocampus Alleviates Spatial Learning Deficits in 3xTg-AD Mice

We obtained four unique siRNA cassettes from GeneCopoeia each in a lentivirus GFP vector, expressing either a non-effective scrambled control (shRNA-Ctl) or one of the three Egr-1 targeting sequences driven by histone H1 promoter. We chose the most effective shRNA-Egr-1 that reduced endogenous Egr-1 expression by 82% in 3T3L1 cells and 85% in mouse hippocampal primary neurons in culture.³⁹ To confirm the specificity of the shRNA and to exclude the possibility of off-targeting effect, we obtained a rescued Egr-1 knockdown phenotype caused by shRNA-Egr-1 in mouse hippocampal primary neurons by overexpressing Egr-1. shRNA-Egr-1 causes accumulation of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor Glu-2 receptor on the surface of neurons. This effect was almost completely rescued by Ln-Egr-1 but not by Ln-vector.³⁹

We next bilaterally injected lentivirus expressing shRNA-Egr-1 or non-specific shRNA-Ctl into the hippocampus of 10-month-old 3xTg-AD and age-matched nTg mice. After 4 months, mice were analyzed for behavior, followed by neuropathology. To determine the effects of Egr-1 inhibition on the behavior, we analyzed mice for hippocampal-dependent spatial memory by the Morris water maze test. One-way analysis of variance, followed by Newman-Keuls post hoc test of data, determined that the swimming speed, percentage of total time floating, percentage of thigmotaxis time, and percentage of thigmotaxis path of 3xTg-AD and nTg mice receiving shRNA-Ctl or shRNA-Egr-1 were not significantly different (Figure 1A). These data suggested that compared to nTg, 3xTg-AD mice do not display any motor defect, anxiety, fear, or depression; and shRNA-Egr-1 or shRNA-Ctl delivery into the hippocampus does not cause any of these symptoms in any of these animals.⁴⁰ In contrast, compared to nTg mice receiving shRNA-Ctl (nTg-shRNA-Ctl), 3xTg-AD mice receiving shRNA-Ctl (3xTg-AD-shRNA-Ctl) displayed significantly longer escape latency during the training (Figure 1B), spent less time in the target quadrant and target swimming path (Figure 2A), and had fewer target platform crossings during the probe trial (Figure 2B). These observations are as expected and indicated that 3xTg-AD mice are cognitively deficient.

3xTg-AD mice receiving shRNA-Egr-1 (3xTg-AD-shRNA-Egr-1) reached the platform faster than 3xTg-AD-shRNA-Ctl on each day 3 to 5 of the trial (Figure 1B). On

Figure 1 Inhibition of Egr-1 in the hippocampus improves learning deficits in 3xTg-AD mice. Lentivirus expressing shRNA-Egr-1 or shRNA-Ctl was injected into the hippocampus of 3xTg-AD and age-matched nTg controls. After 4 months of injection, mice were tested for learning and memory on the water maze. **A:** shRNA-Egr-1 delivery into the hippocampus does not cause motor defect, anxiety, or depression in mice. During the probe trial on day 5, 3xTg-AD injected with shRNA-Ctl (3xTg-AD-Ctl), 3xTg-AD injected with shRNA-Egr-1 (3xTg-AD-Egr-1), nTg mice injected with shRNA-Ctl (nTg-shRNA-Ctl), and nTg injected with shRNA-Egr-1 (nTg-shRNA-Egr-1) were tested for swim speed, percentage of total time floating (swim speed <0.05 m/second), a sign of anxiety and/or depression, thigmotaxis time (percentage of total time spent within 5 cm of the pool wall, a measure of anxiety or fear), and thigmotaxis path (percentage of swim path spent within 5 cm of the pool wall, a measure of anxiety or fear). No statistical differences were observed among groups in all of the above parameters ($P > 0.05$, one-way analysis of variance and Newman-Keuls post hoc analysis). **B:** Escape latency and learning improvement during platform trial. **Left panel:** 3xTg-AD-shRNA-Ctl mice were inferior to nTg-shRNA-Ctl, showing longer escape latency. However, 3xTg-AD-shRNA-Egr-1 mice displayed significantly shorter escape latency than 3xTg-AD-shRNA-Ctl on day 5. **Right panel:** Learning improvement. Learning improvement of a mouse is the reduction in escape latency time in each day of training with respect to day 1 training. All groups of mice displayed progressive improvement in learning with increase in the day of training. Compared to nTg-shRNA-Ctl (blue), 3xTg-AD-shRNA-Ctl mice were significantly slow in learning on day 3, day 4, and day 5. However, 3xTg-AD-shRNA-Egr-1 mice make significantly more progress on day 3, day 4, and day 5 versus 3xTg-AD-shRNA-Ctl mice. Data expressed as means $\pm$ SEM. $n = 10$ nTg-shRNA-Ctl, nTg-shRNA-Egr-1, and 3xTg-AD-shRNA-Ctl (**A** and **B**); $n = 9$ 3xTg-AD-shRNA-Egr-1 (**A** and **B**). * $P < 0.05$ 3xTg-AD-shRNA-Ctl mice versus nTg-shRNA-Ctl mice (repeated measures of one-way analysis of variance and Newman-Keuls post hoc analysis); $^{\dagger}P < 0.05$ 3xTg-AD-shRNA-Egr-1 mice versus 3xTg-AD-shRNA-Ctl mice (repeated measures of one-way analysis of variance and Newman-Keuls post hoc analysis).

the fifth day, 3xTg-AD-shRNA-Egr-1 mice reached the platform 38.5% faster than 3xTg-AD-shRNA-Ctl mice (Figure 1B). Furthermore, on days 3, 4, and 5, although 3xTg-AD-shRNA-Ctl mice reached the platform 5%, 20%, and 37.3% faster, respectively, than on day 1, 3xTg-AD-shRNA-Egr-1 mice reached the target platform 24.3%, 41.7%, and 65.3% faster, respectively, than on day 1 (Figure 1B). These data showed that compared to 3xTg-AD-shRNA-Ctl mice, 3xTg-AD-shRNA-Egr-1 mice were 19.5%, 21.3%, and 28.0% better learners on days 3, 4, and 5, respectively.

