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ApoE4 and Aβ Oligomers Reduce BDNF Expression via HDAC Nuclear Translocation

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Apolipoprotein E4 (ApoE4) is a major genetic risk factor for several neurodegenerative disorders, including Alzheimer's disease (AD). Epigenetic dysregulation, including aberrations in histone acetylation, is also associated with AD. We show here for the first time that ApoE4 increases nuclear translocation of histone deacetylases (HDACs) in human neurons, thereby reducing BDNF expression, whereas ApoE3 increases histone 3 acetylation and upregulates BDNF expression. Amyloid-β (Aβ) oligomers, which have been implicated in AD, caused effects similar to ApoE4. Blocking low-density lipoprotein receptor-related protein 1 (LRP-1) receptor with receptor-associated protein (RAP) or LRP-1 siRNA abolished the ApoE effects. ApoE3 also induced expression of protein kinase C ε (PKCε) and PKCε retained HDACs in the cytosol. PKCε activation and ApoE3 supplementation prevented ApoE4-mediated BDNF downregulation. PKCε activation also reversed Aβ oligomer- and ApoE4-induced nuclear import of HDACs, preventing the loss in BDNF. ApoE4 induced HDAC6–BDNF promoter IV binding, which reduced BDNF exon IV expression. Nuclear HDAC4 and HDAC6 were more abundant in the hippocampus of ApoE4 transgenic mice than in ApoE3 transgenic mice or wild-type controls. Nuclear translocation of HDA6 was also elevated in the hippocampus of AD patients compared with age-matched controls. These results provide new insight into the cause of synaptic loss that is the most important pathologic correlate of cognitive deficits in AD.

Key words: **Alzheimer's disease; ApoE; BDNF; histone deacetylase; LRP1; PKC**

Introduction

The apolipoprotein E ε4 allele (ApoE ε4) is a major risk factor for sporadic and late-onset Alzheimer's disease (LOAD), as well as other neurodegenerative conditions. In AD and healthy aged controls, ApoE ε4 dosage correlates inversely with dendritic spine density in the hippocampus (Buttini et al., 2002). The risk of AD is 2- to 3-fold higher in patients with 1 ApoE ε4 allele and 12-fold higher in patients with two ApoE ε4 alleles (Michaelson, 2014).

ApoE, which in the brain is produced mainly in astrocytes, is a cholesterol-transporting protein and a major determinant of synapse formation and remodeling (Pfrieger, 2003; Bu, 2009). ApoE is also a ligand for lipoprotein receptors and thus may have a role in promoting amyloid-β (Aβ) clearance through the blood–brain barrier or the blood–CSF barrier. There are many functional differences between ApoE3 and ApoE4. ApoE4 increases Aβ deposition (Verghese et al., 2011; Liu et al., 2013). Knock-in transgenic mice containing human ApoE4 allele show reduced synaptic transmission compared to mice with the human ApoE3 allele (Klein et al., 2010). Transcriptome-wide differential gene expression analysis has shown that ApoE4 produces changes in gene expression similar to those found in patients with LOAD (Rhinn et al., 2013). We have also shown that ApoE3 acts via protein kinase C ε (PKCε) to protect primary neurons against Aβ-induced cell death and induce synaptogenesis, whereas ApoE4 does not (Sen et al., 2012).

BDNF is a critical factor in synaptic repair and plasticity. Although evidence for BDNF polymorphisms in AD is still inconclusive, synaptic loss is the single most important correlate of AD. Lower BDNF levels are associated with ApoE4 in AD cases with apathy, a noncognitive symptom common to many forms of dementia (Alvarez et al., 2014). BDNF expression is regulated by at least nine promoters (Aid et al., 2007; Pruunsild et al., 2007), of which promoter IV (PIV) is the most responsive to neuronal activity (Tao et al., 1998). PKCε, which is decreased in AD (Hongpaisan et al., 2011; Khan et al., 2015), also regulates BDNF expression (Lim and Alkon, 2012; Corbett et al., 2013; Hongpaisan et al., 2013; Neumann et al., 2015). BDNF expression is also regulated in part by exon-specific epigenetic modifications.

Recent evidence has shown that histone acetylation and deacetylation are significantly abnormal in several neurodegenerative conditions, including AD (Saha and Pahan, 2006; Kramer and van Bokhoven, 2009; Mai et al., 2009; Fischer et al., 2010; Gräff et al., 2012). Postmortem studies reported that histone deacetylase 2 (HDAC2) is increased in the hippocampus of AD patients (Gräff et al., 2012). Class II HDAC6 levels are also elevated in AD cortex and hippocampus (Ding et al., 2008). Nuclear HDAC4 staining in CA1 neurons increases with increase in AD severity (Herrup et al., 2013). HDAC inhibitors have been reported to improve memory and cognition (Fischer et al., 2007; Kilgore et al., 2010) by inducing histone H3 and H4 acetylation of

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Table 1. Patient ID, Braak stage, and age of human brain tissue

Patient ID	Sex	Age	Average age ± SD	Autopsy diagnosis
AN02930	M	80	77.5 ± 12.34	AD Braak 3
AN14554	F	61		AD Braak 6
AN17726	M	72		AD Braak 2
AN06468	M	98		AD Braak 4
AN16195	F	73		AD Braak 5
AN02773	F	81		AD Braak 5
AN00704	F	82	77.6 ± 6.58	Control
AN00316	F	75		Control
AN17896	M	69		Control
AN12667	M	86		Control
AN08396	M	76		Control

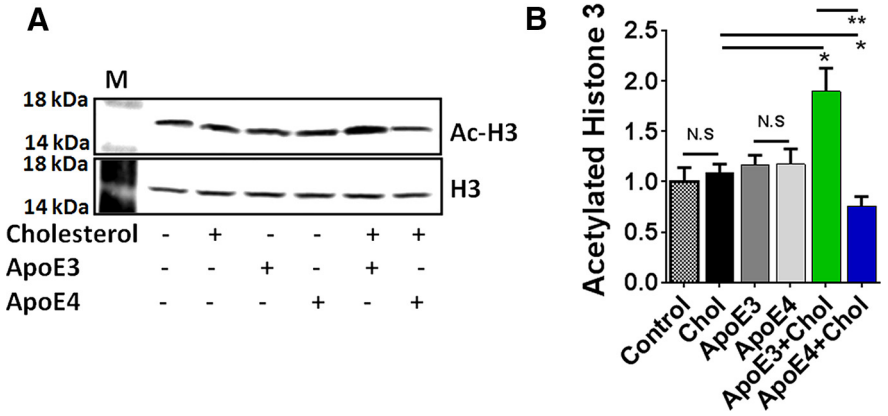


Figure 1. ApoE3, but not ApoE4, induces histone 3 acetylation in the nucleus. SH-SY5Y cells were treated with cholesterol (Chol; 100 μM) and ApoE3 (20 nM) or ApoE4 (20 nM) with or without cholesterol for 24 h. Cells were fractionated and the nuclear lysate used for measuring H3 acetylation. ***A***, Immunoblot showing histone H3K9/14ac (Ac-H3) and H3 levels in the nucleus. M, Molecular weight marker. ***B***, H3K9/14ac expression was normalized against total H3 expression. ApoE3 + Chol induced H3K9/14ac, whereas ApoE4 + Chol markedly reduced acetylation compared with the cholesterol-only cells (Control, Chol). Data are reported as mean ± SEM of 3 independent experiments (Student's *t* test, $*p < 0.05$ and $**p < 0.005$).

BDNF promoters (Bredy et al., 2007; Ishimaru et al., 2010; Boulle et al., 2012). Class II HDAC inhibitors have been shown to induce BDNF PIV activity (Koppel and Timmusk, 2013).

In the context of all of these previous observations, we hypothesized that ApoE3 and ApoE4 differentially regulate gene transcription in AD by modulating histone acetylation through HDACs in the brain. Based on our previous studies showing involvement of PKC in ApoE signaling, we investigated the role of ApoE isoforms and PKC on nuclear translocation of HDACs and BDNF expression in neuronal cells in the presence or absence of amyloid-β amylospheroids (ASPDs) to mimic AD *in vitro*.

Materials and Methods

Materials. Cell culture media were from Invitrogen (F12K, neurobasal, and B27) and K.D. Medical (MEM). Bryostatin-1 was from Biomol International. DCPLA methyl ester (DCPLA-ME) was synthesized in our laboratory using a method described previously (Nelson et al., 2009). ApoE3 (rh-ApoE3), ApoE4 (rh-ApoE4), and other reagents were from Sigma-Aldrich. $A\beta_{1\text{-}42}$ was from Anaspec. Recombinant human receptor-associated protein (RAP) was from Molecular Innovations. Primary antibodies against acetylated histone 3, histone 3, β-actin, lamin B, and PKCε were from Santa Cruz Biotechnology. Primary antibodies against HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, and HDAC6 were from Cell Signaling Technology. All secondary antibodies were from Jackson ImmunoResearch Laboratories.

ASPDs were prepared as described previously (Noguchi et al., 2009; Sen et al., 2012). Briefly, $A\beta_{1\text{-}42}$ was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol and incubated overnight at 4°C and then for 3 h at 37°C. The dissolved $A\beta_{1\text{-}42}$ was lyophilized to 40 nmol/tube. The lyophilized Aβ was dissolved in PBS without Ca^{2+} or Mg^{2+} to a <50 μM concentration and rotated for 14 h at 4°C. The resulting ASPD solution was purified using a 100 kDa molecular weight cutoff filter (Amicon Ultra; Millipore).

Cell culture and treatment. Human SH-SY5Y neuroblastoma cells (Sigma-Aldrich) were cultured in 45% F12K, 45% MEM, and 10% FBS. Cells were treated with cholesterol, ASPD, ApoE3/ApoE4 + cholesterol, or PKC activators for 24 h. ApoE (20 nM) and cholesterol (100 μM) were mixed separately into the cultures. Cholesterol was dissolved in ethanol. To block the ApoE receptors, cells were treated with RAP for 30 min before adding ApoE. Human primary neurons (ScienCell Research Laboratories) were plated on poly-L-lysine-coated plates and were maintained in neuronal medium (ScienCell Research Laboratories) supplemented with the neuronal growth supplement (ScienCell Research Laboratories). For maintenance of neurons, half of the medium was changed every 3 d. Fresh activators were added with every medium change.

