JEM Journal of Experimental Medicine

ARTICLE

Inhibition of PKCδ reduces amyloid-β levels and reverses Alzheimer disease phenotypes

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β-amyloid protein (Aβ) plays a central role in the pathogenesis of Alzheimer disease (AD). Aβ is generated from sequential cleavage of amyloid precursor protein (APP) by β-site APP-cleaving enzyme 1 (BACE1) and the γ-secretase complex. Although activation of some protein kinase C (PKC) isoforms such as PKCα and ε has been shown to regulate nonamyloidogenic pathways and Aβ degradation, it is unclear whether other PKC isoforms are involved in APP processing/AD pathogenesis. In this study, we report that increased PKCδ levels correlate with BACE1 expression in the AD brain. PKCδ knockdown reduces BACE1 expression, BACE1-mediated APP processing, and Aβ production. Conversely, overexpression of PKCδ increases BACE1 expression and Aβ generation. Importantly, inhibition of PKCδ by rottlerin markedly reduces BACE1 expression, Aβ levels, and neuritic plaque formation and rescues cognitive deficits in an APP Swedish mutations K594N/M595L/presenilin-1 with an exon 9 deletion–transgenic AD mouse model. Our study indicates that PKCδ plays an important role in aggravating AD pathogenesis, and PKCδ may be a potential target in AD therapeutics.

Introduction

Alzheimer disease (AD) is the most common neurodegenerative disorder leading to dementia in the elderly worldwide. Pathologically, AD is characterized by neuritic plaques, neurofibrillary tangles, and neuronal loss (Hardy and Selkoe, 2002; Wu et al., 2017). Although the exact etiology of AD has not been fully understood so far, cumulative evidence has demonstrated that β-amyloid (Aβ) peptides play a key role in AD pathogenesis and are generated by sequential endoproteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase enzymes (Xu et al., 1995; Kazim and Iqbal, 2016). The β-site APP-cleaving enzyme 1 (BACE1) is the primary β-secretase in vivo, and BACE1-mediated APP cleavage is an essential rate-limiting step in Aβ generation (Sinha et al., 1999; Yan et al., 1999; Hussain et al., 2000; Querfurth and LaFerla, 2010; Chami and Checler, 2012; Harwell and Coleman, 2016). BACE1 cleaves APP at β-sites to generate a carboxyl-terminal fragment of APP cleaved at the β-site (C99), which is subsequently cleaved by γ-secretase within the transmembrane domain to release Aβ and APP C-terminal fragments (CTFs; Vassar et al., 1999). Suppression of BACE1 by RNA interference or selective BACE1 inhibitors decreases APP β cleavage and Aβ production in APP-transgenic mice, suggesting that even a partial reduction in BACE1 can have dramatically beneficial effects on AD pathology (Kao et al., 2004; Hussain et al., 2007; McConlogue et al., 2007; Neumann et al., 2015; Pigoni et al., 2016).

BACE1 is a type-1 transmembrane aspartyl-protease, which is mainly expressed in neurons and astrocytes (Yan et al., 2001). The expression of BACE1 is tightly regulated transcriptionally and translationally; transcription factors such as NF-κB have been shown to bind to the BACE1 promoter region and consequently regulate its expression. Interestingly, NF-κB mediates BACE1 expression, and increased levels of NF-κB and BACE1 have been identified in AD patients. Thus, increased BACE1 expression through the NF-κB signaling pathway could be a potential pathogenic mechanism underlying AD onset (De Pietri Tonelli et al., 2004; Lammich et al., 2004; Zhang et al., 2007b; Chen et al., 2012).

Protein kinase C (PKC) is a phospholipid-dependent family of Serine/Threonine protein kinases that comprise an extensive signaling net in the brain. Molecular cloning studies have revealed 12 PKC isozymes, which are divided into three subgroups: (1) classical PKCs, (2) novel PKCs, and (3) atypical PKCs. PKC isoforms play a key role in various cognitive functions including learning and memory. Studies so far have revealed that PKC isoforms such as PKCα and -ε signaling pathways closely correlate with pathological damage in AD, and activation of these PKC isoforms can ameliorate Aβ production and associated

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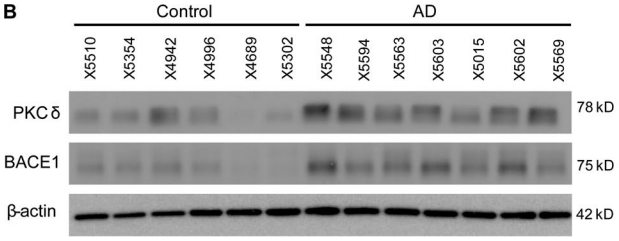
A

Control				AD			
Case	Age/Gender	Case	Age/Gender	Case	Age/Gender	Case	Age/Gender
X5510	92/M	X5783	84/M	X5548	80/M	X5680	84/M
X5354	93/M	X5709	94/M	X5594	83/M	X5789	89/M
X4942	83/M	X5114	87/M	X5563	87/M	X5788	92/M
X4996	91/M	X4954	76/M	X5603	90/M	X5697	88/M
X4689	79/F	X5248	93/F	X5015	77/F	X5569	87/F
X5302	83/F	X5628	80/F	X5602	88/F	X5685	88/F



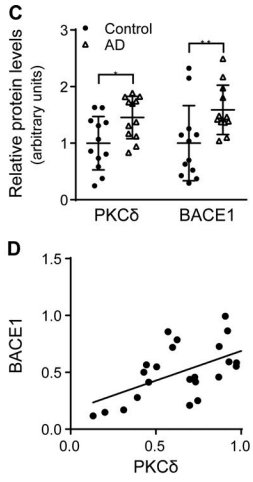


Figure 1. **Expression of PKCδ and BACE1 is elevated in AD patient brains. (A)** Patient information for AD control brain tissues used in this study. **(B)** Representative PKCδ and BACE1 immunoblots from AD and control patient brains. **(C)** Densitometric quantification of protein band intensities indicates that both PKCδ and BACE1 expression are significantly increased in AD brain compared with controls. *, $P < 0.05$; **, $P < 0.01$. Data are means ± SD. **(D)** PKCδ levels positively correlate with BACE1. $R^2 = 0.3089$, $P = 0.0048$, $n = 24$.

dementia in AD double-transgenic mice by enhancing APP α-processing pathways and Aβ degradation (Choi et al., 2006; Khan et al., 2009; Nelson and Alkon, 2009; Nelson et al., 2009). However, PKCδ as a novel PKC may be an exception as curcumin-induced PKCδ degradation was observed to enhance spatial learning in adult and aged rats (Conboy et al., 2009). Further, increased PKCδ expression in brain ischemia leads to delayed neuronal damage in the penumbral area, and selective PKCδ inhibition decreases infarct size and reduces cellular injury in vivo (Phan et al., 2002; Bright et al., 2004; Dave et al., 2011). In Parkinson's disease (PD), blocking proteolytic activation of PKCδ through overexpression of a dominant-negative PKCδ mutant, inhibition of PKCδ by siRNA transfection, or treatment with the PKCδ inhibitor rottlerin prevents MPP^+-induced dopaminergic cell death in primary mesencephalic culture models and MPTP animal models (Yang et al., 2004; Kaul et al., 2005; Zhang et al., 2007a). Together, these studies indicate that inhibition of PKCδ plays an important protective role in brain aging, ischemia, and neurodegenerative disease.

PKCδ has been reported to regulate NF-κB transcription through phosphorylation of the NF-κB inhibitor IκBα (Ser32 and Ser36) and p65 (Ser536) to promote NF-κB–dependent gene expression (Vancurova et al., 2001; Storz et al., 2004; Goon Goh et al., 2008; Lian et al., 2012; Ren et al., 2014; Lee et al., 2016). Recently, PKCδ overexpression was shown to effectively increase LPS-induced expression of inducible nitric oxide synthase and cytokines such as IL-1β, IL-4, and IFN-γ in an NF-κB–dependent manner, whereas the PKCδ inhibitor rottlerin reduced these effects (Burguillos et al., 2011). Moreover, LPS-induced inflammation has also been shown to increase BACE1 expression in the brain by modulating the NF-κB phosphoregulatory pathway, thereby promoting the release of the inhibitory IκBα component from NF-κB and consequently enhancing p65 nuclear translocation (Wang et al., 2015). These results strongly suggest that PKCδ modulates NF-κB signaling to regulate downstream targets such as BACE1. However, whether PKCδ has effects on AD pathogenesis and what relevant mechanisms may be involved remains unclear. In this study, we examined the function of PKCδ in AD pathogenesis and characterized relevant PKCδ-associated mechanisms in vitro and in vivo. Our results show that PKCδ is increased in AD brain and correlates with increased BACE1 expression. PKCδ was found to modulate BACE1 expression through NF-κB phosphorylation to induce BACE1-mediated APP cleavage and Aβ production. Specific inhibition of PKCδ by rottlerin markedly reduced BACE1 expression, Aβ production, and neuritic plaque formation, and rescued cognitive deficits in APP Swedish mutations K594N/M595L (APPswe)/presenilin-1 (PS1) with an exon 9 deletion (PS1dE9) double-transgenic mice. Our work provides evidence that PKCδ plays an important role in AD pathogenesis, and inhibition of PKCδ may be a novel and effective therapeutic target for AD treatment.