Inhibition of Egr-1 in the Hippocampus Improves Memory Deficits in 3xTg-AD Mice

On day 5, mice were subjected to the probe trial for memory. Both nTg-shRNA-Ctl and nTg-shRNA-Egr-1 mice displayed similar preference of various quadrants and spent significantly more time in target than any of the other quadrants (Figure 2A). 3xTg-AD-shRNA-Ctl mice, on the other hand, spent $8.2 \pm 2.08\%$ less ($P < 0.05$) and $7.0 \pm 2.59\%$ more ($P < 0.05$) time in the target and anti-clockwise quadrants, respectively, than the nTg-shRNA-Ctl mice. In contrast,

3xTg-AD-shRNA-Egr-1 mice spent $11.61 \pm 2.59\%$ more ($P < 0.05$) time in the target quadrant and $8.07 \pm 2.6\%$ less ($P < 0.05$) time in the counterclockwise quadrant than 3xTg-AD-shRNA-Ctl mice. 3xTg-AD-shRNA-Egr-1 mice displayed quadrant preference and time spent in the target quadrant similar to nTg-shRNA-Ctl mice.

Next, we compared percentage of total swim path spent by mice in various quadrants (Figure 2A). Again, nTg-shRNA-Ctl and nTg-shRNA-Egr-1 mice had a similar percentage of total swim path in the various quadrants. 3xTg-AD-shRNA-Ctl mice, on the other hand, had significantly reduced swim path in the target quadrant than nTg-shRNA-Ctl mice. However, 3xTg-AD-shRNA-Egr-1 mice had $11.6 \pm 1.8\%$ more ($P < 0.05$) than 3xTg-AD-shRNA-Ctl and similar to nTg-shRNA-Ctl swimming path in the target quadrant. Finally, although nTg-shRNA-Ctl and nTg-shRNA-Egr-1 mice had a similar number of target platform crossings, 3xTg-AD-shRNA-Ctl mice showed significantly fewer number of crossings, as expected. In contrast, 3xTg-AD-shRNA-Egr-1 mice had 1.83 ± 0.4 times more crossings ($P < 0.05$) than 3xTg-AD-shRNA-Ctl mice. Thus, 3xTg-AD-shRNA-Egr-1 mice spent significantly more time in the target quadrant, had more target swimming path, and had

Figure 2 Inhibition of Egr-1 in the hippocampus improves memory deficits in 3xTg-AD mice. On day 5, mice were tested for memory by probe trial. Percentage time and percentage path spent in each quadrant of the pool and number of target platform crossings were recorded. **A: Left panel:** The average percentage of the total time spent in the indicated quadrant is shown. Compared to nTg-shRNA-Ctl, 3xTg-AD-shRNA-Ctl mice spent significantly less time in the target quadrant and more time in the counterclockwise quadrant (one-way analysis of variance and Newman-Keuls post hoc analysis). However, compared to 3xTg-AD-shRNA-Ctl, 3xTg-AD-shRNA-Egr-1 mice spent significantly more time in the target quadrant and less time in the counterclockwise quadrant. **Right panel:** Percentage path in various quadrants. Average percentage path in each quadrant is shown. 3xTg-AD-shRNA-Ctl mice have significantly less percentage target path than nTg-shRNA-Ctl. 3xTg-AD-shRNA-Egr-1, on the other hand, have significantly higher percentage target path than 3xTg-AD-shRNA-Ctl mice (one-way analysis of variance and Newman-Keuls post hoc analysis). **B:** Platform crossing. 3xTg-AD-shRNA-Ctl mice have significantly fewer number of platform crosses than nTg-shRNA-Ctl. 3xTg-AD-shRNA-Egr-1, on the other hand, have significantly higher number of crossings of former platform location than those 3xTg-AD-shRNA-Ctl mice and almost similar number with nTg-shRNA-Ctl controls (one-way analysis of variance and Newman-Keuls post hoc analysis). Data are expressed as average $\pm$ SEM (**A, left panel**) or means $\pm$ SEM (**A, right panel**, and **B**). $n = 10$ nTg-shRNA-Ctl, nTg-shRNA-Egr-1, and 3xTg-AD-shRNA-Ctl (**A and B**); $n = 9$ 3xTg-AD-shRNA-Egr-1 (**A and B**). * $P < 0.05$ (one-way analysis of variance and Newman-Keuls post hoc analysis).

more target platform crossings than 3xTg-AD-shRNA-Ctl mice (Figure 2). The performance of 3xTg-AD-shRNA-Egr-1 mice compared with nTg-shRNA-Ctl mice during the final day of the training session and on the probe trial. This result showed that shRNA-Egr-1 delivery into the hippocampus rescues cognitive defect in 3xTg-AD mice.

shRNA-Egr-1 Delivery Down-Regulates Egr-1 in the Hippocampus

After the memory tests, mice were subjected to neuropathological examinations. All shRNA-Ctl and shRNA-Egr-1 receiving sites displayed GFP fluorescence (shRNA expression) at comparable levels (Figure 3, A and B). Both 3xTg-AD-shRNA-Egr-1 and nTg-shRNA-Egr-1 mice showed significantly reduced Egr-1 immunostaining when compared to mice injected with shRNA-Ctl at injected sites (Figure 3B). Western blot analysis showed that compared to the nTg-shRNA-Ctl, 3xTg-AD-shRNA-Ctl mice had 1.8- and 1.7-fold higher levels of Egr-1 in the hippocampus and the cortex, respectively (Figure 3, C and D). These data determined that 3xTg-AD mice have an elevated level of Egr-1 in the brain, as reported.³⁶ More important, 3xTg-AD-shRNA-Egr-1 mice had an Egr-1 level 70% less than 3xTg-AD-shRNA-Ctl mice in the hippocampus (Figure 3, C and D). Likewise, nTg-shRNA-Egr-1 mice displayed an Egr-1 level reduced by 75% when compared to nTg-shRNA-Ctl mice. This effect was not seen in the cortex of both genotypes, which did not receive lentivirus injection. These results showed that shRNA-Egr-1 had specifically inhibited Egr-1 level in the hippocampus.

Inhibition of Egr-1 Reduces Tau Hyperphosphorylation in the Hippocampus

Accumulation of hyperphosphorylated tau is one of the characteristic pathological features of the AD brain.¹ Therefore, to

determine the effects of Egr-1 inhibition on neuropathology, we analyzed tau phosphorylation in the hippocampus of shRNA-treated mice (Figure 4A). Compared to nTg-shRNA-Ctl, 3xTg-AD-shRNA-Ctl mice had significantly elevated levels of phosphorylated tau on all sites tested (Figure 4A). In addition, these mice also had 1.6-fold more total tau when compared to the nTg-shRNA-Ctl cohort. shRNA-Egr-1 silencing did not affect total tau level in both 3xTg-AD and nTg littermates (Figure 4A). However, compared to 3xTg-AD-shRNA-Ctl mice, tau phosphorylation at Ser⁴⁰⁴, Ser²⁶², Ser²³⁵, Thr²³¹, and Thr¹⁸¹ was significantly reduced in 3xTg-AD-shRNA-Egr-1 mice (Figure 4B). Phosphorylation at Ser²¹⁴, on the other hand, was similar in the two groups (Figure 4B).