Transgenic mice. ApoE target replacement mice were from Taconic Farms. In this strain (C57BL/6), the endogenous murine ApoE gene has been replaced with human alleles of ApoE3 (B6.129P2-Apoe$^{tm2(APOE*3)Mae}$N8) or ApoE4 (B6.129P2-Apoe$^{tm3(APOE*4)Mae}$N8). All experiments were performed on age-matched male animals. All animals were housed in a barrier facility, provided food and water *ad libitum*, and maintained following the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

Human brain tissue. Fresh frozen human brain tissue were obtained from Harvard Brain Tissue Resource Center (McLean Hospital, Boston, MA) after approval for the study from Francine M. Benes (Table 1). Informed consent was obtained from all patients or legal representatives. The pathological diagnosis of AD was conducted according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD). The study was performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (http://www.wma.net/en/30publications/10policies/b3/index.html).

Cell lysis and nuclear fractionation. Briefly, 5×10^6 cells were collected in PBS and washed twice with cold PBS. The cell pellet was resuspended in 500 μl of hypotonic buffer (20 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 1 mM PMSF) and incubated on ice for 15 min. Next, 25 μl of 10% NP-40 was added to the cell suspension and the sample was vortexed for 10 s. The homogenate was centrifuged for 10 min at 1000 × *g* at 4°C to obtain the cytoplasmic fraction (supernatant) and nuclear fraction (pellet). The nuclear pellet was resuspended in 50 μl of complete cell extraction buffer (100 mM Tris-Cl, pH 7.4, 2 mM Na_3VO_4, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 0.5% deoxycholate, 20 mM $Na_4P_2O_7$, and 1 mM PMSF) and incubated on ice for 30 min with vortexing at 10 min intervals. The nuclear lysate was centrifuged at 14,000 × *g* for 30 min at 4°C to obtain the nuclear fraction (supernatant). Protein concentration was measured using the Coomassie Plus (Bradford) Protein Assay kit (Pierce).

Immunoblot analysis. Protein from each sample was separated by SDS-PAGE in a 4–20% gradient Tris-Glycine gel (Invitrogen). The protein was then transferred to nitrocellulose membrane. The membrane was blocked with BSA at room temperature for 15 min and incubated with primary antibody overnight at 4°C. After incubation, the membrane was washed 3× with TBS-T (Tris-buffered saline-Tween 20) and further incubated with alkaline-phosphatase-conjugated secondary antibody (Jackson Immunoresearch Laboratories) at 1:10,000 dilution for 45 min. The membrane was washed 3× with TBS-T and developed using the 1-step NBT-BCIP substrate (Pierce). Lamin B was used as the nuclear

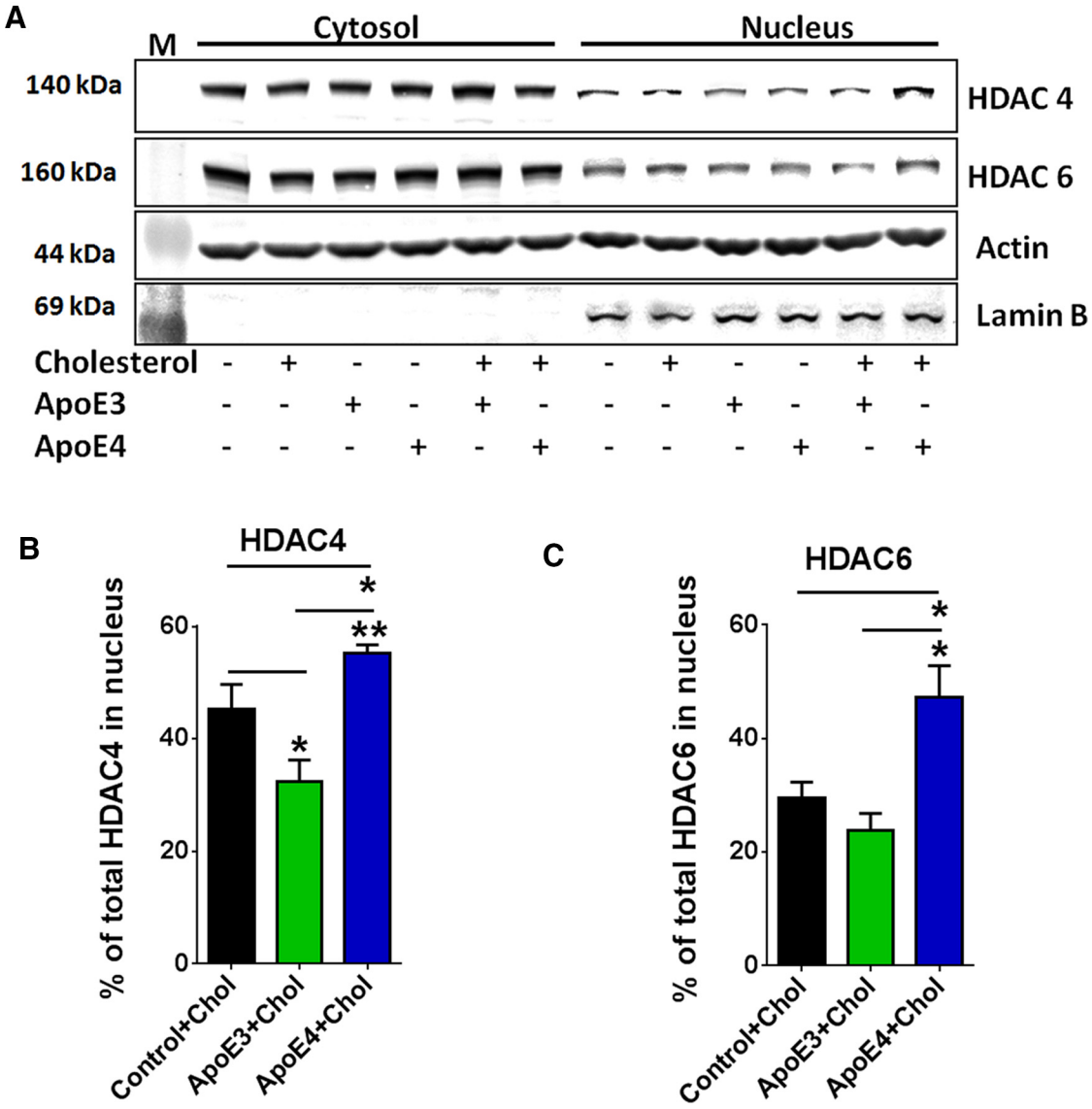


Figure 2. ApoE4 induces translocation of HDAC4 and HDAC6 to the nucleus. SH-SY5Y cells were treated with cholesterol (Chol; 100 μM), ApoE3 (20 nM), ApoE4 (20 nM), ApoE3 (20 nM) + Chol (100 μM), and ApoE4 (20 nM) + Chol (100 μM) for 24 h. Cytosol and nuclear fractions were prepared for immunoblot. ***A***, Immunoblot showing expression levels of HDAC4 and HDAC6 in cytosol and nuclear fractions of SH-SY5Y cells. M, Molecular weight marker. ***B***, ***C***, HDAC nuclear translocation reported as the percentage of total protein in the nucleus [nucleus/(cytosol + nucleus)]. ApoE4+Chol treatment caused a significant increase in nuclear translocation of HDAC4 and HDAC6 compared with ApoE3+Chol treatment. Data are reported as mean ± SEM of 3 independent experiments (Student's *t* test, $^{*}p < 0.05$ and $^{**}p < 0.005$).

loading control and β-actin as the cytosolic loading control. The immunoblot proteins were detected using ImageQuant RT-ECL (GE Life Sciences) and densitometric quantification was performed using IMAL software, which was developed at our institution. For translocation assays, HDAC translocation to the nucleus was represented as the percentage of total protein in the nucleus [nucleus/(cytosol + nucleus)].

Immunofluorescence and confocal microscopy. Cells were grown in eight-chambered slides (Nunc). For immunofluorescence staining, the cells were washed with PBS, pH 7.4, and fixed with 4% paraformaldehyde for 4 min. After fixation, cells were blocked and permeabilized with 5% serum and 0.3% Triton X-100 in 1× PBS for 30 min. Cells were washed 3× with 1× PBS and incubated with primary antibodies for 3 h at 1:100 dilution. After the incubation, slides were again washed 3× in 1× PBS and incubated with the FITC anti-rabbit IgG for 1 h at 1:400 dilution. Cells were further washed and mounted in Pro Long Gold antifade mounting solution (Invitrogen). Stained cells were viewed under an LSM 710 Meta confocal microscope (Zeiss) at 350 and 488 nm excitation and 470 and 525 nm emission for DAPI (a DNA stain) and FITC, respectively. Approximately five to six individual cells from eight different wells were analyzed at 63× lens magnification using Zen 2009 (Zeiss). To measure the percentage of total protein in the nucleus, the whole neuron cell body and the nucleus were separately selected as a region of interest. Mean fluorescence intensity in each channel, DAPI, and HDAC were measured from the nucleus and the whole neuron. Percentage HDAC in nucleus is represented as HDAC (normalized against DAPI) in nucleus/HDAC (normalized against DAPI) in the whole cell body.