Results

PKCδ expression is elevated in human AD

BACE1 levels were previously found to be elevated in AD (Holsinger et al., 2002; Yang et al., 2003; Fukumoto et al., 2004; Zhao et al., 2007; Chen et al., 2012), which prompted us to investigate whether correlations between BACE1 and other neurological mediators such as PKCδ exist. We therefore measured PKCδ and BACE1 levels in AD patient brains (Fig. 1 A) and found that both PKCδ and BACE1 expression increased in AD compared with non-AD controls (Fig. 1, B and C). Interestingly, PKCδ levels positively correlated with BACE1 levels, indicating that PKCδ and BACE1 levels may be linked in vivo (Fig. 1 D).

PKCδ inhibition reduces Aβ production by attenuating BACE1 expression in vitro

We examined whether PKCδ inhibition affects Aβ production by transfecting siRNA oligonucleotides targeting PKCδ in SH-SY5Y cells stably expressing human APP741 (SY5Y-APP741). We found that PKCδ siRNA transfection dramatically reduced the production of Aβ and BACE1 cleavage products including C99 and soluble APPβ (sAPPβ), but had no effect on total APP levels and α-secretase cleavage products including carboxyl-terminal fragment of APP cleaved at the α-site (C83) and sAPPα (Fig. 2 A). To further confirm the role of PKCδ in modulating APP processing, we assayed expression profiles of key APP processing enzymes, including α-secretases A disintegrin and metalloprotease (ADAM) 10 and ADAM17, β-secretase BACE1, and γ-secretase components PS1 and nicastrin (NCT). Our results show that PKCδ knockdown reduced BACE1 expression, with no consequent

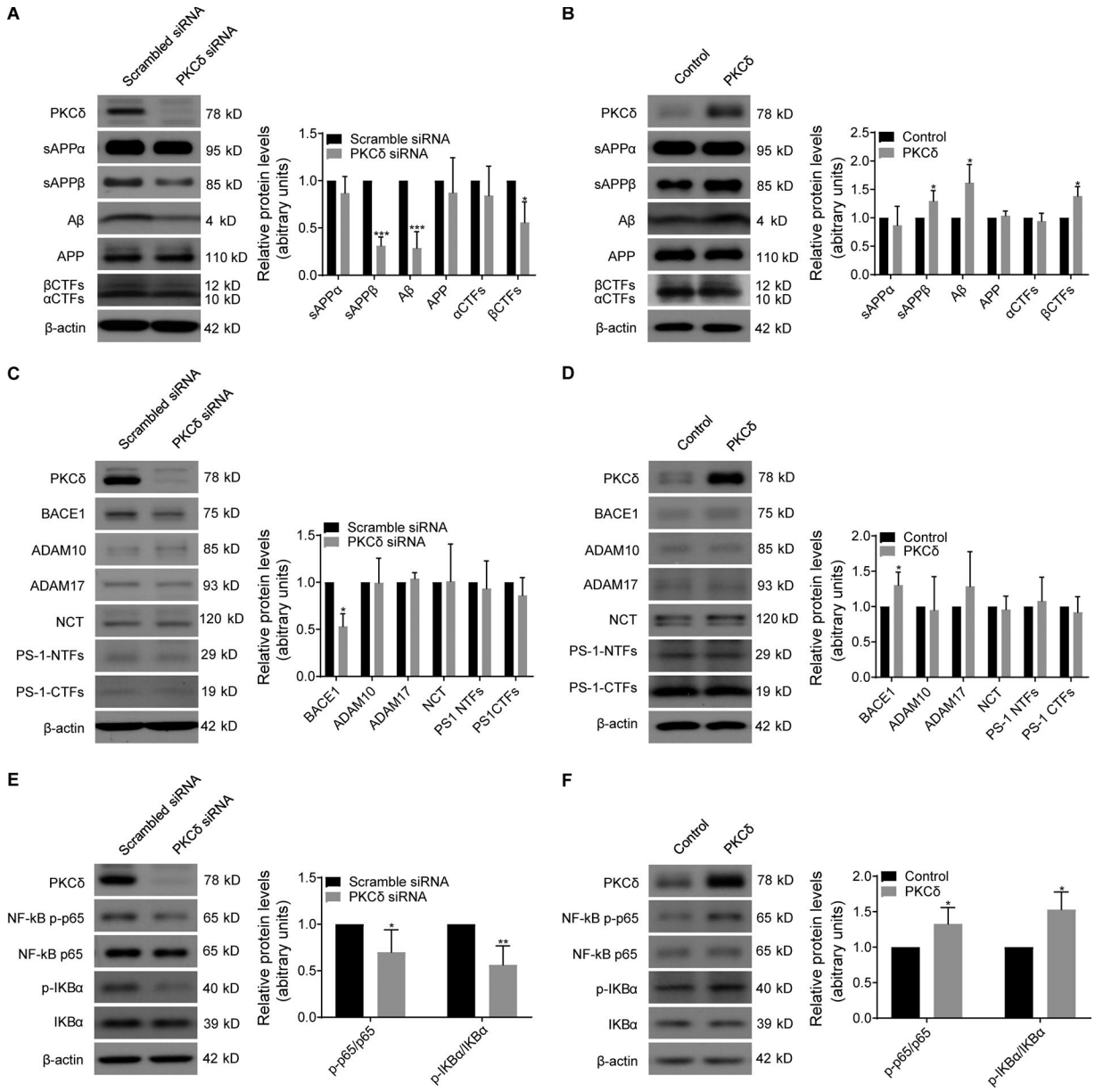


Figure 2. **PKCδ regulates BACE1-mediated APP processing, Aβ production, BACE1 expression, and NF-κB IκBα and p65 phosphorylation in vitro.** **(A)** Human SY5Y-APP741 cells were transfected with scrambled or PKCδ-targeting siRNAs. Full-length APP, C99, and sAPPβ BACE1 cleavage products, C83 and sAPPα α-secretase cleavage products, and Aβ levels were detected by Western blot. PKCδ-specific knockdown significantly reduces the generation production of Aβ, C99, and sAPPβ but has no effect on total APP, C83, and sAPPα. **(B)** PKCδ overexpression increases BACE1 cleavage of APP and Aβ production in mouse N2a-APP695 cells. Full-length APP, C99 and sAPPβ, C83 and sAPPα, and Aβ levels were detected by Western blotting. **(C)** Immunoblots for ADAM10, ADAM17, BACE1, PS1, and NCT with PKCδ knockdown in SY5Y-APP741 cells. **(D)** ADAM10, ADAM17, BACE1, PS1, and NCT levels with PKCδ overexpression were measured by Western blotting in N2a-APP695 cells. **(E)** SY5Y-APP741 cells were transfected with scrambled or PKCδ-targeting siRNAs. IκBα, p65, p-IκBα (pSer32/36), and p-p65 (pSer536) levels were detected by Western blot. **(F)** N2a-APP695 cells transfected with control or PKCδ vectors were immunoblotted for the components as indicated. *n* = 4; *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data are means ± SD.

effect on ADAM10, ADAM17, PS1, and NCT levels (Fig. 2 C). Furthermore, overexpression of PKCδ reversed the reduction of BACE1 and Aβ induced by PKCδ siRNA (Fig. S1 A), suggesting that the change of BACE1 and Aβ is not caused by any potential off-target effects from PKCδ siRNA.