Inhibition of Egr-1 Stabilizes Microtubule Cytoskeleton in the Hippocampus

In the AD brain, tau phosphorylation inhibits tau binding to microtubules and causes microtubule instability.^{1,12} 3xTg-AD-shRNA-Ctl mice had 2.5-fold higher levels of Tyr-tubulin, representing unstable microtubules, and 2.3-fold less Ac-tubulin, representing stable microtubules, compared to nTg-shRNA-Ctl controls (Figure 4C), indicating that microtubules in the hippocampus of 3xTg-AD mice are highly unstable. In 3xTg-AD-shRNA-Egr-1 mice, the amount of Tyr-tubulin was 2.7-fold less and Ac-tubulin 2.8-fold more than in 3xTg-AD-shRNA-Ctl mice. The total tubulin level remained similar in the two experimental groups. These data determined that Egr-1 inhibition stabilizes microtubules in the hippocampus of 3xTg-AD mice.

Inhibition of Egr-1 Down-Regulates the p35 Subunit of Cdk5

The balance of tau phosphorylation in the brain is regulated by the activities of tau kinases and tau-specific

Figure 3 shRNA-Egr-1 delivery reduces Egr-1 level in the hippocampus. The expression of shRNA-Egr-1 and its effect on Egr-1 level were analyzed by immunohistochemistry and Western blot analysis. **A:** Location of lentivirus injection sites in the hippocampus of mice. **B:** Confocal micrographs showing expression of GFP (shRNA-Ctl and shRNA-Egr-1) in the hippocampus. Note that Egr-1 immunostaining is significantly reduced in hippocampus expressing shRNA-Egr-1 in both nTg and 3xTg-AD mice. **C:** Western blots showing Egr-1 levels in the hippocampus expressing shRNA-Egr-1. **D:** Quantification of Western blots. Data expressed as average $\pm$ SEM. $n = 12$ mice per genotype; $n = 6$ mice per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ (one-way analysis of variance and Newman-Keuls post hoc analysis). Scale bars = 80 μ m (**B**).

phosphatases. shRNA-Egr-1 did not affect tau phosphorylation at Ser²¹⁴ (Figure 4, A and B), which is phosphorylated by protein kinase A, Cam kinase 2, and protein kinase C.^{14,44} Likewise, the levels of PP2A and inactive tyrosine phosphorylated pPP2A were comparable between the two groups (Figure 5). These data are consistent with the previous report³² and showed that Egr-1 does not affect tau phosphorylation/dephosphorylation by protein kinase A, Cam kinase 2, protein kinase C, and PP2A.

Cdk5 is one of the major kinases that phosphorylate tau in the brain.¹⁴ It is composed of a catalytic Cdk5 subunit and an activating p35 subunit. Furthermore, p35 is proteolyzed to p25 protein *in vivo*. Cdk5 bound to p25 is more active than that bound to p35.⁴⁵ Egr-1 activates Cdk5 by inducing transcription of p35⁴³ and thus promotes tau phosphorylation in the brain.³² In addition, by promoting Cdk5-catalyzed inhibitory PP1 phosphorylation at Thr³²¹,⁴⁶ Egr-1 causes enhancement in tau phosphorylation at Ser²⁶².³² Consistent with these findings, shRNA-Egr-1 did not affect the ratio of p25/p35 but reduced levels of p35, pPP1 (Figure 5), and tau phosphorylated at Ser⁴⁰⁴, Ser²³⁵, Thr¹⁸¹, and Ser²⁶² in both 3xTg-AD and nTg hippocampi (Figure 4, A and B). However, shRNA-Egr-1 also reduced tau phosphorylation at Thr²³¹ (Figure 4B). Interestingly, among all tau sites tested, phosphorylation at Thr²³¹ is highest in the hippocampus of 3xTg-AD mice (Figure 4A). Studies have shown that tau

phosphorylation at Thr²³¹ plays a critical role in tau dysfunction associated with AD and is preferentially phosphorylated by GSK3 β .^{47–50} These data suggested that Egr-1 also regulates tau phosphorylation by GSK3 β in the brain.

Egr-1 Regulates Tau Phosphorylation by GSK3 β in Neurons

Egr-1 does not directly affect GSK3 β activity,³² and the levels of total and Ser⁹-phosphorylated inactive GSK3 β were unaltered in shRNA-Egr-1 and shRNA-Ctl injected mice (Figure 5). However, a unique feature of GSK3 β is that it phosphorylates some of its substrates only when the substrate is previously phosphorylated (primed) by another kinase.⁴⁹ Previous studies have shown that Cdk5 phosphorylates tau at Ser²³⁵ first. GSK3 β subsequently phosphorylates Ser²³⁵-phosphorylated tau at Thr²³¹.^{48,49} Therefore, it is possible that shRNA-Egr-1 may have reduced tau Thr²³¹ phosphorylation in the hippocampus of 3xTg-AD mice by inhibiting Cdk5 action and thus preventing tau priming. If so, Egr-1-induced Thr²³¹ phosphorylation will be expected to be dependent on actions of both Cdk5 (for Ser²³⁵ phosphorylation) and GSK3 β (for Thr²³¹ phosphorylation). To test this idea, we infected rat hippocampal primary neurons with Ln-Egr-1 on Ln-vector and analyzed infected neurons (Figure 6). Egr-1 activated

A

Figure 4 Inhibition of Egr-1 by shRNA reduces tau phosphorylation and stabilizes microtubules in the hippocampus. Hippocampal extracts of 3xTg-AD and nTg mice receiving shRNA-Egr-1 and shRNA-Ctl were analyzed for tau phosphorylation and microtubule stability by Western blot analyses. **A:** Representative Western blots and a comparison of total and phosphorylated tau in the hippocampus of 3xTg-AD and nTg mice. **Left panel** shows representative Western blots; **right panel**, quantification. **B** and **C:** Quantification of Western blot data showing effects of shRNA-Egr-1 on tau phosphorylation and microtubule stability in the hippocampus of 3xTg-AD and nTg mice. For quantification, total tau band was normalized against respective actin. To quantify tau phosphorylation, phosphorylated tau band intensity was normalized against corresponding total tau bands. Total tubulin band was normalized against actin, whereas Tyr-tubulin band, representing unstable microtubule, and Ac-tubulin bands, representing stable microtubules, were normalized against corresponding total tubulin bands. Data are expressed as means $\pm$ SEM (**A–C**). $n = 12$ mice per genotype; $n = 6$ mice per group. * $P < 0.05$, *** $P < 0.005$ (one-way analysis of variance and Newman-Keuls post hoc analysis); † $P < 0.05$, †† $P < 0.01$ (one-way analysis of variance and Newman-Keuls post hoc analysis).