Knock-down and overexpression. RNAi silencing of LRP-1 was conducted by transfection of double-stranded siRNA oligonucleotides (Trilencer-27) designed and synthesized by Origene. Control transfections included both a proven nontargeting siRNA provided by Origene and transfection reagent only without oligonucleotides. PKCε knock-down was performed using 33 nM three target-specific 19–25 nucleotide PKCε siRNA constructs from Santa Cruz Biotechnology. Overexpression of PKCε was achieved by transfecting a pCMV6-ENTRY vector containing human PKCε cDNA (Origene). Transfection was performed using Lipofectamine 2000 per the manufacturer's instructions (Invitrogen). Medium was changed 6 h after

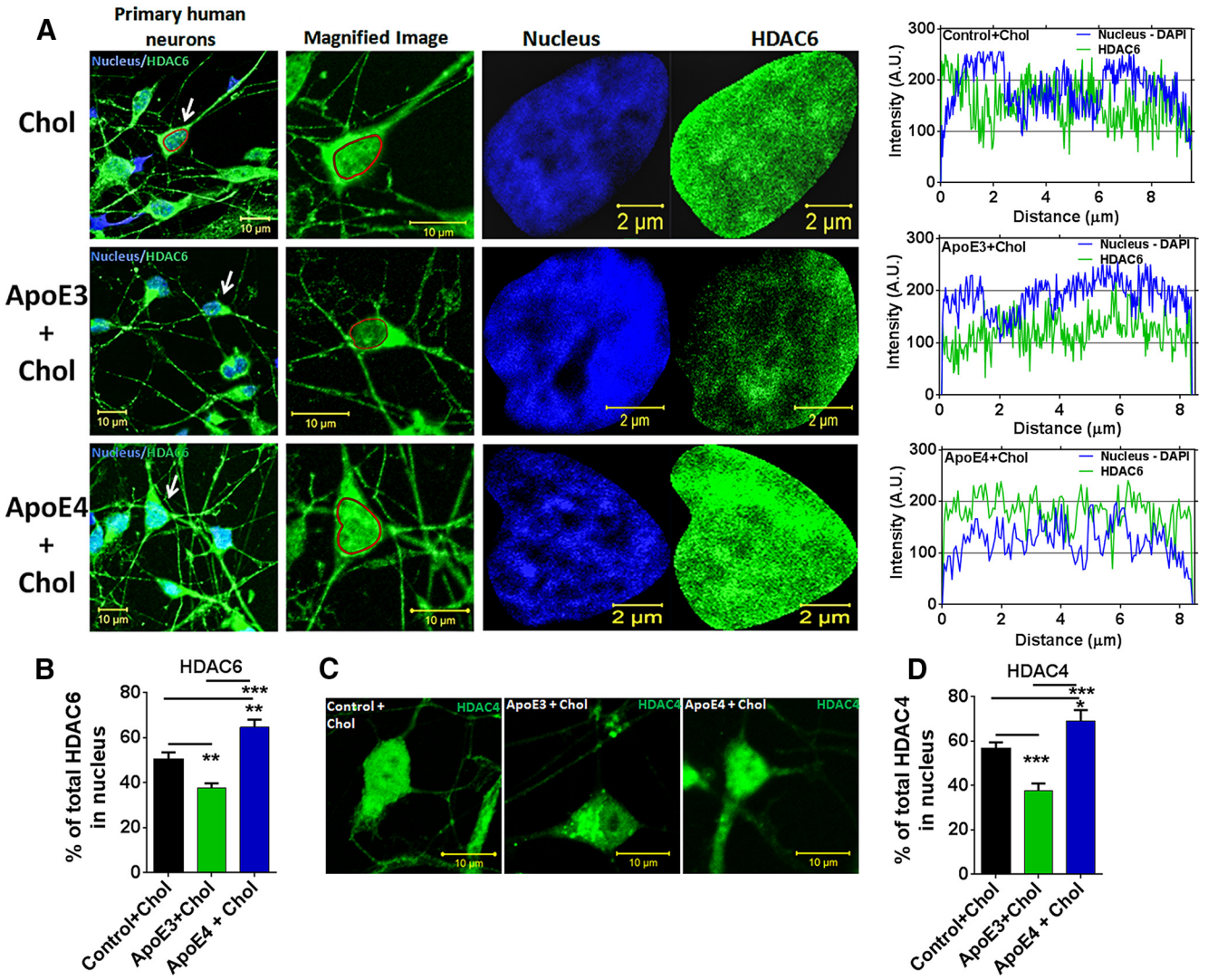


Figure 3. ApoE4 translocates HDAC6 to the nucleus. Primary human neurons were treated with cholesterol (Chol; 100 μM), with or without ApoE3 (20 nM) or ApoE4 (20 nM) for 24 h. ***A***, Representative confocal images of neurons treated with Chol, ApoE3 + Chol or ApoE4 + Chol. First left column represents HDAC6 (green) and DAPI (blue) at low magnification, second column is the magnified image of a single neuron, third column shows the fluorescence intensity of DAPI and HDAC6 in the nucleus, and the fourth column is the graphical profile view of relative intensities along the diameter of the nucleus. ***B***, ApoE4 + Chol (but not ApoE3 + Chol) induce nuclear translocation of HDAC6. ***C***, Confocal images of neurons showing HDAC4 staining. ***D***, ApoE3 + Chol reduces and ApoE4 + Chol induces nuclear import of HDAC4. Data are reported as mean ± SEM (Student's *t* test, $^{*}p < 0.05$ and $^{**}p < 0.005$).

addition of lipofectamine. LRP-1 and PKC expression were measured 72 h after transfection.

qRT-PCR. qRT-PCR was done and analyzed as described previously (Schmittgen and Livak, 2008; Sen et al., 2012). Total RNA (500 ng) was reverse transcribed using oligo(dT) and Superscript III (Invitrogen) at 50°C for 1 h. The cDNA products were analyzed using a LightCycler 480 II (Roche) PCR machine and LightCycler 480 SYBR Green 1 master mix following the manufacturer's protocol. Primers for PKCε (forward primer: TGGCTGACCTTGGTGTTACTCC, reverse primer: GCTGACTTGGATCGGTCGTCTT, PKCα (forward-ACAACCTGGACAGAGTGAAACTC, reverse: CTTGATGGCGTACAGTTCCTCC), PKCδ (forward: ACATTCTGCGGCACTCCTGACT, reverse: CCGATGAGCATTTCGTACAGGAG), GAPDH (forward: GTCTCCTCTGACTTCAACAGCG, reverse: ACCACCCTGTTGCTGTAGCCAA), and BDNF (forward: CATCCGAGGACAAGGTGGCTTG, reverse: GCCGAACTTTCTGGTCCTCATC; Origene). BDNF promoter- and exon-specific primers were used as described previously (Pruunsild et al., 2007). BDNF–promotor I (PI) (forward: GGCACGAACTTTTCTAAGAAG, reverse: CCGCTTTAATAATAATACCAG), BDNF–promotor II (PII) (forward: GAGTCCATTCAGCACCTTGGA, reverse: ATCTCAGTGTGAGCCGAACCT), BDNF–promotor III (PIII) (forward: AGAATCAGGCGGTGGAGGTGGTGTG, reverse: AACCCTCTAAGCCAGCGCCCGAAAC), BDNF–promoter IV (PIV) (forward: AAGCATGCAATGCCCTGGAAC, reverse: TGCCTTGACGTGCGCTGTCAT), BDNF–promoter IX (PIX) (forward: CACTTGCAGTTGTTGCTTA, reverse: GGCTTCAAGTTCTCCTTCTTCCCA) were from Invitrogen. BDNF exons were amplified using BDNF exon-specific forward primer (BDNF–exon III forward: AGTTTCGGGCGCTGGCTTAGAG; exon IV forward: GCTGCAGAACAGAAGGAGTACA) and exon IX reverse primers (exon IX reverse: GTCCTCATCCAACAGCTCTTCTATC).

ChIP. ChIP was conducted using the SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology) following the manufacturer's protocol. Immunoprecipitations were performed at 4°C overnight with primary antibodies (HDAC4, HDAC6, or IgG antibody as a control). Immunoprecipitated DNA was subjected to real-time qRT-PCR using primers specific to the human BDNF promoters. The cumulative fluorescence for each amplicon was normalized to input DNA amplification. Products of ChIP-PCR were separated on a 2% agarose gel with ethidium bromide (Invitrogen) to verify amplification.

Statistical analysis. All experiments were performed at least in triplicate, as noted in the figure legends. For confocal images, six or more random fields from three independent experiments were considered for analysis. Data are

presented as mean ± SEM. All data were analyzed by one-way ANOVA and Newman–Keuls multiple-comparison posttest. Significantly different groups were further analyzed by Student's *t* test using GraphPad Prism 6 software. *p*-values < 0.05 were considered statistically significant.

Results

ApoE4 and ApoE3 affect the acetylation of histone H3 differentially

To investigate whether ApoE3 and ApoE4 regulate acetylation of histones in neurons, we measured and the amount of histone 3 lysine 9/14 acetylation (H3K9/14ac) in SH-SY5Y neuroblastoma cells (Fig. 1*A*,*B*). SH-SY5Y cells were treated with cholesterol, ApoE3, ApoE4, ApoE3 + cholesterol (ApoE3+Chol), or ApoE4 + cholesterol (ApoE4+Chol). After 24 h, ApoE3+Chol treatment increased H3K9/14ac by 89%, whereas ApoE4+Chol reduced H3K9/14ac by 25% compared with cholesterol-treated SH-SY5Y cells ($F_{(5,12)} = 7.33$; ANOVA, $p < 0.0023$). Acetylated H3K9/14 in ApoE3+Chol-treated cells was 2.4-fold higher than in ApoE4+Chol-treated cells (*t* test, $p < 0.005$; Fig. 1*B*). Cholesterol, ApoE3, or ApoE4 alone had no effect on H3K9/14ac.

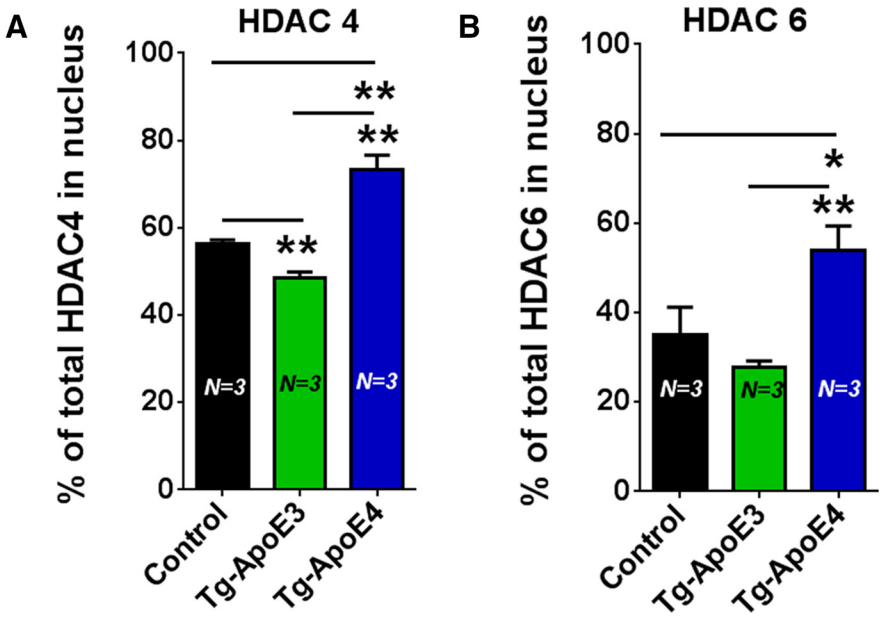


Figure 4. Differential HDAC nuclear localization in the hippocampus of transgenic mice expressing human ApoE3 or ApoE4. HDAC4 and HDAC6 levels in the cytosol and nucleus of hippocampal tissue from control, ApoE3 (Tg-ApoE3), and ApoE4 (Tg-ApoE4) transgenic mice were measured by immunoblot. ***A***, ***B***, HDAC translocation to the nucleus, measured by Western blotting, is reported as the percentage of total protein in the nuclear subcellular fraction [nucleus/(cytosol + nucleus)]. ApoE4 transgenic mice showed a significantly higher amount of nuclear HDAC4 ($F_{(2,8)} = 34.5$; ANOVA, $p < 0.001$) and HDAC6 ($F_{(2,8)} = 7.83$; ANOVA, $p < 0.03$) compared with ApoE3 transgenic mice. Data are reported as mean ± SEM of 3 independent experiments (Student's *t* test, $^*p < 0.05$ and $^{**}p < 0.005$, $n = 3$ animals for each group).