To further confirm whether PKCδ inhibition could also reduce Aβ production and BACE1-mediated APP processing in mouse cell models, we generated several PKCδ KO monoclonal cell lines using a CRISPR-Cas9 targeting system in N2a cells stably expressing human APP695 (N2a-APP695). We observed a significant reduction of Aβ, BACE1, and sAPPβ in a PKCδ KO cell line, where total APP levels, α- and γ-secretase components (e.g., ADAM10, ADAM17, PS1, and NCT), and sAPPα remain unaltered (Fig. S1, B, C, and E). We also observed reduced BACE1 and Aβ levels in two additional PKCδ KO cell lines (Fig. S1 F). Thus, our findings indicate that PKCδ down-regulation reduces BACE1 expression, BACE1-mediated APP processing, and Aβ production in different cell lines in vitro.

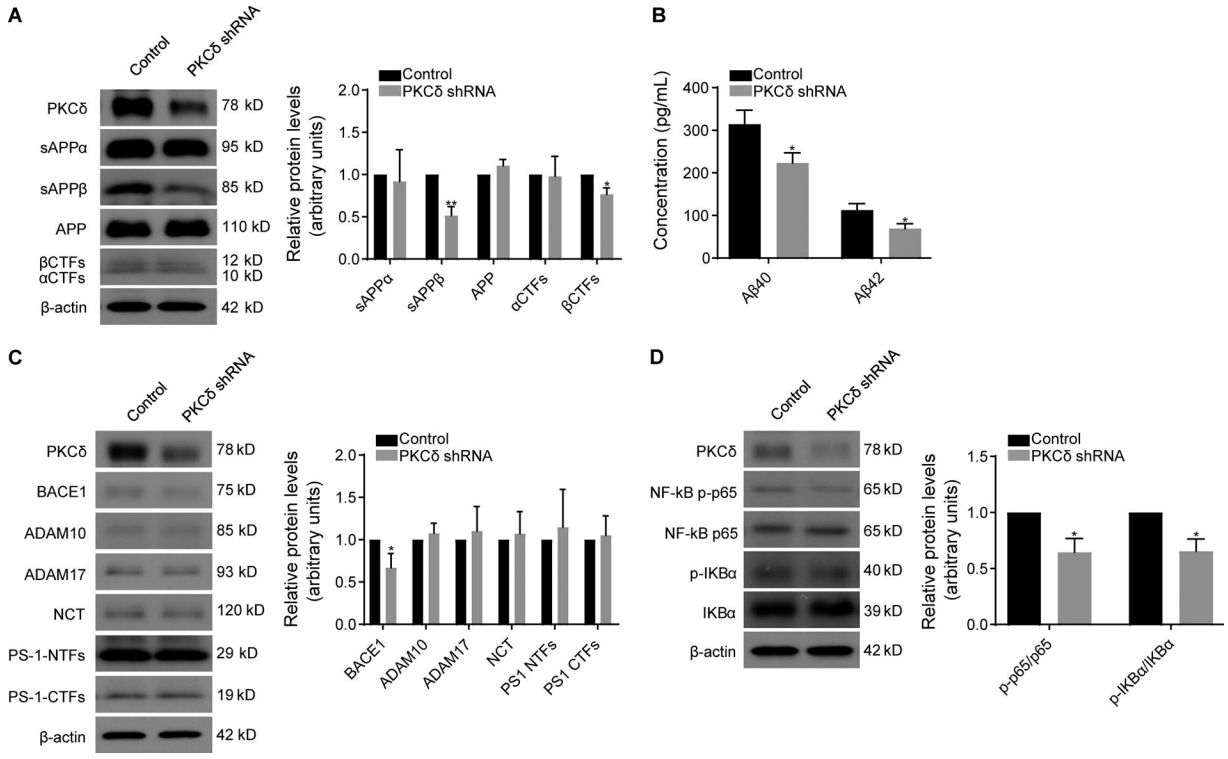


Figure 3. **shRNA-mediated PKCδ knockdown with lentiviral transduction reduces BACE1 expression, BACE1-dependent APP cleavage, Aβ production, and IκBα/p65 phosphorylation in Tg2576 primary neurons. (A)** Full-length APP, C99, and sAPPβ BACE1 cleavage products and C83 and sAPPα α secretase cleavage products were detected by Western blot. **(B)** $Aβ_{1–40}$ and $Aβ_{1–42}$ levels detected in conditioned medium from shRNA-transduced primary neuronal cultures by ELISA. **(C)** ADAM10, ADAM17, BACE1, PS1, and NCT were detected by Western blotting in lysates from primary neurons as indicated. **(D)** IκBα, p65, p-IκBα (Ser32/36), and p-p65 (Ser536) levels were detected in cell lysates from primary neurons transduced with control or PKCδ-targeting shRNAs by Western blotting. $n = 4$; *, $P < 0.05$; **, $P < 0.01$. Data are means ± SD.

PKCδ overexpression up-regulates BACE1 expression and Aβ production in vitro

Because PKCδ expression positively correlates with BACE1 levels in AD patients, this suggests that PKCδ may up-regulate BACE1 expression and consequent Aβ generation. To confirm this, we overexpressed PKCδ in N2a-APP695 cells to examine consequent effects on APP metabolism and secretase levels. Overexpression of PKCδ significantly increased the production of Aβ, C99, and sAPPβ, without significantly affecting total APP levels or C83 and sAPPα (Fig. 2 B). Moreover, PKCδ overexpression markedly increased BACE1 expression with no effect on ADAM10, ADAM17, PS1, and NCT levels (Fig. 2 D). Collectively, these results demonstrate that PKCδ plays an important role in regulating BACE1-mediated APP processing and Aβ production through BACE1 expression in vitro.

PKCδ phosphorylates IκBα and p65 NF-κB subunits

PKCδ has been previously shown to regulate NF-κB activation through phosphorylation of the NF-κB inhibitor IκBα at Ser32 and Ser36 (Vancurova et al., 2001). Phosphorylation of IκBα results in consequent polyubiquitination at Lys22 and degradation by the 26S proteasome. This results in translocation of the transcriptional p65 NF-κB subunit to the nucleus to promote downstream gene expression (Vancurova et al., 2001; Storz et al., 2004). PKCδ has also been shown to phosphorylate p65 at Ser536 to initiate transcriptional activation of NF-κB–dependent genes (Goon Goh et al., 2008; Ren et al., 2014). Recently, increased phosphorylation of IκBα (at Ser32 and Ser36) and p65 (at Ser536) has been shown to enhance BACE1 expression in the brain after LPS-induced inflammation (Wang et al., 2015). These results suggest that PKCδ may modulate NF-κB phosphorylation to regulate downstream BACE1 expression.

To further confirm the mechanism underlying PKCδ in BACE1-dependent APP processing and Aβ generation, we used siRNA, shRNA, and overexpression vectors to modulate PKCδ levels in SY5Y-APP741 and N2a-APP695 cells and examined changes in NF-κB phosphoregulation. In SY5Y-APP741 and N2a-APP695 cells, PKCδ knockdown significantly decreased IκBα (pSer32/36) and p65 (pSer536) phosphorylation, with no effect on total IκBα and p65 levels (Fig. 2 E). CRISPR/Cas9-mediated PKCδ deletion in N2a-APP695 cells similarly resulted in a reduction in phosphorylated NF-κB IκBα (pSer32/36) and p65 (pSer536) levels (Fig. S1 D). In contrast, PKCδ overexpression in mouse N2a-APP695 cells increased p-IκBα (Ser32/36) and p-p65 (Ser536) levels whereas total IκBα and p65 levels remained unchanged (Fig. 2 F).

PKCδ down-regulation attenuates Aβ production by reducing BACE1 expression in primary neurons

We next examined whether PKCδ down-regulation could affect Aβ production by lentivirus-based delivery of PKCδ-targeting shRNAs in primary neurons derived from Tg2576 mouse embryos.