B **C**

Cdk5, whereas the activity of GSK3 β remained at the basal level in Ln-Egr-1 infected neurons (Figure 6C). These data are as expected and showed that Egr-1 activates Cdk5 and has no effect on GSK3 β activity. Inhibition of Cdk5 by roscovitine blocked tau phosphorylation at both Thr²³¹

and Ser²³⁵ (Figure 6, A and B). Inhibition of GSK3 β by lithium chloride, on the other hand, inhibited only Thr²³¹ phosphorylation (Figure 6B). Thus, Egr-1 promotes tau phosphorylation at Ser²³⁵ and Thr²³¹. Egr-1-induced phosphorylation at Ser²³⁵ is Cdk5 dependent. Egr-1-induced

Figure 5 Inhibition of Egr-1 reduces level of p35 subunit of Cdk5 in the hippocampus of 3xTg-AD mice. Representative Western blots and their quantification. For quantification, GSK3β, Cdk5, PP1, and PP2A bands were normalized against corresponding actin bands. pS⁹-GSK3β, pT³²⁰ PP1, and pY-PP2A bands were normalized against corresponding total protein bands. Total p35 is the sum of p35 and p25 normalized against corresponding actin band. Data are expressed as average ± SEM. *n* = 12 mice per genotype; *n* = 6 mice per group. **P* < 0.05, ****P* < 0.005 (one-way analysis of variance and Newman-Keuls post hoc analysis).

phosphorylation of Thr²³¹, on the other hand, is dependent on the activities of both Cdk5 and GSK3β. Moreover, in cells treated with lithium chloride, Cdk5 was active but Thr²³¹ phosphorylation was blocked (Figure 6, A and B). This indicates that Cdk5 does not phosphorylate Thr²³¹ in neurons.

Inhibition of Egr-1 Reduces Aβ Pathology in the Hippocampus of 3xTg-AD Mice

3xTg-AD mice displayed extensive Aβ₄₂ immunoreactive plaques in both the hippocampus and the cortex (Figure 7A). Interestingly, compared to 3xTg-AD-shRNA-Ctl controls, 3xTg-AD-shRNA-Egr-1 mice had 57.1% fewer plaques in the hippocampus (Figure 7A). Moreover, the level of soluble Aβ₄₂ was 84.9% lower in the hippocampus of 3xTg-AD-shRNA-Egr-1 than 3xTg-AD-shRNA-Ctl mice (Figure 7B). Likewise, 3xTg-AD-shRNA-Egr-1 mice had soluble Aβ₄₀ 78% lower in the hippocampus than 3xTg-AD-shRNA-Ctl mice. This effect was not observed in the cortex, which did not receive shRNA injection (Figure 7). These data determined that inhibition of Egr-1 also reduces Aβ pathology in 3xTg-AD mice.

Inhibition of Egr-1 Down-Regulates BACE-1 Level in the Hippocampus

Aβ level in the brain is mainly controlled by its production and clearance. The proteolytic degradation is a major route of Aβ clearance.⁵¹ Neprilysin and insulin-degrading enzyme are among the main Aβ-degrading enzymes in the brain.^{51–53} Levels of both neprilysin and insulin-degrading enzyme were similar in all experimental groups (Figure 8A). These data indicated that levels of neprilysin

and insulin-degrading enzyme are not altered in 3xTg-AD mice and injection of shRNA-Egr-1 does not affect levels of these enzymes in the hippocampus.

Aβ is produced by a sequential cleavage of APP by BACE-1 and γ-secretase, a multimeric enzyme with PS1 or PS2 being the catalytic subunit.^{2,54} Western blot analysis showed that compared to nTg-shRNA-Ctl, the level of PS1 was 1.45-fold higher in 3xTg-AD-shRNA-Ctl mice (Figure 8A). The level of PS2, on the other hand, was similar in the two groups. More important, levels of both PS1 and PS2 were comparable in 3xTg-AD-shRNA-Egr-1 and 3xTg-AD-shRNA-Ctl mice. A similar observation was made in the nTg mice. This result determined that inhibition of Egr-1 does not affect levels of PS1 and PS2 in the hippocampus.

We then analyzed the level of BACE-1. 3xTg-AD-shRNA-Egr-1 mice had a BACE-1 level 60.1% lower than 3xTg-AD-shRNA-Ctl mice in the hippocampus (Figure 8B). Likewise, in the hippocampus of nTg-shRNA-Egr-1 mice, the BACE-1 level was 55.2% of nTg-shRNA-Ctl. This effect was not apparent in the cortex of both genotypes, which did not receive viral injection. Immunohistochemical data showed that compared to shRNA-Ctl-injected animals, the level of BACE-1 in shRNA-Egr-1-injected counterparts was 35.2% lower in 3xTg-AD and 25.3% lower in nTg (Figure 8C). Again, no such effect on BACE-1 level was observed in the cortex.

In the brain, APP is cleaved by either α-secretase or BACE-1, followed by γ-secretase. BACE-1 cleavage generates C-terminal C99 and N-terminal sAPPβ. α-Secretase cleavage, on the other hand, generates C83 and sAPPα². The levels of both C99 and sAPPβ were 45.3% and 58.9% lower, respectively, in 3xTg-AD-shRNA-Egr-1 mice when compared to the 3xTg-AD-shRNA-Ctl cohort (Figure 9A).

Figure 6 Egr-1–promoted tau phosphorylation at Thr²³¹ requires activities of both Cdk5 and GSK3β. Rat hippocampal primary neurons infected with Ln-Egr-1 or Ln-vector for 48 hours were treated with 400 nmol/L Cdk5 inhibitor roscovitine, 10 mmol/L GSK3β inhibitor lithium chloride (LiCl₂), or vehicle for 15 minutes. Treated neurons were then analyzed by Western blotting for proteins and activity assays for Cdk5 and GSK3β. **A**: Representative Western blots. **B** and **C**: Quantification. Data are expressed as means ± SEM. *n* = 3 independent experiments using independent cultures (**B** and **C**). **P* < 0.05, ***P* < 0.01 (one-way analysis of variance and Newman-Keuls post hoc analysis).

The level of C83, on the other hand, was similar in the two experimental groups. The amount of C99/C83 ratio, which is an indicator of amyloidogenic APP metabolism, was 47.8% lower in 3xTg-AD-shRNA-Egr-1 than 3xTg-AD-shRNA-Ctl (Figure 9A). Similar observations were made in nTg littermates. Thus, shRNA-Egr-1 reduced levels of BACE-1 and BACE-1–cleaved APP metabolites (sAPPβ and C99) and lowered the C99/C83 ratio in the hippocampus.