ApoE4 induces nuclear import of class II HDACs

To determine whether the effect of ApoE isoforms on histone 3 acetylation is related to modulation of HDAC localization, we measured the levels of HDACs in the cytosol and nucleus of SH-SY5Y cells treated with cholesterol, ApoE3, ApoE4, ApoE3+Chol, or ApoE4+Chol for 24 h. Cytosolic and nuclear fractions were prepared for immunoblot analysis of HDAC1, HDAC2, and HDAC3 (class I) and HDAC4, HDAC5, and HDAC6 (class II), the HDAC isoforms reported to be expressed in the brain (Simões-Pires et al., 2013). Consistent with previous reports, we found that the class I HDACs, HDAC1 (90%), HDAC2 (80%), and HDAC3 (50%), are primarily localized in the nucleus and showed no significant change in localization in response to ApoE3+Chol or ApoE4+Chol (data not shown). Among the class II HDACs (Fig. 2*A*), HDAC4 showed a significant increase in nuclear translocation ($F_{(2,8)} = 11.01$; ANOVA, $p < 0.01$) in ApoE4+Chol-treated cells (55.3 ± 1.4%) compared with ApoE3+Chol (32.4 ± 3.8%; $p < 0.005$) and cholesterol (45.3 ± 4.4%; $P < 0.05$)-treated cells (Fig. 2*B*). HDAC5 showed no significant change in localization with treatment (data not shown); however, ApoE4+Chol induced a 2-fold increase in nuclear import of HDAC6 (47.2 ± 5.6%, $F_{(2,8)} = 9.2$; ANOVA, $p < 0.01$) compared with ApoE3+Chol (23.8 ± 3.1%; $p < 0.01$) and cholesterol alone (29.6 ± 2.8%; Fig. 2*C*). There was no change in translocation of HDAC4 or HDAC6 by cholesterol, ApoE3, or ApoE4 alone (Fig. 2*A*).

We verified the ApoE4+Chol-induced HDAC6 and HDAC4 nuclear translocation in primary human neurons by confocal microscopy. Primary human neurons were treated with either cholesterol, ApoE3+Chol or ApoE4+Chol for 24 h. ApoE4+Chol increased the nuclear intensity of HDAC6 (155.8 ± 18.28, $n = 30$ cells) by 32%, whereas ApoE3+Chol reduced HDAC6 by 41% (69.22 ± 5.87, $n = 31$ cells) compared with cholesterol (117.4 ± 10.39, $n = 28$; Fig. 3*A*). In terms of percentage of total HDAC6 in the nucleus, ApoE3+Chol-treated neurons showed a lower nuclear HDAC6 percentage than ApoE4+Chol (37.7 ± 2.14%; $p < 0.005$, $n = 8$ experiments, 40 cells vs 64.7 ± 3.39%; $p < 0.001$, $n = 8$ experiments, 40 cells) compared with cells treated with cholesterol alone (50.4 ± 3.0%; $F_{(2,21)} = 21.6$; ANOVA, $p < 0.0001$, $n = 8$ experiments, 40 cells; Fig. 3*B*). Similar results were observed with HDAC4 (Cholesterol alone = 57.7 ± 2.8%; ApoE4+Chol = 69.2 ± 4.8%; ApoE3+Chol = 37.8 ± 3.1%, $F_{(2,21)} = 18.2$; ANOVA, $p < 0.0001$, $n = 8$ experiments, 40 cells; Fig. 3*C*,*D*).

In ApoE4 transgenic mice, nuclear HDAC4 in hippocampus was higher (73.3 ± 3.3% nuclear, $n = 3$) than in ApoE3 transgenic mice (48.5 ± 1.4%, $n = 3$; *t* test, $p < 0.003$) and control mice (56.6 ± 0.7%, $n = 3$; *t* test, $p < 0.004$; Fig. 4*A*). Nuclear localization of HDAC6 was also higher in ApoE4 transgenic mice (54.5 ± 5.4%) compared with ApoE3 transgenic mice (27.9 ± 1.3%; *t* test, $p = 0.005$) and control mice (34.9 ± 6.3%; *t* test, $p < 0.05$; Fig. 4*B*). Together, these results suggest that ApoE3 and ApoE4 have differential effects on nucleo-cytoplasmic shuttling of the class II HDACs, HDAC4 and HDAC6.

ApoE regulates HDAC through LRP-1

In the brain, ApoE produced by astrocytes transports cholesterol to neurons via ApoE receptors, which are members of the low-density lipoprotein receptor (LDLR) family. LDLR and LRP-1 are the main ApoE lipoprotein metabolic receptors in the brain (Koryakina et al., 2009; Holtzman et al., 2012; Liu et al., 2013), and we previously showed that ApoE3 protects against ASPD-induced synaptic damage through LRP-1 (Sen et al., 2012). To test the involvement of LRP-1 in ApoE-mediated HDAC nuclear translocation, we pretreated SH-SY5Y cells for 30 min with the ApoE receptor binding protein RAP (100 nM; Migliorini et al., 2003), then treated the cells with ApoE3+Chol or ApoE4+Chol for 24 h

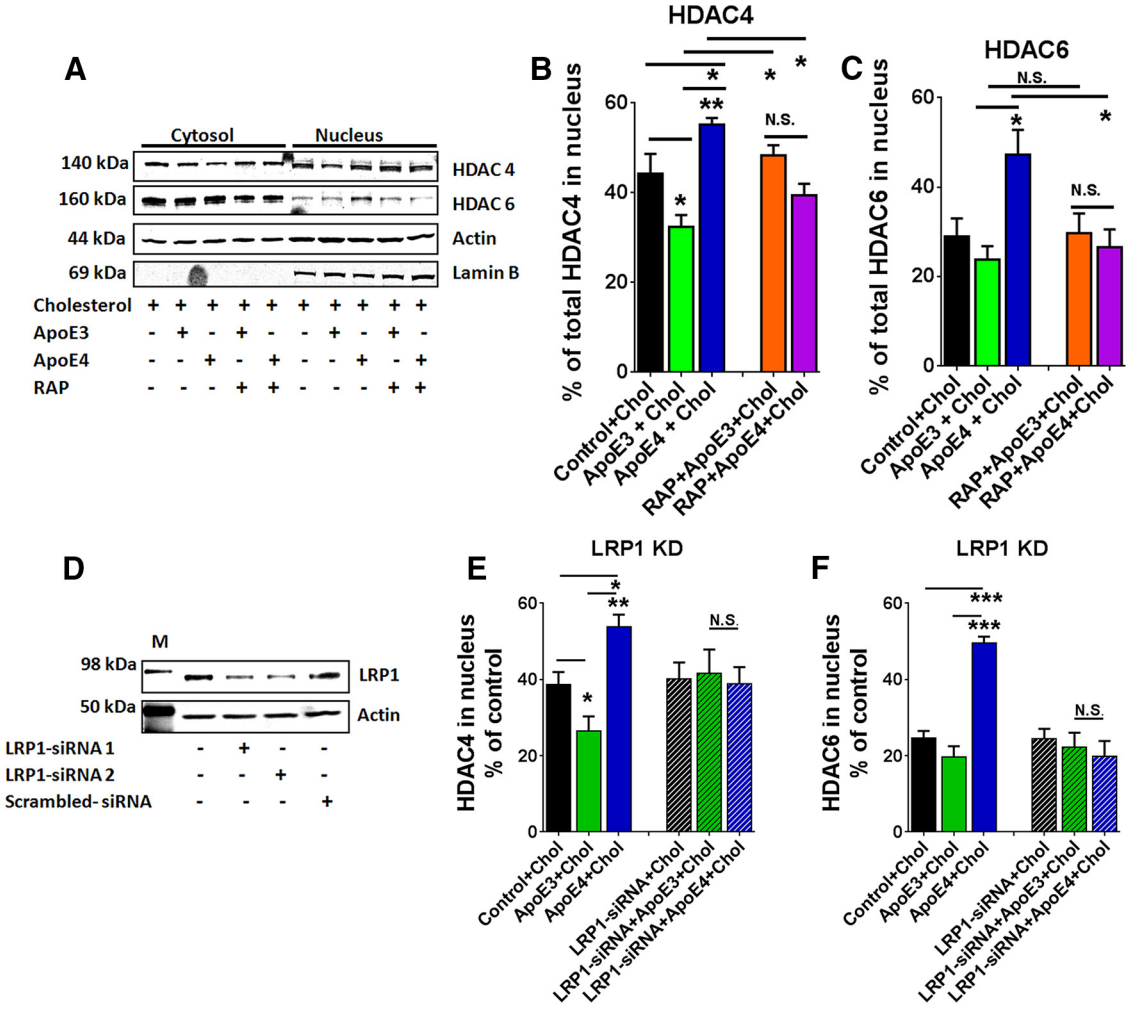


Figure 5. Blocking LRP-1 prevents ApoE-mediated nuclear translocation of HDAC4 and HDAC6. ***A***, HDAC4 and HDAC6 protein levels in the cytosol and nuclear fractions of SH-SY5Y cells treated with the indicated combinations of cholesterol (Chol; 100 μM), ApoE3 (20 nM), ApoE4 (20 nM), and RAP (100 nM) for 24 h. ***B***, ***C***, HDAC translocation reported as the percentage of total protein in the nucleus [nucleus/(cytosol + nucleus)]. HDAC4 nuclear translocation was significantly higher in ApoE4+Chol-treated cells compared with ApoE3+Chol-treated cells ($F_{(4,10)} = 9.5$; $p = 0.002$), but this difference was lost in cells pretreated with RAP. HDAC6 ($F_{(4,10)} = 4.6$; ANOVA, $p = 0.02$) nuclear protein levels were also not significantly different between ApoE3- and ApoE4-treated cells that were pretreated with RAP (ApoE4+Chol vs RAP+ApoE4+Chol, $p = 0.04$; ApoE4+Chol vs RAP+ApoE3+Chol, $p < 0.03$). ***D***, Immunoblot showing the protein levels of LRP-1 and β-actin in LRP-1-siRNA-transfected SH-SY5Y cells. ***E***, ***F***, ApoE3+Chol and ApoE4+Chol showed no differential nuclear translocation of HDAC4 (***E***) and HDAC6 (***F***) in LRP-1 (LRP-1 KD)-downregulated cells. Data are reported as mean ± SEM of 3 independent experiments (Student's *t* test, *$p < 0.05$ and **$p < 0.005$).

and measured the nuclear translocation of HDACs (Fig. 5*A*). Blocking LRP-1 with RAP abolished the effect of ApoE3+Chol and ApoE4+Chol on nuclear translocation of HDAC4 (Fig. 5*B*) and HDAC6 (Fig. 5*C*).