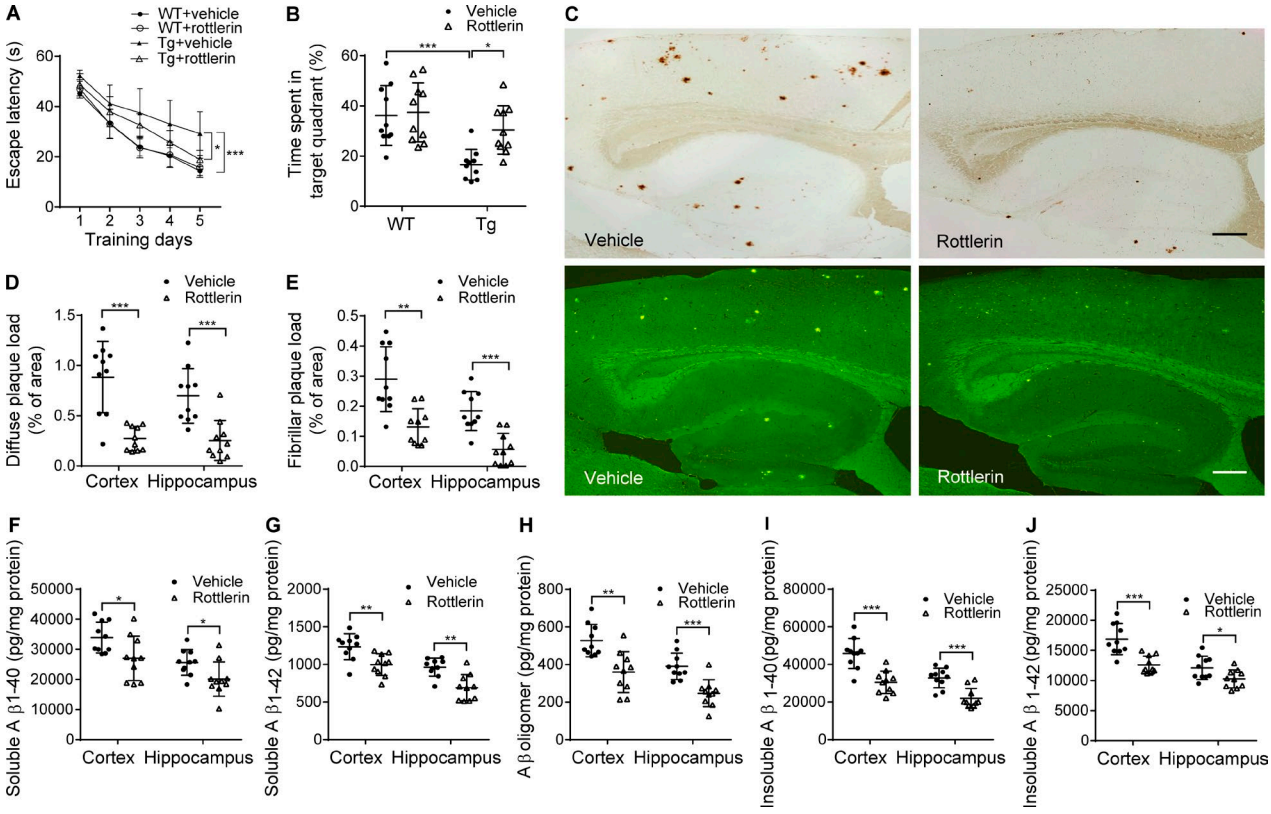


Figure 4. **Rottlerin treatment improves cognitive deficits and reduces diffuse and fibrillar plaque burden and Aβ levels in APPswe/PS1dE9 mice.** **(A)** Hidden-platform tests performed over 5 d in the Morris water maze. Significance was determined by repeated-measures ANOVA; vehicle-treated APPswe/PS1dE9 mice exhibited a longer escape latency compared with rottlerin- and vehicle-treated WT mice, whereas rottlerin-treated APPswe/PS1dE9 mice exhibited a shorter escape latency compared with vehicle-treated APPswe/PS1dE9 mice. **(B)** Probe trial 24 h after the last hidden-platform test in the Morris water maze. Percentage of time spent in the target quadrant is shown for APPswe/PS1dE9 and WT groups treated with rottlerin or vehicle. **(C)** Representative histological images from sagittal brain sections from APPswe/PS1dE9 mice treated with vehicle (top left) and rottlerin (top right) immunostained with monoclonal anti-Aβ antibody (DE2) for diffuse plaques (brown) and treated with vehicle (bottom left) and rottlerin (bottom right) stained with thioflavin S for fibrillar plaques (green). Bar, 300 µm. **(D and E)** Quantification of the percent area occupied by diffuse plaques (D) and fibrillar plaques (E) in APPswe/PS1dE9 mouse brain treated with vehicle and rottlerin. **(F–H)** Plaques from rottlerin-treated APPswe/PS1dE9 mice were found to be significantly lower than the vehicle-treated transgenic control group. Levels of soluble $A\beta_{1-40}$ (F), $A\beta_{1-42}$ (G), and Aβ oligomers (H) obtained in RIPA buffer in rottlerin-treated animals are significantly lower than in vehicle-treated controls. **(I and J)** Levels of insoluble $A\beta_{1-40}$ (I) and $A\beta_{1-42}$ (J) obtained by using formic acid extraction in rottlerin-treated animals are significantly lower than in vehicle-treated controls. *n* = 10 in each group. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data are means ± SD. Tg, transgenic.

Similar to our observations in SY5Y-APP741 and N2a-APP695 cells, PKCδ knockdown markedly reduced levels of C99 and sAPPβ, with no effect on total APP levels and C83 and sAPPα (Fig. 3 A). PKCδ shRNA transduction also decreased $A\beta_{1-40}$ and $A\beta_{1-42}$ levels (Fig. 3 B). Although PKCδ knockdown reduced BACE1 expression, no effect on ADAM10, ADAM17, PS1, and NCT levels were observed in neurons (Fig. 3 C). Interestingly, p-IκBα (Ser32/36) and p-p65 (Ser536) levels were also markedly decreased without any changes in total IκBα and p65 levels with PKCδ shRNA transduction (Fig. 3 D). Collectively, our findings indicate that PKCδ-dependent IκBα and p65 phosphorylation up-regulates BACE1 expression to enhance Aβ production in vitro.

Rottlerin-mediated PKCδ inhibition rescues cognitive deficits in APPswe/PS1dE9 mice

To investigate whether pharmacological PKCδ inhibitors such as rottlerin can affect cognitive deficits in AD pathogenesis, we used the Morris water maze to test spatial learning and memory in APPswe/PS1dE9 mice 12 wk after rottlerin treatment (see Materials and methods). In visible-platform tests, both APPswe/PS1dE9 mice and WT mice in vehicle- or rottlerin-treated groups exhibited similar swimming speeds and escape latencies (unpublished data; P > 0.05), indicating that rottlerin treatment did not affect locomotion or visual function. In hidden-platform tests, vehicle-treated APPswe/PS1dE9 mice showed severe spatial learning impairment, indicated by greater escape latencies compared with vehicle- and rottlerin-treated WT mice (P < 0.001). However, rottlerin treatment significantly improved deficiencies in spatial learning in APPswe/PS1dE9 mice, where rottlerin treatment resulted in shorter escape latencies compared with vehicle-treated APPswe/PS1dE9 mice (P < 0.05; Fig. 4 A). In the probe trial, vehicle-treated APPswe/PS1dE9 mice exhibited severe spatial memory impairment as demonstrated by a shorter duration spent in the target quadrant (P < 0.001). In contrast, rottlerin treatment significantly improved the spatial memory defects in APPswe/PS1dE9 mice, with longer time periods spent in the target quadrant compared with vehicle-treated APPswe/PS1dE9 controls (P < 0.05; Fig. 4 B).

Because synaptic markers have been previously noted to be depleted in AD models (Harwell and Coleman, 2016; Yan et