The above data suggested that Egr-1 is either an activator or a stabilizer of BACE-1. To substantiate the hippocampal data and to evaluate the above possibilities, we infected hippocampal primary neurons with shRNA-Egr-1 or shRNA-Ctl and analyzed them. In neurons infected with shRNA-Egr-1, the level of Egr-1 was 72.2% lower when compared to those infected with shRNA-Ctl (Figure 9B). The level of BACE-1 was also 64.2% lower in neurons infected with shRNA-Egr-1 than those infected with shRNA-Ctl. Thus, as in the hippocampus, knockdown of

Egr-1 reduced BACE-1 level in neurons. We then infected neurons with Ln-Egr-1 or Ln-vector. Western blot analysis showed that neurons overexpressing Egr-1 had a 1.9-fold higher level of BACE-1 protein than those expressing vector (Figure 9C). Likewise, BACE-1 immunostaining was 1.8-fold higher in neurons expressing Ln-Egr-1 than those expressing Ln-vector (Figure 9C). This result determined that Egr-1 is an activator of BACE-1 in neurons.

Egr-1 Activates Cdk5 and BACE-1 via a Transcriptional Mechanism

Egr-1 is a transcription factor and regulates expressions of target genes by binding to their promoters. To determine the mechanism by which Egr-1 activates Cdk5 and BACE-1, we infected rat hippocampal primary neurons with Ln-Egr-1 or Ln-vector and analyzed them (Figure 10, A and B). Western blot and qPCR analyses showed that compared to Ln-vector–infected neurons, Ln-Egr-1–infected neurons

Figure 7 Inhibition of Egr-1 by shRNA reduces Aβ pathology in the hippocampus of 3xTg-AD mice. The hippocampus and cortex of 3xTg-AD and nTg mice receiving shRNA-Ctl and shRNA-Egr-1 were analyzed by immunohistochemistry and Western blotting. **A:** Immunohistochemistry. Representative confocal images of hippocampus and cortex of 3xTg-AD and nTg mice, showing plaques. DAPI (blue) stained brain slices were counterimmunostained with human Aβ42 antibody. Abundant Aβ42-positive plaques are seen in both the hippocampus and the cortex of 3xTg-AD mice. A significant reduction in plaques is observed in the hippocampus of 3xTg-AD receiving shRNA-Egr-1 when compared to 3xTg-AD hippocampus receiving shRNA-Ctl. Quantification of immunohistochemical staining was performed using Spectrum Analysis algorithm package and ImageScope analysis software version 11.2 (Aperio Technologies). Data are expressed as the area of hippocampus or cortex occupied by Aβ immunoreactivity. One-way analysis of variance and Newman-Keuls post hoc analysis were performed. **B:** Western blot analysis. Samples were separated on 4% to 20% gradient Tricine gel (Invitrogen). Western blotting was performed using anti-Aβ40 or anti-Aβ42 antibody. *t*-Test was performed. Data are expressed as means ± SEM. *n* = 12 randomly selected micrographs from 12 animals in each genotype (**A**); *n* = 6 per group (**A**); *n* = 12 animals per genotype (**B**); *n* = 6 per group (**B**). ***P* < 0.01. Scale bar = 50 μm (**A**).

had 1.9-fold more BACE-1 protein and 2.1-fold higher BACE-1 mRNA. Likewise, Ln-Egr-1-expressing neurons showed 2.6- and 2.1-fold higher p35 protein and mRNA, respectively, in Ln-vector-infected neurons. The level of Cdk5 protein was comparable between the two groups. This result determined that in neurons, overexpression of Egr-1 induces expressions of BACE-1 and the p35 subunit of Cdk5.

We next performed a chromatin immunoprecipitation assay of infected neurons using antibody against Egr-1 and primers flanking the Egr-1 binding site on promoter of *BACE1*⁴² or p35⁴³ (Table 1). We observed 5.6-fold *BACE1* promoter amplification from anti-Egr-1 antibody than from IgG control (Figure 10B). More important, compared to Ln-vector-infected neurons, Ln-Egr-1-infected neurons had 5.2-fold higher *BACE1* promoter amplification (Figure 10B). Likewise, neurons expressing Ln-Egr-1 displayed 6.3-fold more p35 promoter amplification than from Ln-vector-infected neurons (Figure 10C). This result showed that compared to Ln-vector-infected neurons, those infected with Ln-Egr-1 had 5.2- and 6.3-fold more Egr-1 bound to *BACE1* promoter and p35 promoter,

respectively. Based on this result, we concluded that Egr-1 is a transcriptional activator of BACE-1 and p35 in neurons.

Egr-1 Activates BACE-1 and Promotes APP Processing Independent of Cdk5 and GSK3β

Silencing Egr-1 in the hippocampus of both nTg and 3xTg-AD mice reduced levels of BACE-1 and amyloidogenic APP metabolites (sAPPβ, C99, Aβ40, and Aβ42). Overexpression of Egr-1 in the hippocampal primary neurons activated BACE-1. These data suggested that Egr-1 promotes amyloidogenic APP processing via activating BACE-1. However, Cdk5 was reported to activate BACE-1 and promote aberrant APP processing.^{55–57} Likewise, inhibition of GSK3β was reported to inhibit BACE-1 expression in 20E3 cells.⁵⁸ Egr-1 activates Cdk5 by inducing transcription of p35,⁴³ whereas Cdk5 primes tau for GSK3β.⁴⁹ Therefore, to determine whether Egr-1 promotes APP processing via BACE-1, Cdk5, or GSK3β, we treated mouse hippocampal primary neurons infected with Ln-Egr-1 or Ln-vector with

Figure 8 Inhibition of Egr-1 by shRNA down-regulates BACE-1 in the hippocampus. **A: Left panels:** Effect of inhibition of Egr-1 on the levels of enzymes involved in A β degradation. Western blot analysis of insulin-degrading enzyme (IDE) and neprilysin. **Right panels:** Western blot analysis of PS1 and PS2. One-way analysis of variance and Newman-Keuls post hoc analysis were performed. **B:** Inhibition of Egr-1 down-regulates BACE-1. Western blot and quantification of BACE-1. One-way analysis of variance and Newman-Keuls post hoc analysis were performed. **C: Top panel:** Immunohistochemistry. Micrographs of hippocampus and cortex. Higher-magnification images of the boxed areas (top row) of hippocampus (middle row) and cortex (bottom row) show BACE-1 in hippocampus and cortex of nTg and 3xTg-AD mice. **Middle and bottom panels:** Quantification of immunohistochemical staining, which was performed using Spectrum Analysis algorithm package and ImageScope analysis software version 11.2 (Aperio Technologies). One-way analysis of variance and *t*-test were performed. Data are expressed as means $\pm$ SEM. *n* = 12 mice per genotype (A); *n* = 6 mice per group (A and B); *n* = 12 randomly selected micrographs of 12 mice per genotype (C); *n* = 6 mice per group (C, middle and bottom panels). Scale bars: 500 μ m (C, top row); 50 μ m (C, middle row); 20 μ m (C, bottom row). **P* < 0.05, ***P* < 0.01, and ****P* < 0.005.