To further test whether LRP-1 regulates ApoE-mediated HDAC translocation, we decreased the LRP-1 levels in SH-SY5Y cells using LRP-1 siRNA. As shown in Figure 5*D*, LRP-1 siRNA 1 and 2 both decreased LRP-1 levels by ~80% in SH-SY5Y cells compared with control. ApoE4+Chol (38.8 ± 4.5%) and ApoE3+Chol (41.6 ± 6.3%) had no effect on HDAC4 translocation to the nucleus in LRP-1 downregulated cells (Fig. 5*E*) compared with cholesterol-treated control cells (40.2 ± 4.3%). LRP-1 downregulation also prevented the ApoE4+Chol-mediated nuclear translocation of HDAC6 (Fig. 5*F*), indicating that ApoE acts via LRP-1 receptors to modulate HDAC nucleo-cytoplasmic shuttling.

ApoE-mediated HDAC nucleo-cytoplasmic shuttling is regulated by PKCε

We previously showed that the neuroprotective and synaptogenic effects of ApoE3 are mediated by PKCε and LRP-1, and that ApoE3, but not ApoE4, induces PKCε transcription and increases PKCε levels in both control and ASPD-treated cells (Sen et al., 2012). To investigate whether PKCε regulates ApoE-mediated HDAC nuclear translocation, we determined the amount of PKCε, α, and δ mRNA in SH-SY5Y cells treated with cholesterol with or without ApoE3 or ApoE4. ApoE3+Chol induced the expression of PKCε mRNA by 2.5-fold compared with cholesterol-treated cells, whereas ApoE4+Chol reduced the PKCε mRNA level (Fig. 6*A*). ApoE3+Chol failed to increase the PKCε mRNA in LRP-1 downregulated cells (Fig. 6*B*). We found no change in the transcript levels of either PKCα or δ in ApoE3+Chol or ApoE4+Chol treated cells (Fig. 6*C*,*D*).

PKCε overexpression (Fig. 6*E*) reduced nuclear HDAC4 by 1.83-fold compared with control cells (40.9 ± 2.8% vs 22.3 ± 1.6%; *t* test, $p < 0.005$; Fig. 6*F*) and reduced nuclear HDAC6 by 54% compared with control cells (29.9 ± 1.4% vs 16.3 ± 3.2%; *t* test, $p < 0.005$; Fig. 6*G*). Conversely, PKCε knock-down had no effect on HDAC4 but increased HDAC6 levels in the nucleus by 1.4-fold compared with control cells (43.3 ± 3.8% vs 29.9 ±

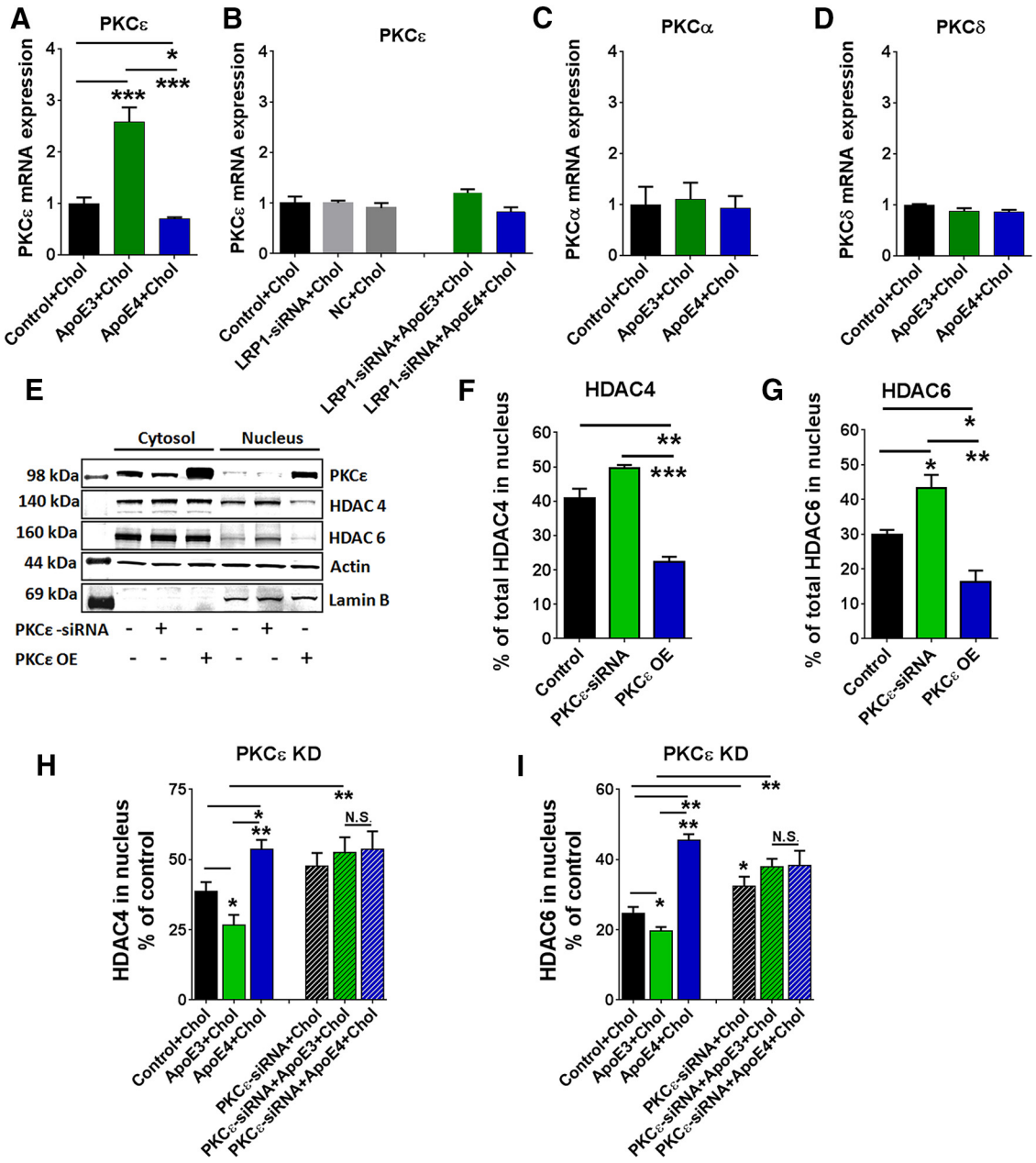


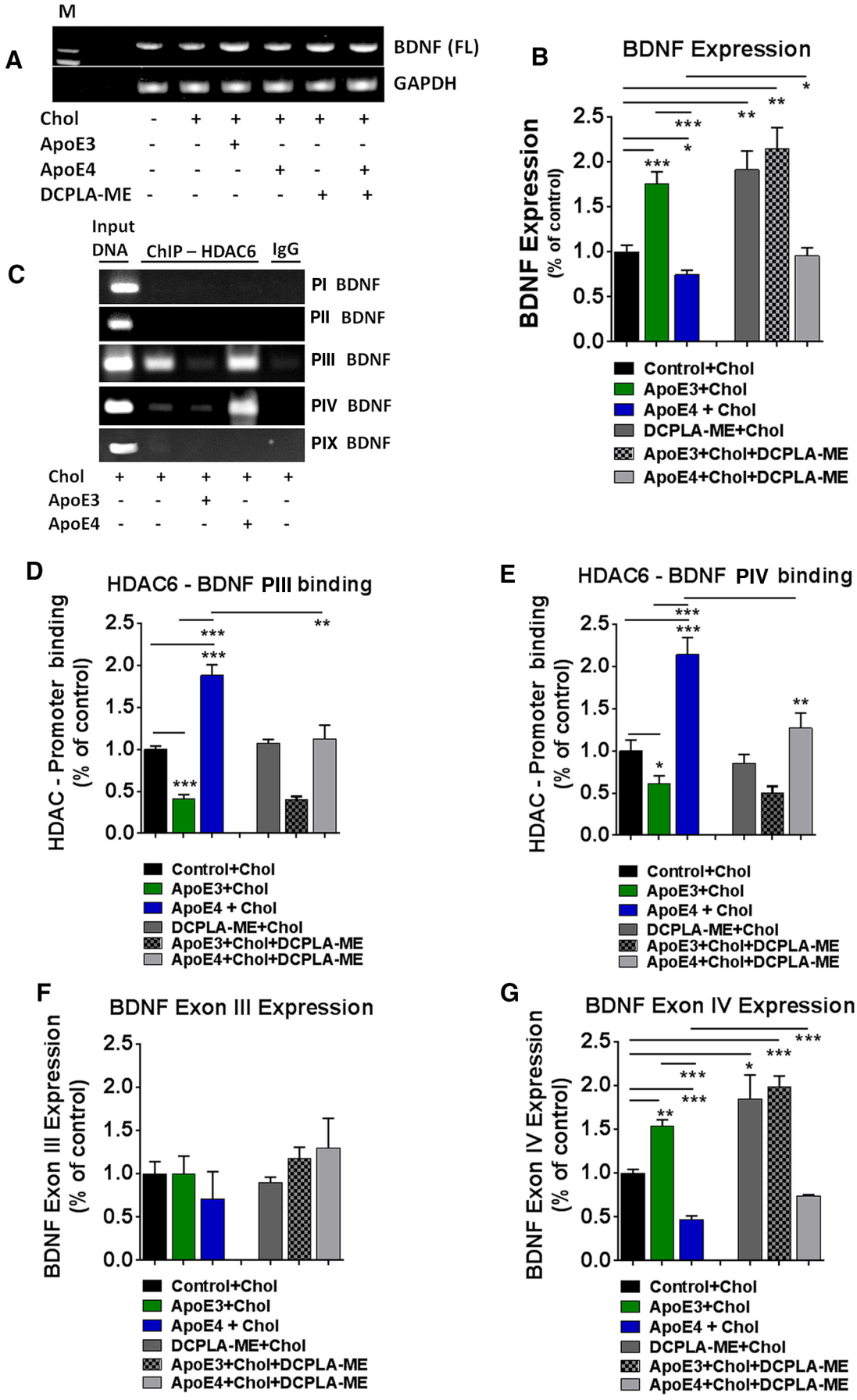
Figure 6. ApoE-induced HDAC translocation is regulated by PKCε. ***A***, ApoE3, but not ApoE4, induce PKCε mRNA levels ($F_{(2,15)} = 32.8$; $p < 0.0001$) as measured by qRT-PCR in SH-SY5Y cells treated with cholesterol (Chol; 100 μM) with or without ApoE3 (20 nM) or ApoE4 (20 nM) for 24 h. ***B***, Blocking LRP-1 blocked the ApoE3+Chol-mediated increase in PKCε expression. LRP-1 siRNA itself and the nonspecific scrambled-siRNA (NC) had no effects on PKCε expression. ***C***, ***D***, ApoE3+Chol- and ApoE4+Chol-treated cells showed no change in mRNA levels of PKCα (***C***) or PKCδ (***D***). ***E***, Immunoblot showing PKCε, HDAC4, HDAC6, β-actin, and lamin B expression in the cytosol and nucleus of SH-SY5Y cells transfected with empty vector (control), PKCε siRNA, or a PKCε expression vector (PKCε OE). ***F***, ***G***, PKCε overexpression prevents whereas PKCε knock-down induces HDAC4 ($F_{(2,8)} = 52.64$; ANOVA, $p < 0.0005$) and HDAC6 ($F_{(2,8)} = 20.8$; ANOVA, $p < 0.005$) translocation to the nucleus. ***H***, ***I***, HDAC4 (***H***) and (***I***) HDAC6 nuclear protein levels were not significantly different in ApoE3- and ApoE4-treated cells in which PKCε (PKCε KD) was downregulated. Data are mean ± SEM of 3 independent experiments (Student's *t* test, $^*p < 0.05$ and $^{**}p < 0.005$).