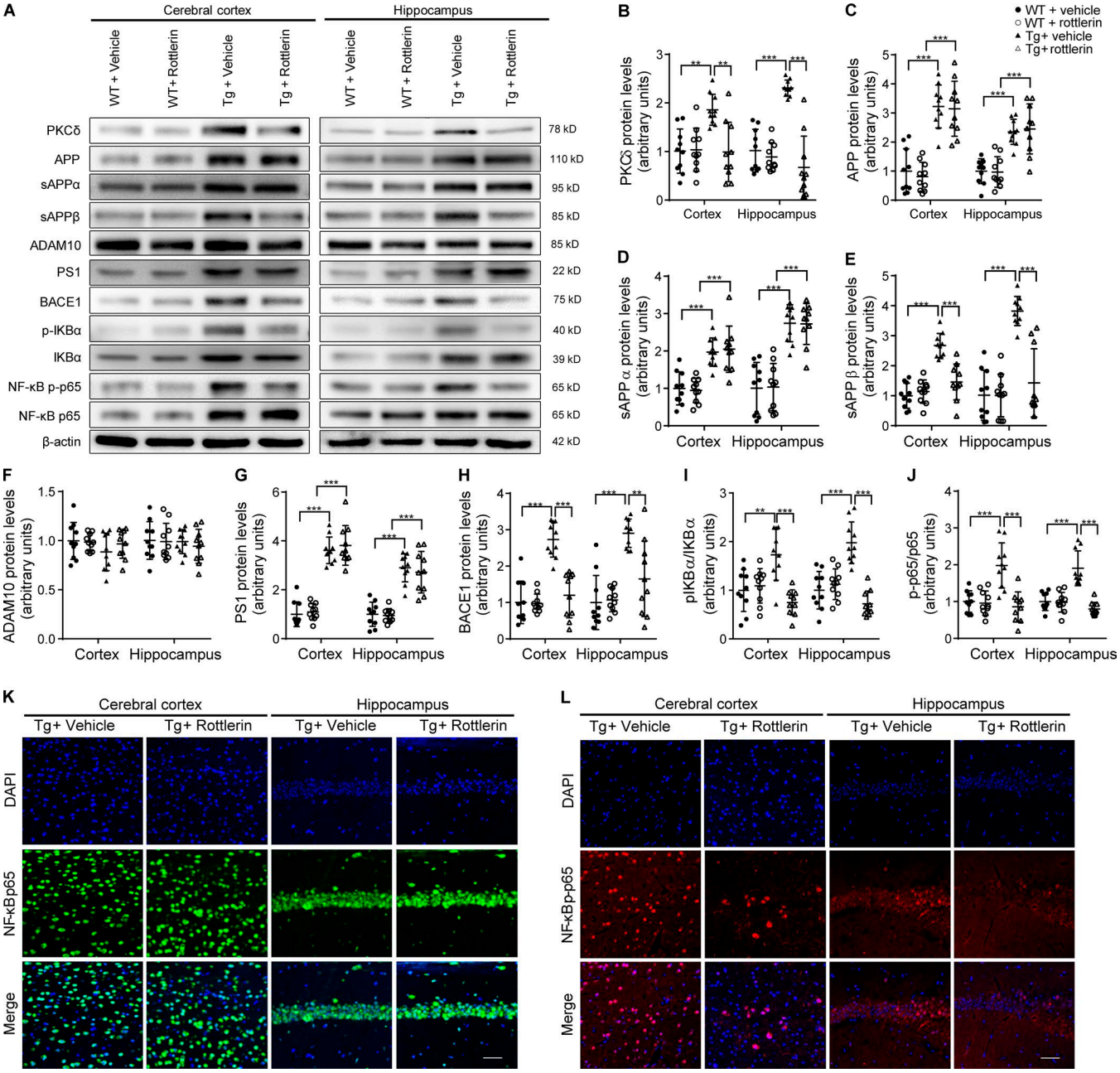


Figure 5. **Rottlerin treatment attenuates amyloidogenic APP processing, reduces BACE1 expression and IκBα and NF-κB p65 phosphorylation in APPswe/PS1dE9 mice. (A)** Representative Western blots to detect PKCδ, full-length APP, sAPPα, sAPPβ, ADAM10, PS1, BACE1, p-IκBα, IκBα, NF-κB p-p65, and NF-κB p65 in homogenates from the cerebral cortex and hippocampus in mice treated with rottlerin or vehicle. **(B–J)** Densitometric quantification of protein band optical densities for PKCδ (B), APP (C), sAPPα (D), sAPPβ (E), ADAM10 (F), PS1 (G), and BACE1 (H) expression levels and densitometric quantification of protein ratios for p-IκBα/IκBα (I) and p-p65/p65 (J) are shown. $n = 10$ in each group. **, $P < 0.01$; ***, $P < 0.001$. Tg, transgenic. **(K and L)** Representative immunofluorescence and laser scanning confocal images of NF-κB p65 (K) and NF-κB p-p65 (L) in brain sections from APPswe/PS1dE9 mice treated with vehicle and rottlerin. Data are means ± SD. Bars, 50 µm.

al., 2016), we looked for changes in synaptic components such as synaptophysin (SYP) and postsynaptic density-95 (PSD-95), and we found that both SYP and PSD-95 levels were significantly decreased in the cerebral cortex and hippocampus in vehicle-treated APPswe/PS1dE9 mice compared with vehicle/rottlerin-treated WT mouse groups. Rottlerin treatment increased SYP and PSD-95 staining in these brain regions compared with vehicle-treated APPswe/PS1dE9 controls, indicating restoration of synaptic markers with PKC inhibition (Fig. S2, A, D, and E). Together, these results demonstrate that PKCδ inhibition with rottlerin treatment significantly improved spatial learning and memory deficits in APPswe/PS1dE9 mice.

Rottlerin-mediated PKCδ inhibition reduces Aβ generation and deposition in APPswe/PS1dE9 mouse brain

We next investigated whether PKCδ inhibition through rottlerin treatment altered Aβ generation and deposition in APPswe/PS1dE9 mice. Aβ plaque deposition as visualized through

immunohistochemical and histological staining was performed in the APPswe/PS1dE9 mouse brain to examine effects of PKCδ on plaque formation (Fig. 4 C); the area occupied by diffused and fibrillar plaques was found to be markedly decreased in the rottlerin-treated APPswe/PS1dE9 mice compared with vehicle controls (Fig. 4, D and E). Rottlerin treatment significantly decreased soluble $A\beta_{1-40}/A\beta_{1-42}$, Aβ oligomers, insoluble $A\beta_{1-40}/A\beta_{1-42}$ in the APPswe/PS1dE9 mouse brain (Fig. 4, F–J). We also characterized changes in components that mediate Aβ turnover such as insulin-degrading enzyme (IDE) and neprilysin (NEP), although IDE and NEP levels were significantly reduced in vehicle-treated APPswe/PS1dE9 and vehicle/rottlerin-treated WT mouse cortex and hippocampus. Rottlerin treatment had no effect on IDE and NEP levels compared with vehicle controls APPswe/PS1dE9, indicating that effects of rottlerin were not mediated through the modulation of Aβ degradation (Fig. S2, A–C). Collectively, our findings demonstrate that inhibition of PKCδ by rottlerin treatment remarkably reduced Aβ production and deposition in the APPswe/PS1dE9 mouse brain.

Rottlerin-mediates PKCδ inhibition and reduces BACE1 expression/BACE1-mediated APP processing in APPswe/PS1dE9 mice

To further investigate the mechanism underlying rottlerin-mediated reduction in Aβ generation, we characterized potential perturbations in PKCδ expression and APP processing components with rottlerin treatment by Western blotting (Fig. 5 A). We found that PKCδ levels were markedly increased in the cerebral cortex and hippocampus in vehicle-treated APPswe/PS1dE9 mice compared with vehicle/rottlerin-treated WT mouse groups, whereas rottlerin treatment resulted in a decrease in PKCδ levels in these regions compared with vehicle-treated APPswe/PS1dE9 controls (Fig. 5 B). Although rottlerin treatment had no effect on total APP levels or sAPPα (Fig. 5, C and D), sAPPβ levels were found to be dramatically reduced (Fig. 5 E) in APPswe/PS1dE9 mouse brain compared with vehicle controls. Further, ADAM10 and PS1 levels showed little change (Fig. 5, F and G), whereas BACE1 levels were markedly decreased relative to vehicle-treated transgenic controls (Fig. 5 H). In addition, we characterized changes in mRNA expression in BACE1, PS1, ADAM10, and other PKC isoforms (including PKCα, β, ε, γ, and ζ) reported to affect cognition and memory. Our results indicate that changes in BACE1, PS1, and ADAM10 mRNA expression were consistent with protein expression (Fig. S2, F–H). Moreover, PKCα, β, ε, γ, and ζ levels were all significantly decreased in the cerebral cortex and hippocampus in vehicle-treated APPswe/PS1dE9 mice compared with vehicle/rottlerin-treated WT mouse groups; rottlerin treatment had no effect on these PKC isoforms compared with vehicle-treated APPswe/PS1dE9 controls (Fig. S3). These results demonstrate that rottlerin-mediated PKCδ inhibition specifically reduced PKCδ and BACE1 expression and attenuated consequent APP β-secretase cleavage in the APPswe/PS1dE9 mouse brain.