Cdk5 inhibitor rocovitine or GSK3 β inhibitor lithium chloride and analyzed them (Figure 11).

A kinase activity assay determined that compared to neurons infected with Ln-vector, those infected with Ln-Egr-1 had 2.5-fold higher Cdk5 activity, but the GSK3 β activity was comparable (Figure 11B). Tau phosphorylation was 2.8- and 2.5-fold higher at Ser^{396/404} and Thr²³¹, respectively, in neurons overexpressing Ln-Egr-1 than those expressing Ln-vector. Roscovitine inhibited Egr-1-induced Cdk5 activity but showed no effect on GSK3 β activity. Egr-1-induced tau phosphorylations at Ser^{396/404} and Thr²³¹ were almost completely inhibited by roscovitine. Lithium chloride inhibited GSK3 β activity and tau phosphorylation on Thr²³¹, but had no effect on Cdk5 activity and tau phosphorylation at Ser^{396/404}. Thus, Egr-1 activated Cdk5 and promoted tau phosphorylation at Ser^{396/404} and Thr²³¹ (Figure 6). Blocking Cdk5 action blocked phosphorylation at both Ser^{396/404} and Thr²³¹. Egr-1 did not activate GSK3 β , but blocking GSK3 β action blocked tau phosphorylation at Thr²³¹.

We next analyzed BACE-1 and APP processing (Figure 11, A and C). Western blot analysis showed that

compared to Ln-vector-infected neurons, BACE-1 protein level was 1.8-fold higher in Ln-Egr-1-infected neurons. A fluorogenic BACE-1 activity assay substantiated Western data and determined that BACE-1 activity was 1.7-fold higher in neurons expressing Ln-Egr-1 than those expressing Ln-vector (Figure 11C). Moreover, compared to Ln-vector-infected neurons, those infected with Ln-Egr-1 had 3.3-fold more BACE-1-cleaved C99 but a comparable level of α -secretase-specific C83. Similarly, Ln-Egr-1-infected neurons secreted 1.9- and 1.5-fold more sAPP β and A β 42, respectively, in medium than those infected with Ln-vector. Thus, overexpression of Egr-1 activated BACE-1 and accelerated amyloidogenic APP processing in mouse hippocampal primary neurons.

Compared to Ln-Egr-1-infected neurons treated with vehicle, Ln-Egr-1-infected neurons treated with roscovitine had comparable levels of BACE-1 protein, BACE-1 activity, C99, sAPP β , and A β 42 (Figure 11C). Similar observations were made in Ln-Egr-1-infected neurons treated with lithium chloride or vehicle and Ln-Egr-1-infected neurons treated with roscovitine plus lithium chloride or vehicle. These data indicated that blocking actions of Cdk5,

A **B** **C**

Figure 9 **A:** Inhibition of Egr-1 by shRNA reduces BACE-1-generated C99 and sAPP β in the hippocampus. Hippocampal and cortical extracts of mice injected with shRNA-Egr-1 or shRNA-ctl were separated either on 8% SDS gel for sAPP β and actin or 4% to 12% Bis-Tris gradient gel for C99 and C83. Blots were probed against sAPP β or APP-C-terminal antibody. Representative Western blots and their quantification. To determine the C99/C83 ratio, the C99 value of a sample was divided by the C83 value of that sample. One-way analysis of variance and Newman-Keuls post hoc analysis were performed. **B:** Inhibition of Egr-1 by shRNA reduces BACE-1 level in mouse hippocampal primary neurons. Mouse neurons infected with shRNA-Egr-1 or shRNA-ctl were analyzed by Western blotting. *t*-Test was performed. **C:** Overexpression of Egr-1 up-regulates BACE-1 in hippocampal primary neurons. Mouse hippocampal primary neurons were infected with Ln-Egr-1 or Ln-vector and then analyzed by Western blotting and immunocytochemistry. Western blot analysis. *t*-Test was performed. Immunocytochemistry: GFP shows infected neurons. Quantification of BACE-1 level is from micrographs of 15 randomly chosen infected neurons from four different cultures in each group along at least 30 μ m of each neurite. *t*-test was performed. Data are expressed as means $\pm$ SEM. *n* = 12 mice per genotype (**A**); *n* = 6 mice per group (**A**); *n* = 3 independent cultures (**B**); *n* = 4 independent experiments (**C**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.005. Scale bars: 30 μ m (**C**, first, second, and third columns); 2 μ m (**C**, fourth column).

GSK3 β , or both do not affect Egr-1-induced BACE-1 activation and amyloidogenic APP processing in neurons. Based on these data, we concluded that Egr-1-induced BACE-1 activation and amyloidogenic APP processing are independent of Cdk5 and GSK3 β .

Egr-1 Promotes Tau Phosphorylation Independent of BACE-1 and A β

Egr-1 activates Cdk5 and promotes tau phosphorylation in neurons (Figures 4 and 6). Because Cdk5 phosphorylates tau, these data suggested that Egr-1 promotes tau phosphorylation by activating Cdk5. However, Egr-1 also promotes A β production (Figure 11). Several studies have shown that A β promotes tau phosphorylation by activating

intracellular kinases, including Cdk5.^{59,60} Therefore, Egr-1 may promote tau phosphorylation via A β . To test this possibility, we silenced BACE-1 by shRNA in neurons infected with Ln-Egr-1 and analyzed them (Figure 12). Compared to neurons infected with Ln-vector and treated with shRNA-ctl, those infected with Ln-Egr-1 and treated with shRNA-ctl showed increased BACE-1 level, elevated BACE-1 activity, increased A β 42, and increased phosphorylated tau, as expected (Figure 12, A–C). More important, neurons infected with Ln-Egr-1 and treated with shRNA-BACE-1 displayed 65% and 63% reductions in the levels of BACE-1 and A β 42, respectively, when compared to those infected with Ln-Egr-1 and treated with shRNA-ctl. These data determined that Egr-1 promotes A β production via BACE-1. The levels of tau phosphorylated at all