1.4%; *t* test, $p < 0.05$; Fig. 6*G*). These data indicate that PKCε is involved in the retention of HDAC4 and required for retention of HDAC6 in the cytosol.

Next we measured the percentage of nuclear HDAC4 and HDAC6 SH-SY5Y cells transfected with PKCε-siRNA. PKCε knock-down abolished the HDAC4 nuclear export by ApoE3+Chol (52.6 ± 5.4%; *t* test, $p < 0.01$ vs 26.6 ± 3.8% in normal cells; Fig. 6*H*). PKCε knock-down also abolished the effect of ApoE3+Chol on HDAC6 in the nucleus (37.9 ± 2.1%; *t* test, $p < 0.002$ vs 19.7 ± 1.1% in normal cells; Fig. 6*I*).

ApoE4 downregulates BDNF expression

Recent studies have shown additive effects of ApoE and BDNF in memory-related disorders (Kauppi et al., 2014; Lim et al., 2014). ApoE4 carriers have reduced BDNF levels (Maioli et al., 2012; Alvarez et al., 2014). We have found that ApoE3 induces PKCε expression in rat primary neurons (Sen et al., 2012) and in human SH-SY5Y cells, as described above. PKCε is known to regulate BDNF expression (Hongpaisan et al., 2011; Lim and Alkon, 2012; Hongpaisan et al., 2013; Neumann et al., 2015). To delineate the link between ApoE isoforms, PKCε, HDACs


A
M
BDNF (FL)
GAPDH
Chol - + + + + +
ApoE3 - - + - - -
ApoE4 - - - + - +
DCPLA-ME - - - - + +
B
BDNF Expression
BDNF Expression (% of control)
Control+Chol
ApoE3+Chol
ApoE4 + Chol
DCPLA-ME+Chol
ApoE3+Chol+DCPLA-ME
ApoE4+Chol+DCPLA-ME
C
Input DNA
ChIP – HDAC6
IgG
PI BDNF
PII BDNF
PIII BDNF
PIV BDNF
PIX BDNF
Chol + + + + +
ApoE3 - - + - -
ApoE4 - - - + -
D
HDAC6 - BDNF PIII binding
HDAC - Promoter binding (% of control)
E
HDAC6 - BDNF PIV binding
HDAC - Promoter binding (% of control)
F
BDNF Exon III Expression
BDNF Exon III Expression (% of control)
G
BDNF Exon IV Expression
BDNF Exon IV Expression (% of control)

and BDNF, we measured the mRNA expression of BDNF by semiquantitative RT-PCR (full-length; Fig. 7*A*) and qRT-PCR (Fig. 7*B*) using non-full-length primers in SH-SY5Y cells. Cholesterol-only-treated cells showed no change in BDNF expression (Fig. 7*A*). ApoE3+Chol increased BDNF expression (1.76 ± 0.13; *t* test, $p < 0.001$) and ApoE4+Chol downregulated BDNF expression (0.74 ± 0.05; $p < 0.02$; Fig. 7*B*). DCPLA-ME, a PKCε specific activator (Nelson et al., 2009; Sen et al., 2012), upregulated BDNF expression in cholesterol-only treated cells (1.91 ± 0.21-fold). DCPLA-ME prevented BDNF downregulation in ApoE4+Chol+DCPLA-ME (0.95 ± 0.1; $p < 0.05$) treated cells. These results indicate that PKCε activator induces BDNF expression to the same extent as ApoE3 and reverses the effect of ApoE4 on BDNF. The effects of ApoE3 and DCPLA-ME were not additive.

ApoE4 induces HDAC6–BDNF PIII/PIV association

Next we investigated the role of BDNF promoters in regulation of BDNF expression by ApoE. Chromosome immunoprecipitation was performed using HDAC4, HDAC6 or IgG (as a control) from SH-SY5Y cells treated with cholesterol, ApoE isoforms, and DCPLA-ME. A fixed fraction (2%) of the total unprecipitated DNA (used a positive control, used to normalize the results) or 100% of the HDAC-or IgG-immunoprecipitated DNA were amplified by PCR against BDNF promoters PI, PII, PIII, PIV and PIX. HDAC4 and HDAC6 (Fig. 7*C*) showed no association to PI, PII or PIX. ApoE3+Chol reduced HDAC6-PIII association (0.41 ± 0.05; $p < 0.001$) and ApoE4+Chol increased it (1.9 ± 0.13; $p < 0.001$) compared with cholesterol-only treated cells (1.0 ± 0.04; $F_{(5,24)} = 51.8$; ANOVA, $p < 0.0001$; Fig. 7*D*). ApoE4+Chol also increased HDAC6-PIV association and ApoE3+Chol reduced it. (Cholesterol-only = 1 ± 0.13; ApoE3+Chol = 0.61 ± 0.10, $p < 0.05$; ApoE4+Chol = 2.1 ± 0.20; $p < 0.001$; $F_{(5,30)} = 18.6$; ANOVA, $p < 0.0001$; Fig. 7*E*). DCPLA-ME blocked the ApoE4+Chol induced increase in HDAC6-PIII and HDAC6-PIV association, but had no effect on ApoE3 (Fig. 7*D*,*E*). This is consistent with the idea that ApoE4 either directly or indirectly inhibits PKCε synthesis, and PKCε inhibits HDAC6 transport to the nucleus where it can bind the promoter.

HDAC4 also coimmunoprecipitated with BDNF PIII and PIV, but no significant difference was noticed in binding among treated groups (HDAC4-PIII = $F_{(5,24)} = 1.1$; ANOVA, $p = 0.4$; HDAC4-PIV = $F_{(5,24)} = 0.98$; ANOVA, $p = 0.46$; results not shown). These results suggest that PKCε activity blocks HDAC4 transport to the nucleus, thereby preventing HDAC4 from binding to BDNF promoters III and IV, whereas HDAC4 does not bind the promoters directly, but binds indirectly via transcription factors such as MEF2C and MEF2D. We conclude that ApoE4 does not induce PKCε, thereby allowing HDAC to enter the nucleus, bind (indirectly) to the BDNF promoter, and thereby repress BDNF expression.

To determine the effect of HDAC6–PIII/PIV association on BDNF expression, we analyzed the expression of BDNF–exon III and IV by qRT-PCR. BDNF–exon IV expression was increased by ApoE3+Chol and decreased by ApoE4+Chol (1.54 ± 0.07-fold; $p < 0.0027$, and 0.47 ± 0.04-fold; $p < 0.0005$, respectively; Fig. 7*G*). BDNF–exon III expression showed a trend toward lower expression when ApoE4 was added, but it was not statistically significant (Fig. 7*F*). DCPLA-ME increased expression of exon IV by approximately the same percentage in all three treatments ($F_{(5,21)} = 40.0$; ANOVA, $p < 0.0001$; DCPLA-ME+Chol = 1.85 ± 0.27-fold, $p < 0.035$; ApoE3+Chol+DCPLA-ME = 1.98 ± 0.13, $p < 0.05$ vs ApoE3+Chol; ApoE4+Chol+DCPLA-ME = 0.74 ± 0.01, $p < 0.0005$ vs ApoE4+Chol; Fig. 7*G*). These results indicate that ApoE4 induces an interaction between HDAC6 and BDNF–PIV that leads to reduced BDNF expression. PKC activators increased BDNF exon IV expression but had little effect on exon III expression, indicating that exon IV is responsive to PKCε but exon III is not.