Rottlerin-mediated PKCδ inhibition reduces IκBα and p65 phosphorylation in APPswe/PS1dE9 mice

To further determine whether PKCδ inhibition could influence BACE1 expression through NF-κB regulation, we examined phosphoregulatory changes in the NF-κB pathway in APPswe/PS1dE9 mouse models with rottlerin treatment (Fig. 5 A). Rottlerin-mediated PKCδ inhibition significantly decreased phosphorylation of IκBα (Ser32/36) and p65 (Ser536) with no effect on IκBα and p65 expression levels in the APPswe/PS1dE9 mouse brain (Fig. 5, I and J). Moreover, immunofluorescence with laser scanning confocal microscopy also confirmed that rottlerin treatment had no effect on p65 expression (Fig. 5 K), with significant reductions in p65 (Ser536) levels in the cerebral cortex and hippocampal region in APPswe/PS1dE9 mice (Fig. 5 L). Collectively, our findings demonstrate, in both AD-cultured cell and mouse models, that PKCδ inhibition reduced BACE1 expression through IκBα/p65 phosphorylation, thereby attenuating BACE1-mediated APP processing and Aβ production in cells, neurons, and APPswe/PS1dE9 mouse models.

Discussion

PKC isoforms including PKCα, β, δ, ε, γ, and ζ are ubiquitously distributed in neuronal structures throughout the brain and are involved in a broad range of vital functions such as neuronal plasticity, metabolism, inflammation, and gene expression; neuroprotection and neurodegeneration; and behavior, learning, and memory (Hama et al., 2004; Sun and Alkon, 2012, 2014). Given the diversity of PKC signaling in neuronal function, PKCs provide a potential molecular link, which connects cognitive deficiency and amyloidogenesis in AD. Previous studies have characterized changes in several PKC isoforms in postmortem AD brain tissue, and immunocytochemical results have revealed either no change or reductions in these PKC isoform levels compared with controls. PKCβ and ε isoforms potentially exhibit the most significant reduction of all isoforms characterized in AD (Masliah et al., 1990; Clark et al., 1991; Shimohama et al., 1993; Wang et al., 1994; Matsushima et al., 1996). Further studies demonstrate that activation of PKCα and ε enhance the generation of C83 and sAPPα α-secretase cleavage products with little neurotoxicity. PMA-induced activation of PKC isoforms can promote accumulation of APP cleavage products such as sAPPβ at late Golgi vesicles (Xu et al., 1995). Moreover, PKCε overexpression has been shown to selectively increase the activity of endothelin-converting enzyme to enhance Aβ degradation (Eckman et al., 2001; Choi et al., 2006; da Cruz e Silva et al., 2009; Khan et al., 2009; Nelson and Alkon, 2009; Nelson et al., 2009). However, specific pathological changes in the PKCδ isoform are yet unclear; only one study so far reports increases in membrane-associated PKCδ levels in AD (Matsushima et al., 1996). In agreement with these results, we observed elevations in both PKCδ and BACE1 levels in AD brain. More interestingly, PKCδ levels correlated tightly with BACE1 expression, implicating an interdependent relationship between PKCδ and BACE1 in AD.

PKCδ is expressed in cortical and hippocampal regions of the brain and phosphorylates a variety of target proteins. Phosphorylation of target proteins can alter stability, protein–protein interactions, cellular distribution, or catalytic activity, which in turn propagates signals from the plasma membrane to molecular targets in the cytoplasm and nucleus (Sun and Alkon, 2012, 2014; Lucke-Wold et al., 2015; Talman et al., 2016). PKC-mediated

signal transduction is often isoform- and cell type–specific and is triggered in response to a specific stimulus. Moreover, different PKC isoforms can have opposing effects, which converge on a single pathway (Chen et al., 2001). Evidence so far indicates that, in contrast with other PKC isoforms, increased PKCδ plays a role in enhancing neurodegenerative signaling pathways associated with neuroinflammation, oxidative stress, and apoptosis and in the context of aging, brain ischemia, and neurotoxicity (Kanthasamy et al., 2003; Steinberg, 2004; Yoshida, 2007; Shin et al., 2012, 2014). Given that PKCδ is also expressed in microglia (Zhang et al., 2014), modulation of PKCδ in microglia may have other effects in addition to amyloidogenic APP processing as we describe in this study.

Although little is known with regard to the relationship between PKCδ and AD pathogenesis, functional links between PKCδ and PD has been previously reported. Elevations in microglial PKCδ expression and kinase activity and consequent neuroinflammation and neurotoxicity are observed in both in vitro and in vivo PD models. Mechanisms underlying neurotoxicity in these models appear to be associated with ROS, MPTP, and a diverse variety of inflammogens, including TNFα, LPS, and aggregated α-synuclein. Thus, enhanced PKCδ activity could potentially facilitate increased IκBα and p65 phosphorylation, thereby enhancing consequent expression of various proinflammatory mediators in these models. Because rottlerin treatment or PKCδ gene silencing/genetic ablation attenuated proinflammatory cytokine levels, this suggests that a potential positive-feedback loop involving the PKCδ–NF-κB pathway could be involved in pathogenic mechanisms in neurodegenerative disease such as PD (Vancurova et al., 2001; Burguillos et al., 2011; Gordon et al., 2012, 2016).

Inflammation induced by Aβ exposure is commonly associated with AD brain pathology, which may involve various proinflammatory transcriptional regulators such as NF-κB in mediating or aggravating Aβ-mediated degeneration (Liu et al., 2015; Sachdeva and Chopra, 2015). Furthermore, previous studies have reported increased NF-κB p65 activity and BACE1 expression in the postmortem AD brain; given that NF-κB promoter elements can be found within the BACE1 locus, up-regulation of NF-κB could be potentially linked in AD. Indeed, NF-κB p65 overexpression increased transcription from the human BACE1 promoter region, whereas inhibiting NF-κB signaling reduced BACE1 expression (Bourne et al., 2007; Chami et al., 2012; Chen et al., 2012). Under nonpathological conditions, the absence of an NF-κB activator would have little influence on BACE1 up-regulation. However, in the presence of Aβ, neuroinflammatory activation of NF-κB would up-regulate BACE1 expression and consequent amyloidogenic Aβ production (Bourne et al., 2007; Buggia-Prevot et al., 2008; Chami et al., 2012). Given that BACE1 is potentially up-regulated in response to Aβ-dependent NF-κB activation, this may represent a feed-forward system where Aβ exposure potentially enhances amyloidogenic processing pathways.

Interestingly, exogenous application of Aβ peptides has been found to increase PKCδ levels and NF-κB activity (Valerio et al., 2006; Meng et al., 2013), and various studies indicate that PKCδ expression is regulated in an NF-κB–dependent manner (Vancurova et al., 2001; Storz et al., 2004; Goon Goh et al., 2008; Burguillos et al., 2011; Ren et al., 2014; Wang et al., 2015). Our results indicate that PKCδ overexpression enhanced IκBα and p65 phosphorylation, up-regulated BACE1 expression, and increased BACE1-mediated APP cleavage and Aβ production, whereas PKCδ knockdown reduced IκBα and p65 phosphorylation, down-regulated BACE1 expression, and decreased BACE1-mediated APP cleavage and Aβ production in vitro. Collectively, our results present a model where PKCδ–NF-κB signaling up-regulates BACE1 expression in AD.

Given that PKCδ could enhance AD pathogenesis through BACE1 up-regulation, PKCδ inhibitors may be potentially beneficial in reversing AD phenotypes. Rottlerin is a compound derived from the kamala tree (*Mallotus philippinensis*) with a low toxicity profile (lowest lethal dose: 750 mg/kg per rat), which inhibits PKCδ with an IC_{50} of 3–6 μM. K_i values for other PKC isoforms (α, β, ε, γ, and λ) are 5–10 times higher, indicating that the biological effects of rottlerin are largely via PKCδ inhibition (Varma et al., 1959; Gschwendt et al., 1994; Samokhin et al., 1999; Davies et al., 2000). In this study, we found that rottlerin treatment greatly attenuated increased PKCδ levels with markedly decreased IκBα and p65 phosphorylation, BACE1 expression, BACE1-mediated APP cleavage, and Aβ production/deposition in the APPswe/PS1dE9 mouse brain, with corresponding amelioration of cognitive deficits.

In conclusion, our results demonstrate that PKCδ regulates BACE1 expression, thereby enhancing Aβ production. PKCδ inhibition reduces Aβ neuropathology and rescues cognitive deficits in APPswe/PS1dE9 AD mouse models, indicating that PKCδ inhibition may be a viable treatment strategy in AD.

Materials and methods

PKCδ and BACE1 levels in human brains

Brain cortical region samples from patients with AD and from controls were provided by E. Masliah, (University of California, San Diego, La Jolla, CA). Information pertaining to these patients and controls are listed in Fig. 1 A. Usage of human brain tissues in this study falls into exemption category 4 according to the policy of Institutional Review Board. Samples were lysed in radioimmunoprecipitation assay (RIPA) buffer, and equal protein amounts from the cell lysates were subjected to SDS-PAGE and Western blotting.