Figure 10 Egr-1 activates BACE-1 and Cdk5 by a transcriptional mechanism. Rat hippocampal primary neurons were infected with Ln-vector of Ln-Egr-1. Infected neurons were analyzed by Western blotting for proteins, qPCR for mRNAs, and chromatin immunoprecipitation (ChIP) assay for promoter binding. **A:** Western blotting, qPCR, and quantification. **B:** ChIP assay for *BACE1* promoter. Neurons infected with Ln-Egr-1 or Ln-vector were immunoprecipitated using anti-Egr-1 or IgG. *BACE1* promoter in the immunoprecipitate was amplified by qPCR using primers specific to Egr-1-binding site of *BACE1* promoter (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as the internal control. Bar graphs were generated from qPCR data. *BACE1* promoter amplification between Ln-vector- and Ln-Egr-1-infected neurons. To make the comparison, the qPCR value obtained for the IgG from the ChIP data of a sample was subtracted from the corresponding value for the anti-Egr-1. Resulting values were used to generate the graph. Values are the average of three determinations. **C:** ChIP assay for p35 promoter. ChIP assay was performed as in **B** except primers flanking p35 promoter were used. Data are expressed as average $\pm$ SEM (**A**). $n = 3$ independent cultures (**A**); $n = 4$ independent cultures (**B**). * $P < 0.05$, *** $P < 0.005$ (t -test).

sites examined were comparable in neurons infected with Ln-Egr-1 and treated with shRNA-BACE-1 and those infected with Ln-Egr-1 and treated with shRNA-Ctl. Furthermore, activities of both Cdk5 and GSK3 β were also similar between the two groups (Figure 12D). Thus, blocking BACE-1 action blocked Egr-1-induced A β production but had no effect on tau phosphorylation and activities of Cdk5 and GSK3 β . This result determined that Egr-1 promotes tau phosphorylation independent of BACE-1 and A β .

Discussion

The accumulation of A β and hyperphosphorylated tau in the brain is regarded as central to AD pathogenesis.^{1,2} So far, all of the A β - or tau-targeted therapies have been unsuccessful in clinical trials.¹ The reasons for these failures are not completely clear. However, A β and tau pathologies coexist in AD, and A β induces tau filament formation in mouse brain.⁶¹ Mice expressing mutated human APP and mutated human tau develop severalfold increased neurofibrillary tangles, compared with mice expressing only mutated human tau.⁶² Neurons without tau are resistant to A β toxicity, and APP transgenic mice lacking tau develop A β pathology but remain cognitively normal.^{63,64} A combined

reduction of both A β and tau, but not A β alone, was found to improve cognition in a mouse model with both plaques and tangles.⁶⁵ Increasing evidence suggests that A β and tau interact to either cause or enhance AD.⁶⁶ A therapeutic strategy targeting both pathologies is currently being sought for the effective treatment of AD.⁶⁶

BACE-1 and γ -secretase catalyze the sequential cleavage of APP to generate A β .² Likewise, Cdk5 and GSK3 β are recognized as the major kinases that phosphorylate tau in the AD brain.^{12,14} Repeated failures of clinical trials targeting γ -secretase³ or GSK3 β ^{17–19,67} in mild to moderate AD patients have shifted the focus of drug development against AD to BACE-1 and Cdk5.^{1,20} However, although BACE-1 knockout mice do not develop plaques and knockdown of BACE-1 in the hippocampus reduces A β in the APP mouse model,^{68,69} a direct pharmacological inhibition of BACE-1 impairs synaptic plasticity and cognition in mice.^{6,7} Cdk5, on the other hand, is involved in brain development and adult hippocampal neurogenesis, and Cdk5 knockout mice die perinatally.^{15,16} These observations suggest that a direct pharmacological inhibition of BACE-1 or Cdk5 is associated with mechanism-based adverse effects.

Cdk5 requires the p35 subunit to become active. Egr-1 is the main inducer of p35 expression and activator of Cdk5 in the brain.^{32,43} Previously, we showed that Egr-1 knockout mice have reduced levels of phosphorylated tau and

Figure 11 Egr-1 promotes amyloidogenic APP processing independent of Cdk5 and tau phosphorylation. Mouse hippocampal primary neurons infected with Ln-Egr-1 or Ln-vector were treated with 400 nmol/L roscovitine, 10 mmol/L lithium chloride (LiCl₂), or vehicle for 15 minutes and analyzed for Cdk5 and GSK3β activities, tau phosphorylation (Western blotting), BACE-1 protein (Western blotting), and BACE-1 activity (fluorogenic BACE-1 activity assay). Western blotting was performed for C99 and C83 using mouse anti-APP-C-terminal fragment antibody. Culture medium was analyzed for mouse sAPPβ (Western blotting) and Aβ42 (enzyme-linked immunosorbent assay). **A:** Representative Western blots. **B and C:** Quantification. $n = 4$ independent cultures. * $P < 0.05$, ** $P < 0.01$ (one-way analysis of variance).

overexpression of Egr-1 promotes Cdk5-mediated tau phosphorylation in neurons and in rat hippocampus.³² In this study, we showed that Egr-1 also promotes tau phosphorylation by GSK3β at Thr²³¹. We also demonstrated that Egr-1, by activating BACE-1, accelerates amyloidogenic APP processing in neurons. Our studies suggest that Egr-1 is a physiological regulator of tau phosphorylation and Aβ production in the brain. More important, targeted inhibition of Egr-1 in the hippocampus of cognitively deficient 3xTg-AD mice reduces Aβ load and hyperphosphorylated tau, and rescues cognitive impairment (this study). Egr-1 mice are healthy and do not display any undesirable phenotype, except female infertility.⁷⁰ Our data suggest that Egr-1 is a potential therapeutic candidate for treatment of AD.

Extracellular Aβ promotes intracellular tau hyperphosphorylation by activating several kinases, including Cdk5.^{59,60} BACE-1 is the rate-limiting enzyme in Aβ generation, and its activation triggers amyloidogenic APP processing.² This implies that BACE-1 activation will activate Cdk5 and promote both tau phosphorylation and amyloidogenic APP processing. Studies have also shown that Cdk5 activates BACE-1, leading to aberrant APP processing.^{55–57} These observations suggest that activation of BACE-1 alone or Cdk5 alone can promote both amyloidogenic APP processing and tau phosphorylation. Egr-1 activates both Cdk5 and BACE-1 and promotes Aβ production and tau phosphorylation in neurons and mouse brain (this study). However, we showed that in neurons, inhibition of

Figure 12 Egr-1 promotes tau phosphorylation independent of BACE-1 and Aβ. Mouse hippocampal primary neurons were coinfectd with Ln-Egr-1 and shRNA-BACE-1 and analyzed. **A:** Representative Western blots. **B:** Quantification of BACE-1 and Aβ42. **C:** Quantification of tau phosphorylation. **D:** Quantification of Cdk5 and GSK3β activities. Data are expressed as average ± SEM. *n* = 3 cultures. **P* < 0.05, ***P* < 0.01 (one-way analysis of variance).