ApoE4 increases ASPD-induced nuclear translocation of HDACs

We used immunoblots to examine the effect of ASPDs, a neurotoxic form of Aβ present in the AD brain, on HDAC4 and HDAC6 nuclear import. Human SH-SY5Y cells were treated with cholesterol and ApoE3 or ApoE4 in the presence or absence of ASPDs. ASPDs increased the nuclear import of both HDAC4 and HDAC6. Addition of ApoE3+Chol significantly reduced the percentage of HDAC4 and HDAC6 staining in the nucleus (−48.4 ± 9.7%; $p = 0.026$ and −29.3 ± 6.9%; $p = 0.01$; Fig. 8*A*). In contrast, ApoE4+Chol had no effect on the nuclear import of HDAC4 (+5.7 ± 13% change; Fig. 8*B*) and increased the import of HDAC6 (+39.4 ± 16.9% change, $p < 0.04$) in the presence of ASPD (Fig. 8*C*). Cells treated with both ApoE3 and ApoE4 (10 nM) and cholesterol showed intermediate levels of nuclear HDAC4 and HDAC6 (HDAC4 = $F_{(2,8)} = 14.2$; ANOVA, $p < 0.005$ and HDAC6 = $F_{(2,8)} = 7.15$; ANOVA; $p < 0.03$; Fig. 8*B*,*C*). These results show that ApoE3 inhibits the effect of ASPDs on nuclear translocation of HDACs, but ApoE4 does not. We also analyzed the amount of nuclear HDAC6 from human brain hippocampus. Autopsy-confirmed AD cases showed increased nuclear HDAC6 compared with age-matched controls (AD = 70.4 ± 5.3% and non-AD = 56.9 ± 2.9; $p < 0.05$; Fig. 8*D*).

PKCε activation inhibits ApoE4-induced nuclear translocation of HDACs

To investigate the effect of PKCε activation on ApoE4+ASPD-induced nuclear translocation of HDAC4 and HDAC6, we treated SH-SY5Y cells for 24 h with combinations of ASPDs, cholesterol, and ApoE4 and measured HDAC4 and HDAC6 levels in the cytosol and nucleus by immunoblotting. ASPD and ApoE4 had a synergistic effect on HDAC6 (Chol = 12.9 ± 3.0%; ASPD+APOE4+Chol = 39.7 ± 3.3%, $p < 0.005$; ASPD+Chol = 21.7 ± 2.8%, $p < 0.05$), but not HDAC4 (Fig. 8*E*,*F*). PKCε activation by DCPLA-ME or bryostatin 1 reduced the nuclear import of HDAC4 by 35 ± 6.9% ($p = 0.015$) and 40 ± 8.4% ($p = 0.013$), respectively ($F_{(6,14)} = 11.6$; ANOVA, $p < 0.0001$; Fig. 8*E*). Similar reductions in nuclear HDAC6 also occurred with DCPLA-ME (56 ± 5.9% reduction, $p < 0.005$) or bryostatin-1 (46 ± 9.2% reduction, $p < 0.05$; Fig. 8*F*). In each

←

Figure 7. ApoE4 induces HDAC6–PIII and HDAC6–PIV association. ***A***, Agarose gel image showing full-length BDNF and GAPDH expression from untreated, cholesterol (Chol; 100 μM) with or without ApoE3 (20 nM) or ApoE4 (20 nM) for 24 h. ***B***, qRT-PCR analysis of BDNF expression. ApoE4+Chol downregulated BDNF, whereas ApoE3+Chol and DCPLA-ME upregulated BDNF expression ($F_{(4,25)} = 18.7$; ANOVA, $p < 0.0001$). ***C***, Agarose gel image of HDAC6–BDNF promoter binding. The number of cycles in this image is greater than in the qRT-PCR experiments, so this image cannot be compared with the bar graphs below. ***D***, ApoE4+Chol increases HDAC6–PIII binding. ***E***, ApoE4 increases HDAC6–PIV binding. ***F***, Effects of ApoE3 and ApoE4 BDNF exon III expression. ***G***, Effects of ApoE3 and ApoE4 on BDNF exon IV expression. ApoE4+Chol downregulate exon IV and DCPLA-ME blocks the decrease. Data are reported as mean ± SEM of 3 independent experiments (Student's *t* test, *$p < 0.05$; **$p < 0.005$; and ***$p < 0.0005$).

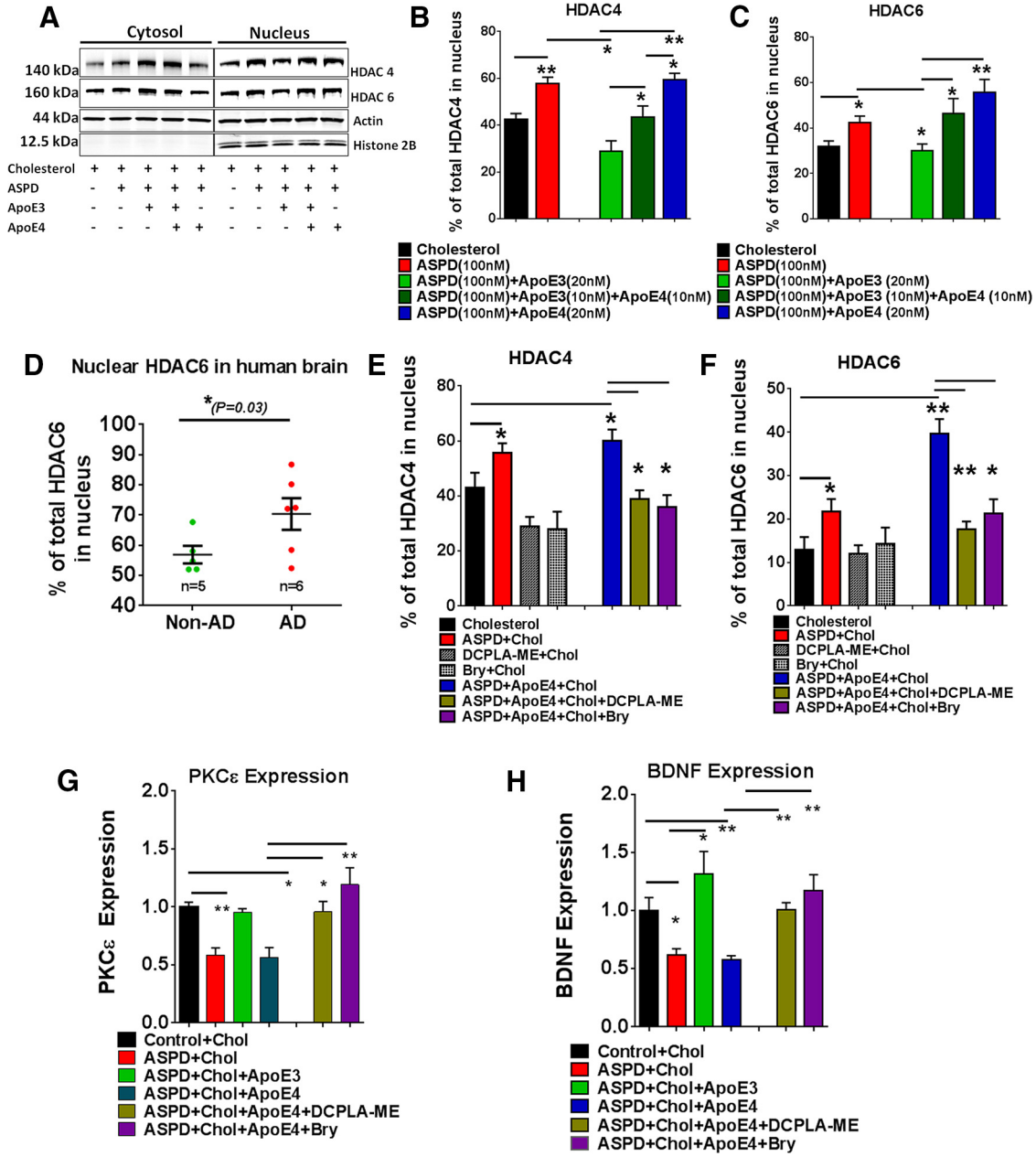


Figure 8. ApoE3 prevents nuclear import of HDAC4 and HDAC6 in ASPD-treated neurons. SH-SY5Y cells and primary human neurons were treated with cholesterol (Chol;100 μM), ASPD (100 nM) + Chol (100 μM), ASPD (100 nM) + ApoE3 (20 nM) + Chol (100 μM), ASPD (100 nM) + ApoE3 (10 nM) + ApoE4 (10 nM) + Chol (100 μM), and ASPD (100 nM) + ApoE4 (20 nM) + Chol (100 μM) for 24 h. ***A***, Immunoblot of HDAC4, HDAC6, β-actin, and histone 2B protein in the cytosol and nucleus of treated SH-SY5Y cells. HDAC translocation to the nucleus is reported as the percentage of total protein in the nucleus [nucleus/(cytosol + nucleus)]. ***B***, Nuclear HDAC4 was significantly reduced in cells treated with ApoE3+Chol+ASPD compared with either ASPD alone or ApoE4+Chol+ASPD. ***C***, Nuclear HDAC6 was also lower in cells treated with ApoE3+Chol+ASPD compared with cells treated with either ASPD alone or ApoE4+Chol+ASPD. Cells treated with both ApoE3 and ApoE4 showed an intermediate level of nuclear HDAC4 and HDAC6. ***D***, Percentage of nuclear HDAC6 in the hippocampus of autopsy-confirmed human AD and age-matched controls. AD cases ($n = 6$) showed higher nuclear HDAC6 than non-AD cases ($n = 5$). ***E***, PKCε activation significantly reduces the nuclear import of HDAC4. SH-SY5Y cells were treated with the indicated combinations of cholesterol (Chol; 100 μM), ASPD (100 nM), ApoE4 (20 nM), DCPLA-ME (100 nM), and bryostatin 1 (0.27 nM) for 24 h. ***F***, HDAC6 in SH-SY5Y cells treated with ASPD and ApoE4. ***G***, ApoE3+Chol (but not ApoE4+Chol) prevents ASPD+Chol-induced loss of PKCε ($F_{(5,12)} = 8.73$; ANOVA, $p < 0.002$). ***G***, Effect of ASPD and ApoE on BDNF expression ($F_{(5,12)} = 9.7$; ANOVA, $p < 0.001$). Data are reported as mean ± SEM of three independent experiments (Student's *t* test, $^*p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$).

case, regardless of whether ASPDs, ApoE4, or a combination of both was used, PKC activation reduced HDAC import to normal or below normal levels.

Finally, we measured the levels of PKCε and BDNF mRNA expression (using qRT-PCR) in these ASPD-treated cells. PKCε levels were downregulated by ASPD+Chol (0.58 ± 0.06-fold; $p < 0.005$). ApoE3 restored PKCε expression to normal, but ApoE4 did not (0.56 ± 0.09-fold; $p < 0.02$). DCPLA-ME and bryostatin-1 counteracted the ASPD+ApoE4-mediated loss of PKCε (0.95 ± 0.09-fold and 1.19 ± 0.14%, respectively; Fig. 8*G*).