Cell culture and transfection

All cell lines were maintained at 37°C in an incubator containing 5% CO_2. Human neuroblastoma SY5Y-APP741 and mouse neuroblastoma N2a-APP695 were cultured in 50% DMEM (HyClone) mixed with 50% OptiMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco), in the presence of 400 μg/ml G418 (Sigma-Aldrich).

The target sequence for human PKCδ for siRNA oligonucleotide duplexes was 5′-CCGCTTCAAGGTTCACAACTA-3′, and human PKCδ and control siRNA were both purchased from QIAGEN. shRNA for mouse PKCδ comprised a hairpin targeting 5′-CAGAGT TCCTGAATGAGAA-3′, and mouse PKCδ shRNA, scrambled shRNA, PKCδ plasmid control, and PKCδ plasmid WT were all purchased from Thermo Fisher Scientific. Cells were transfected with PKCδ siRNA or plasmid WT by using Lipofectamine 2000 (Invitrogen).

Generation of PKCδ KO cell lines

The CRISPR-Cas9 system was used to generate PKCδ KO cell lines. 20-nt sequences with 5′-NGG protospacer-adjacent motif in the mouse PKCδ gene were selected by using an online tool (http://crispr.mit.edu/). A PKCδ sgRNA (forward: 5′-ACC GAGGCCGCTTCGAACTCTAC-3′; reverse: 5′-AACGTAGAGTTC GAAGCGGCCTC-3′) was inserted into PX552 (provided by F. Zhang, Massachusetts Institute of Technology, Cambridge, MA; 60958; Addgene) pAAV-U6sgRNA(SapI)_hSyn-GFP-KASH-bGH vector by following a protocol described previously (Swiech et al., 2015).

N2a-APP695 cells were cotransfected with PX552-PKCδ sgRNA and PX458 (provided by F. Zhang; 48138; Addgene; Ran et al., 2013). 24 h after transfection, GFP-positive cells were plated into 96-well plates (one cell per well) and subjected to subsequent expansion. Expanded monoclonal cell lines were validated by Western blot and sequencing.

Primary neuronal culture and transduction

Dissociated cortical cells from Tg2576 mice were seeded into 6-well plates (800,000 cells per 6-well plate) and maintained in Neurobasal A medium supplemented with B27, 2 mM glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin.

Premade lentiviruses encoding mouse PKCδ shRNA and scrambled shRNA were purchased from the Sanford Burnham Prebys Viral Vector Core facility (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA). All lentiviruses were amplified in HEK293A cells. Cells were transduced with lentiviruses encoding PKCδ shRNA or scrambled shRNA by using Lipofectamine 2000 (Invitrogen).

Transgenic mice and rottlerin treatment

Animals used in this study were homozygous APPswe/PS1dE9 double-transgenic mouse lines, which harbor human APPswe (Swedish mutations K594N/M595L) and PS1 with an exon 9 deletion (PS1dE9) under the control of the mouse prion promoter, purchased from Beijing HFK Biotechnology. Animal procedures were approved by the Institutional Animal Experiment Committee of Fourth Military Medical University, China, and performed in accordance with the University Policies on the Use and Care of Animals.

6-mo-old male APPswe/PS1dE9 mice and their nontransgenic WT littermates were randomly assigned into four groups (n = 10 in each group): rottlerin-treated APPswe/PS1dE9 mice, vehicle-treated APPswe/PS1dE9 mice, rottlerin-treated WT, and vehicle-treated WT mice. Male mice were used exclusively to exclude possible contributive effects from estrogen. Each rottlerin-treated mouse received 8 mg/kg rottlerin (Sigma-Aldrich) diluted in 2% DMSO in PBS via intraperitoneal injection once per day for 12 wk. Concurrently, mice in the control groups were injected only with an equal volume of 2% DMSO in PBS. The treatment dose of rottlerin in this study was optimized based on previous research (Zhang et al., 2007a).

Throughout the study, mice had free access to food and water and were housed in a pathogen-free environment on a 12-h light/dark cycle. Body weight, food and water intake, and overall general health were assessed every week.

Morris water maze test

The Morris water maze test was performed as described previously (Zhou et al., 2015). In brief, the test was performed in a 1.5-m-diameter pool with a 10-cm diameter platform placed in the southeast quadrant of the pool. Trajectories of all animals were monitored and acquired by using a computerized tracking system (Water 2020; HVS Image). The procedure comprised a 1-d visible platform test, 5 d of training trials, and lastly, a probe trial 24 h after the last training trial. In the visible-platform test, mice were tested for four continuous trials, with an intertrial interval of 60 min. In the training trials, the hidden platform was kept within the southeast quadrant and submerged 1.5 cm below the water surface. Mice were given a maximum of 60 s to escape onto the hidden platform. If a mouse could not locate the platform within 60 s, the experimenter guided it to the platform and allowed the mouse to rest there for 15 s. The animals were trained for four trials per day for 5 d. The time needed for an individual mouse to reach the hidden platform was recorded as the escape latency. In the probe trial, the platform was removed, and mice were given 60 s to locate the original location of the platform. The percentage of time that an individual mouse spent in the target quadrant was recorded as a measure of the spatial memory score. Male mice were used exclusively to exclude possible contributive effects from female hormones, which are known to regulate APP trafficking and Aβ generation (Xu et al., 1998).

Brain tissue preparation

After the behavioral tests, all animals were deeply anesthetized with sodium pentobarbital (100 mg/kg intraperitoneally) and transcardially perfused with 100 ml ice-cold normal saline. Brains were removed and dissected into two hemispheres through the midsagittal plane. One hemisphere was placed in 4% paraformaldehyde in PBS, followed by xylene treatment and embedding in paraffin for immunohistochemical and histological analyses. The entire hippocampus and cerebral cortex were quickly isolated from the remaining hemisphere on ice and snap-frozen in liquid nitrogen and stored at −80°C to be used for the biochemical analyses. Whole brain, cerebral cortex, and hippocampus weights were measured, respectively.

Immunohistochemical staining

Immunohistochemical and histological staining was performed as previously described (Zhao et al., 2015, 2018; Zhou et al., 2015). Sagittal brain sections (5-µm-thick) were deparaffinized and rehydrated. Antigen retrieval was performed by treatment with proteinase K (0.2 mg/ml) for 10 min at room temperature for Aβ staining. Nonspecific binding sites were blocked by incubation in 0.1% Triton X-100 and 2% bovine serum (Sigma-Aldrich) for 20 min at room temperature. Sections were then incubated with mouse monoclonal anti-Aβ antibody (DE2) targeting residues 1–16 of human Aβ (1:200; EMD Millipore) overnight at 4°C. The primary antibody was detected with a horseradish peroxidase–conjugated secondary antibody and visualized with a stable diaminobenzidine solution (Vector Laboratories). Thioflavin-S staining for fibrillar plaques was performed with 1% thioflavin-S (Sigma-Aldrich); and green fluorescence from plaques was visualized by using fluorescence microscopy.

For quantification, images were acquired by using an Olympus microscope connected to a digital camera. The percentage of area occupied by DE2-positive plaques (total Aβ plaque load) or thioflavin-S–positive plaques (fibrillar Aβ plaque load) in the frontoparietal cortex and hippocampus was calculated, respectively. Mean values for each parameter were recorded from six equidistant sections (at 150-µm intervals) per mouse in each group. All measurements were analyzed in a blinded fashion.

Aβ immunoblot and ELISA assay

SY5Y-APP741 cells in six-well plates were transfected with PKCδ or scrambled siRNA, and N2a-APP695 cells in six-well plates were transfected with PKCδ plasmid WT or PKCδ plasmid control. Cells were transfected for 24–48 h and incubated with 1 ml OptiMEM for 4 h. To detect total levels of secreted sAPPα, sAPPβ, and Aβ, conditioned media was collected and incubated with trichloroacetic acid (1:9 vol/vol) overnight at 4°C to precipitate proteins. Precipitates were subjected to immunoblotting with the 2B3 antibody to detect sAPPα, 6A1 antibody to detect sAPPβ, and 6E10 antibody to detect Aβ. Signals were developed by using an enhanced chemiluminescence kit (EMD Millipore), and relative band intensity was normalized relative to sAPPα, sAPPβ, and Aβ in PKCδ control siRNA or plasmid WT by using an automated image analysis system (Olympus).