Cdk5 blocks Egr-1–induced tau phosphorylation without affecting BACE-1 activation and Aβ generation (Figure 11). Likewise, BACE-1 inhibition blocks Egr-1–induced Aβ production but has no influence on Cdk5 activity and tau phosphorylation (Figure 12). Our data indicate that in neurons, Egr-1 promotes tau phosphorylation and Aβ production, activating, respectively, Cdk5 and BACE-1. However, in a previous study, removal of extracellular Aβ was shown to clear tau pathology in the brains of 3xTg-AD mice, indicating that tau pathology is downstream of Aβ in these mice.⁶⁵ The reduction in the level of phosphorylated tau in the hippocampus of 3xTg-AD mice receiving shRNA-Egr-1 may be because of the inhibition of both Cdk5 and BACE-1 activities.

Among all tau sites tested, Thr²³¹ is most phosphorylated in the hippocampus of 3xTg-AD mice (Figure 4A). Previous studies have shown that tau is phosphorylated at Thr²³¹ in AD but not in the normal brain,^{71,72} and phosphorylation at this site significantly inhibits ability of tau to bind and stabilize microtubules.⁵⁰ In addition, phosphorylation at Thr²³¹ causes a conformational change that exposes the C-terminal region of tau for further phosphorylation.⁴⁷ Furthermore, prolyl isomerase 1 specifically binds to phosphorylated Thr²³¹ of tau and facilitates the *cis-trans* configuration

change of the pThr-Pro bond, thereby enabling PP2A to dephosphorylate tau and to restore tau function to bind to microtubules.⁷³ Accumulation of Thr²³¹ phosphorylated tau in the AD brain was suggested to sequester prolyl isomerase 1 and cause depletion of soluble prolyl isomerase 1.⁷³ Tau phosphorylation at Thr²³¹ is implicated to play an important role in the development of tau dysfunction associated with AD.

GSK3β preferentially phosphorylates tau Thr²³¹ in the brain.^{47,49,50} In this study, we find that inhibition of Egr-1 by shRNA reduces tau phosphorylation at Thr²³¹. However, Egr-1 does not activate GSK3β (Figure 6).³² It is, therefore, likely that Egr-1 regulates tau phosphorylation by GSK3β at Thr²³¹ indirectly. Some of GSK3β substrates are first phosphorylated (primed) by another kinase at position *n* + 4 to generate a sequence S/T XXXpS/T motif, recognized by GSK3β.^{49,50} Previous studies have shown that Cdk5 phosphorylates tau at Ser²³⁵ and primes tau for phosphorylation at Thr²³¹ by GSK3β.^{48,49} In this study, we showed that in rat hippocampal primary neurons in culture, overexpression of Egr-1 promotes tau phosphorylation at both Ser²³⁵ and Thr²³¹. Although the phosphorylation at Ser²³⁵ is Cdk5 dependent, phosphorylation at Thr²³¹ requires activity of both Cdk5 and GSK3β. More important,

blocking Ser²³⁵ phosphorylation by inhibition of Cdk5 blocks phosphorylation at Thr²³¹. Previous reports^{47,49} and these data together suggest that Egr-1 activates Cdk5, which phosphorylates tau at Ser²³⁵ and primes tau for Thr²³¹ phosphorylation by GSK3 β . GSK3 β subsequently phosphorylates tau at Thr²³¹.

3xTg-AD mice, in addition to human tau, also express endogenous murine tau. A previous study showed that in 3xTg-AD mice, endogenous tau is phosphorylated at Ser^{396/404} and Thr¹⁸¹, and the authors suggested that these phosphorylations trigger phosphorylation and aggregation of human tau.⁷⁴ In the current study, we found that silencing Egr-1 reduces tau phosphorylation at several sites, including Ser⁴⁰⁴ and Thr¹⁸¹ in both 3xTg-AD and nTg mice (Figure 4). A previous study⁷⁴ and our data together suggest that Egr-1 knockdown may have reduced some phosphorylation of human tau in 3xTg-AD mice by inhibiting phosphorylation of endogenous tau at Ser⁴⁰⁴ and Thr¹⁸¹. Phosphorylation of endogenous tau does not affect cognition of 3xTg-AD mice.⁷⁴ Improvements in the learning and memory of 3xTg-AD mice because of knockdown of Egr-1 in the hippocampus may have occurred independent of endogenous tau phosphorylation and endogenous tau-induced phosphorylation of human tau.

Knockdown of Egr-1 reduces tau phosphorylation at several sites, including Ser²⁶², in both 3xTg-AD and nTg mice. Ser²⁶² is a non-proline-directed site and is not phosphorylated by Cdk5.⁴⁶ Previously, we showed that Cdk5 phosphorylates and inactivates PP1, which dephosphorylates Ser²⁶² phosphorylated tau.⁴⁶ Thus, Cdk5, by phosphorylating and inhibiting PP1 activity, reduces tau phosphorylation at Ser²⁶². Both nTg and 3xTg-AD mice receiving shRNA-Egr-1 display reduced phosphorylated PP1 when compared to those receiving shRNA-Ctl (Figure 5). These data indicate that silencing Egr-1 activates PP1. It is, therefore, possible that in mice, silencing Egr-1 reduces tau phosphorylation at Ser²⁶² via PP1 activation.

The hypothesis that vascular disorders may play a role in AD was criticized on the basis of neuropathological evidence supporting an additive effect of cerebral infarction on neurodegenerative lesions.⁷⁵ In addition, stroke or transient ischemic attacks are exclusion criteria for an AD diagnosis.⁷⁶ However, epidemiological studies have indicated that AD and vascular dementia share common risk factors.^{20,21} In addition, in most cases of dementia, vascular lesions and degenerative processes of AD coexist.⁷⁷ Hypoxic ischemia is a powerful promoter of cerebral amyloidogenesis and tau hyperphosphorylation in humans, rats, and mice.^{23,24} Increasing evidence suggests that there is a molecular link between AD and vascular dementia.^{20,21}

In the brain, Egr-1 is induced by injury, transient focal ischemia, and hypoxia, and plays a key role in the induction of both cardiovascular and cerebrovascular pathologies.^{27,28} BACE-1 expression is also increased during transient ischemia, hypoxia, inflammation, and head trauma.²⁰ Likewise, Cdk5 is activated during hypoxia and ischemic stroke in the brain.^{78,79} It is plausible that vascular insults induce

Egr-1, which activates BACE-1 and Cdk5 and promotes amyloidogenesis and tau hyperphosphorylation observed in cerebrovascular pathologies.

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