BDNF was also downregulated by ASPD+Chol (0.62 ± 0.05-fold; $p < 0.04$; Fig. 8*H*). The addition of ApoE4 did not increase the effect of ASPD. ApoE3 prevented BDNF downregulation by ASPDs (1.32 ± 0.19-fold; $p < 0.025$ vs ASPD+Chol). PKCε activation also prevented BDNF loss in these cells (Fig. 8*H*). These results suggest that PKCε activa-

tion reverses the ApoE4-mediated nuclear translocation of HDAC, thereby restoring BDNF synthesis to normal levels.

Discussion

Histone acetylation is associated with active transcription of genes implicated in synaptic plasticity and learning behavior (Levenson et al., 2004; Fischer et al., 2007). Previous work using whole-transcriptome gene expression analysis showed that ApoE4 carrier status is associated with a consistent transcriptomic shift that resembles the LOAD profile (Rhinn et al., 2013). We previously reported that PKCε and synaptic proteins including PSD-95 and synaptophysin are differentially regulated by ApoE3 and ApoE4 (Sen et al., 2012). We found that ApoE4 inhibits histone H3K9 and H3K14 acetylation, whereas ApoE3 induces it. We also found that ApoE4 increased the nuclear import of HDAC4 and HDAC6, which leads to histone deacetylation and epigenetic changes in BDNF gene transcription, and that PKCε reverses the ApoE effect. This is a novel mechanism by which ApoE3 and ApoE4 can regulate HDAC nuclear translocation differentially. If these epigenetic changes in BDNF gene transcription occur in the human brain, they could predispose ApoE4 carriers toward LOAD.

Our data show that ApoE4 increases the nuclear abundance of class IIa HDAC4 and class IIb HDAC6 in primary human neurons and SH-SY5Y cells. We found no difference in HDAC translocation between untreated SH-SY5Y cells and cholesterol-only-treated cells, further confirming that the ApoE-isoform-specific differential regulation of HDAC is not effective in the absence of cholesterol. This is consistent with previous reports showing that the effects of ApoE isoforms are determined by their lipidation state (Strittmatter et al., 1993a; Strittmatter et al., 1993b; LaDu et al., 1994; Tokuda et al., 2000; Sen et al., 2012). We found no significant change in HDAC1, HDAC2, HDAC3, or HDAC5 localization, indicating a signal-specific regulation of HDACs by ApoE.

ApoE4 is the strongest genetic risk factor for and also lowers the age of LOAD (Chartier-Harlin, 1994; Houlden, 1998) compared with the common ApoE3 and the rare ApoE2. ApoE2 has very low binding affinity to LDLR (1–2% that of ApoE3) (Simmons et al., 1997; Dong et al., 1998) and lower affinity than ApoE3 for LRP1 (40% of ApoE3 or ApoE4) (Weisgraber et al., 1982; Kowal et al., 1990). We also found that blocking LRP-1 abolishes ApoE regulation of HDAC nucleocytoplasmic shuttling, suggesting that ApoE's effect is mediated by LRP-1. Therefore, it could be expected that ApoE2 would have less effect on HDAC translocation than ApoE3. A low affinity of LRP-1 for ApoE2 may explain its protective effects in AD (Harris-White and Frautschy, 2005). Transgenic mice containing human ApoE4 are known to have reduced LRP-1 and defective PKC signaling (Yong et al., 2014).

Our earlier studies showed that PKCε in ApoE3 mice is activated constitutively compared with ApoE4 mice. We also showed that, in rat primary neurons, ApoE3 induced PKCε, whereas ApoE4 did not (Sen et al., 2012). PKC activation increases the acetylation of histone H3, but not histone H4 (Levenson et al., 2006). Therefore, ApoE3-mediated increases in PKCε may induce positive gene regulation by restricting HDACs from nuclear import, leading to histone acetylation, whereas negative gene regulation by ApoE4 promotes histone deacetylation through HDAC translocation to the nucleus (Fig. 9). Our experiments also indicate that PKCε is required for retaining HDAC4 and HDAC6 in the cytosol, opposing the effect of ApoE4. We also found that PKCε reduces the expression of HDAC6. This is consistent with previous findings showing that HDAC6 is increased by 91% in AD hippocampus (Simões-Pires et al., 2013) and PKCε is lower in the AD brain (Matsushima et al., 1996; Khan et al., 2015) compared with the non-AD brain. Our findings are also consistent with previous work indicating that HDAC activity is regulated by phosphorylation (Vega et al., 2004; Zhang et al., 2005; Yang and Seto, 2008). However, it remains to be established whether PKCε phosphorylates HDAC4 or HDAC6 directly.

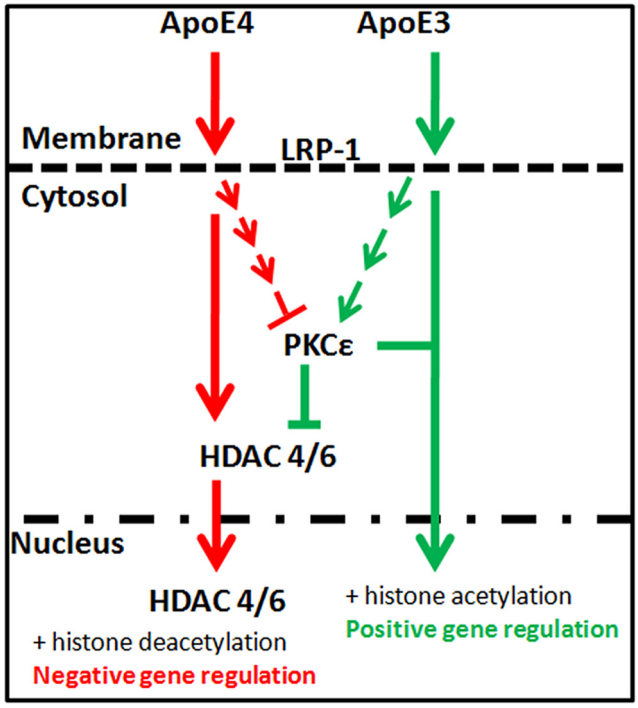


Figure 9. ApoE-isoform-mediated regulation of gene expression.

BDNF is the most abundant neurotrophin in the brain and is an important player in synaptic plasticity and cognitive function. Both PKCε (Hongpaisan et al., 2011; Lim and Alkon, 2012; Hongpaisan et al., 2013) and ApoE (Maioli et al., 2012; Alvarez et al., 2014; Neumann et al., 2015) are known to be associated with the expression of BDNF. Here, we found that ApoE4 reduces BDNF expression and therefore may interfere with the synaptic repair or maturation. ApoE4 also increases HDAC6–PIII/PIV binding and reduces BDNF exon IV expression. HDAC4 and HDAC5 repress BDNF PIV activity and class II HDAC inhibitors induce BDNF exon IV expression (Koppel and Timmusk, 2013). We also found that ApoE4 promotes the association of HDAC4 and HDAC6 with BDNF PIV, confirming these previous findings. Exon IV and VI overexpression increases the number of secondary dendrites and regulate synaptic plasticity in adulthood (Baj et al., 2011; Baj et al., 2012). Loss of BDNF–PIV-driven expression causes a deficit in functioning of GABAergic interneurons in hippocampus and frontal cortex (Sakata et al., 2009; Sakata and Duke, 2014). BDNF exons I, II, and IV are downregulated in the AD brain (Allen et al., 2011). Despite resistance of GABAergic system to neurodegeneration, GABAergic currents show an age-dependent reduction in AD brain (Rissman et al., 2007; Rissman and Mobley, 2011; Limon et al., 2012). GABAergic interneurons are dysfunctional and impair hippocampal neurogenesis in adult ApoE4 knock-in mice (Li et al., 2009). Alternative polyadenylation sites result in long 3′UTR BDNF transcripts localized to dendrites and short 3′UTR transcripts localized to the cell soma (An et al., 2008). These different forms may have different func-

tions. Therefore, ApoE4-mediated loss in BDNF exon IV could contribute to lower neurotrophin levels in proximal synapses, causing dysfunction of GABAergic inhibitory synapses in AD (Megías et al., 2001).

Considerable evidence suggests that a decrease in BDNF is associated with AD. BDNF expression is greatly decreased in the hippocampus and temporal and frontal cortex of AD patients (Siegel and Chauhan, 2000). BDNF mRNA is decreased in parietal cortex and hippocampus by 3- to 4-fold in AD autopsy samples (Holsinger et al., 2000); both precursor and mature BDNF are decreased dramatically in preclinical stages of AD and correlate with behavioral scores (Peng et al., 2005). The decreases in BDNF noted in these studies are comparable to those observed here (Fig. 8*F*). Our results shed new light on the mechanisms by which ApoE3 phenotype, by upregulating BDNF, may protect against AD.

Further, we found that ASPDs, the most toxic form of β-amyloid oligomers (Sen et al., 2012), increase nuclear transport of HDAC4 and HDAC6. ApoE3 counteracts this effect, but ApoE4 does not. We also found that, in human AD hippocampus, HDAC6 is significantly higher in the nuclear fraction than non-AD cases. Onset of LOAD occurs in dose-dependent manner, starting at 78.4 years in patients without the *ApoE* ε4 allele, 75.3 years in carriers of one *ApoE* ε4 allele, and 72.9 years in carriers of two *ApoE* ε4 alleles (Sando et al., 2008). Future studies will determine whether the genes affected by ApoE3 and ApoE4 are essential for synaptic repair, Aβ clearance, and degradation. Future studies will also be required to identify the molecules involved in nuclear import and signaling.

Finally, we found that the PKCε activators bryostatin-1 and DCPLA-ME prevent the nuclear import of HDACs by ApoE4 and ASPD and also prevent the ApoE4+ASPD-induced BDNF loss. Therefore, it is possible that PKCε activators may prevent or reverse synaptic loss by opposing the effects of ApoE4 in AD by derepressing gene expression essential for neuroprotection. PKC activators have been proposed as therapeutic agents for memory impairment and neurodegenerative diseases such as AD and PKC is involved in several aspects of AD pathobiology (Nelson and Alkon, 2009).

This study expands our understanding of the mechanism by which ApoE4 influences epigenetic modifications of gene expression leading to synaptic loss, which is the hallmark of Alzheimer's disease. These studies also add to our understanding of the observed neuroprotective effects of ApoE3 and the role of PKCε and may explain the therapeutic effects of HDAC inhibitors and PKC activators in neurodegenerative disease models.

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