Levels of secreted $Aβ_{1–40}$ and $Aβ_{1–42}$ from Tg2576-derived primary neurons were measured by using commercial human $Aβ_{1–40}$ and $Aβ_{1–42}$ ELISA systems (Invitrogen) according to the manufacturer's instructions.

APPswe/PS1dE9 mouse brain homogenates were sequentially extracted in RIPA buffer containing a cocktail of protease inhibitors (Sigma-Aldrich) to obtain soluble Aβ fractions and subsequently extracted in 70% formic acid for insoluble Aβ as previously described (Zhou et al., 2015). Soluble and insoluble levels of $Aβ_{1–40}$ and $Aβ_{1–42}$ in all samples were determined by using commercial human $Aβ_{1–40}$ and $Aβ_{1–42}$ ELISA kits (Invitrogen) according to the manufacturer's instructions. In addition, oligomeric Aβ levels in RIPA-soluble fractions from mouse brain were measured by using a commercial human Aβ oligomer (82E1-specific) assay ELISA kit (Immuno-Biochemical Laboratories).

Western blot analyses

RIPA fractions from brain tissues or cells were used for Western blot analyses as previously described (Zhou et al., 2015; Yan et al., 2016). In brief, samples were diluted in 4× SDS-sample buffer, boiled, and resolved on Novex 4–20% Tris-glycine protein gels (Invitrogen) and subsequently transferred to polyvinylidene fluoride membranes. Nonspecific binding sites were blocked for 1 h in PBS containing 5% nonfat dried milk, and membranes were probed with the following primary antibodies diluted in the blocking medium overnight at 4°C: mouse anti–PKCδ (1:500; Santa Cruz Biotechnology, Inc.), rabbit anti–PKCα (1:1,000; Abcam), anti-PKCβ1 (1:1,000; Abcam), anti-PKCβ2 (1:1,000; Abcam), anti-PKCε (1:1,000; Abcam), anti-PKCγ (1:1,000; Abcam), anti-PKCζ (1:1,000; Abcam), anti-IDE (1:1,000; Abcam), anti-NEP (1:1,000; Abcam), anti-SYP (1:1,000; Abcam), anti–PSD-95 (1:1,000; Abcam), mouse anti–APP (1:500; EMD Millipore), anti-APP CTFs (1:1,000; Cell Signaling Technology), anti-APP N-terminal fragment (1:1,000; 22C11; EMD Millipore), mouse anti–human Aβ 6E10 (1:1,000; Covance), mouse anti–human sAPPα (2B3; 1:100; Immuno-Biological Laboratories), rabbit anti–human sAPPβ (6A1; 1:100; Immuno-Biological Laboratories); mouse anti–BACE1 (1:1,000; clone 3D5; provided by R. Vassar, Northwestern University, Evanston, IL), mouse anti–ADAM10 (1:2,000; EMD Millipore), rabbit anti–ADAM17 (1:2,000; EMD Millipore), rabbit anti–PS1 N-terminal fragment (1:1,000; homemade polyclonal antibodies), mouse anti–PS1 CTFS (1:1,000; from G. Thinakaran, University of Chicago, Chicago, IL), mouse anti–NCT (1:1,000; Abcam), mouse anti-IκBα (1:1,000; Cell Signaling Technology), mouse anti–p-IκBα (Ser32/36; 1:1,000; Cell Signaling Technology), mouse anti–NF-κB p65 (1:1,000; Cell Signaling Technology), rabbit anti–NF-κB p-p65 (Ser536; 1:1,000; Cell Signaling Technology), and mouse anti–β-actin (1:5,000; Sigma-Aldrich). Membranes were washed with Tris-buffered saline at 0.1%, followed by incubation with horseradish peroxide–conjugated second antibodies (1:5,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Signals were developed using an enhanced chemiluminescence kit (EMD Millipore), and relative band intensity was normalized relative to β-actin using an automated image analysis system (Olympus).

Quantitative RT-PCR analyses

Total RNA was extracted from brain tissue by using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA was reverse transcribed by using a PrimeScript Double Strand cDNA Synthesis kit (Takara Bio Inc.), and first-strand products were used as PCR templates. Quantitative RT-PCR was performed by using a QuantiNava SYBR Green PCR kit and performed by using the iQ5 multicolor RT-PCR detection system (Bio-Rad Laboratories). The thermal cycling profile was as follows: 55°C for 30 min, 95°C for 15 min, and then 40 cycles of 95°C for 30 s, and 55°C for 30 s. The primer sequences used were as follows: BACE1 forward, 5′-TACTACTGCCCGTGTCCACC-3′; BACE1 reverse, 5′-ACA ACCTGAGGGGAAAGTCC-3′; PS1 forward, 5′-CTCATGGCCCTG GTATTTATCAA-3′; PS1 reverse, 5′-GAGCCATGC GGTCCATTC-3′; ADAM10 forward, 5′-GTTGCCGCCTCCTAAACCA-3′; ADAM10 reverse, 5′-GGCGGTCTCCTCCTCTTTAAAG-3′; β-actin forward, 5′-AATGTGTCCGTCGTGGA TCT-3′; and β-actin reverse, 5′-GGT CCTCAGTGTAGCCCAAG-3′.

Immunofluorescence and laser scanning confocal microscopy

Sagittal brain sections (5-µm thickness) were deparaffinized and rehydrated. Antigen retrieval was performed by using a 10-mM sodium citrate solution, pH 6.0, for 30 min at 90°C in a water bath for NF-κB p65 and phosphorylated NF-κB p65 (pSer536) staining. Nonspecific binding sites were blocked by incubation with 0.1% Triton X-100/2% bovine serum (Sigma-Aldrich) for 20 min at room temperature. Sections were then incubated with mouse anti–NF-κB p65 (1:800; Cell Signaling Technology) and rabbit anti–NF-κB p-p65 (pSer536; 1:100; Cell Signaling Technology), respectively at 4°C overnight. Sections were then stained with fluorescently conjugated secondary antibodies at room temperature for 2 h and counterstained with DAPI (1:800; Sigma-Aldrich) for 10 min. All sections were washed with PBS, and images were acquired using a confocal microscope (C2 Si; Nikon).

Statistical analysis

All data are presented as means ± SD. In Morris water maze tests, differences in escape latency and swimming speed between different groups were analyzed by two-way ANOVA with repeated measures followed by post hoc least significant difference tests for multiple comparisons. All other data were analyzed with a one-way ANOVA followed by post hoc least significant difference or Student *'s t* tests. All statistical analyses were performed by using SPSS 17.0 software (SPSS), and statistical significance was achieved at $P < 0.05$.

Online supplemental material

Fig. S1 shows PKCδ overexpression rescues changes in BACE1 and Aβ levels induced by PKCδ siRNA, and PKCδ deletion by CRISPR-Cas9 editing reduces BACE1-mediated APP processing, Aβ production, BACE1 expression, and NF-κB IκBα and p65 phosphorylation in vitro. Fig. S2 shows rottlerin increases expression of SYP and PSD-95 and attenuates BACE1 mRNA in APPswe/PS1dE9 mice, with no effect on IDE, NEP, ADAM10 mRNA, and PS1 mRNA expression. Fig. S3 shows rottlerin treatment has no effect on other PKC isozyme levels (PKCα, β, ε, γ, and ζ) in APPswe/PS1dE9 mice.

Acknowledgments

This work was supported by research grants from the National Natural Science Foundation of China (81371209, 81701041, 81372984, 81771163, and U1405222), the Natural Science Foundation of Shaanxi Province (S2014JC9313), Tangdu Hospital Innovation Development Foundation (2016LYJ007), and the National Institutes of Health (R21 AG048519, R01 AG021173, R01 AG038710, R01 AG044420, RF1 AG056130, FR1 AG056114, and R01 NS046673) as well as from the Tanz Family Fund and the Cure Alzheimer's Fund.

The authors declare no competing financial interests.

Author contributions: W. Zhang, Y. Zhao, and H. Xu conceived this study and designed the experiments. Y. Du and C. Li performed the experiments. J. Tian characterized the CRISPR/Cas9 deletion cell lines. T.Y. Huang and Y. Zhao provided essential discussion. Q. Zheng and Z. Li analyzed the data. Y. Zhao, T.Y. Huang, W. Zhang, and H. Xu wrote the manuscript.

Submitted: 5 July 2017
Revised: 11 January 2018
Accepted: 29 March 2018

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