Retinoic Acid-Elicited RARα/RXRα Signaling Attenuates Aβ Production by Directly Inhibiting γ-Secretase-Mediated Cleavage of Amyloid Precursor Protein

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ABSTRACT: Retinoic acid (RA)-elicited signaling has been shown to play critical roles in development, organogenesis, and the immune response. RA regulates expression of Alzheimer's disease (AD)-related genes and attenuates amyloid pathology in a transgenic mouse model. In this study, we investigated whether RA can suppress the production of amyloid-β (Aβ) through direct inhibition of γ-secretase activity. We report that RA treatment of cells results in significant inhibition of γ-secretase-mediated processing of the amyloid precursor protein C-terminal fragment APP-C99, compared with DMSO-treated controls. RA-elicited signaling was found to significantly increase accumulation of APP-C99 and decrease production of secreted Aβ40. In addition, RA-induced inhibition of γ-secretase activity was found to be mediated through significant activation of extracellular signal-regulated kinases (ERK1/2).

Treatment of cells with the specific ERK inhibitor PD98059 completely abolished RA-mediated inhibition of γ-secretase. Consistent with these findings, RA was observed to inhibit secretase-mediated proteolysis of full-length APP. Finally, we have established that RA inhibits γ-secretase through nuclear retinoic acid receptor-α (RARα) and retinoid X receptor-α (RXRα). Our findings provide a new mechanistic explanation for the neuroprotective role of RA in AD pathology and add to the previous data showing the importance of RA signaling as a target for AD therapy.

KEYWORDS: Alzheimer's disease, retinoic acid, γ-secretase, amyloid-β, APP-C99, ERK, amyloid precursor protein
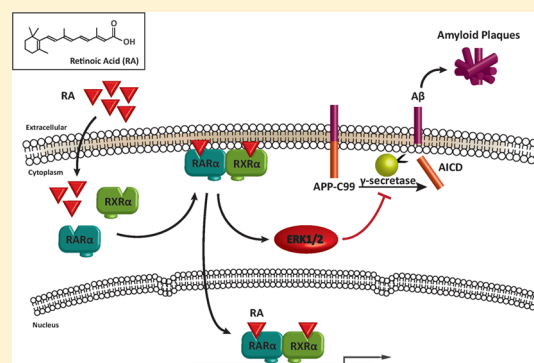
Alzheimer's disease (AD) poses a serious global health problem, which is expected to worsen if effective therapies are not identified.[1] AD is caused by a deposition of cytotoxic amyloid-β (Aβ) peptides in the brain, which are generated through sequential cleavages of amyloid precursor protein (APP) by a group of membrane-localized enzymes, known as secretases.[2−4] In the amyloidogenic pathway, APP is first cleaved by the β-site APP-cleaving enzyme 1 (BACE1), leading to the release of the extracellular APP domain as soluble APPβ (sAPPβ) and generating a membrane-bound C-terminal fragment of 99 amino acids, C99 (β-CTF). APP-C99 is further cleaved by γ-secretase to generate Aβ and the APP intracellular domain (AICD). The γ-secretase enzyme complex consists of four core subunits: presenilin (PS1 or PS2), nicastrin (NCT), anterior pharynx defective-1 (Aph-1), and presenilin enhancer-2 (Pen-2).[5,6] In the nonamyloidogenic pathway, α-secretase cleaves APP within the Aβ domain, thereby precluding Aβ

generation. Such cleavage releases the extracellular domain of APP in the form of soluble APPα (sAPPα), leaving behind a membrane-bound C-terminal fragment of 83 amino acids, C83 (α-CTF). APP-C83 is subsequently cleaved by γ-secretase to generate a small 24−26-amino acid peptide, p3, and AICD.[7] AICD forms a ternary complex with Fe65 and Tip60 and translocates into the nucleus to regulate gene expression.[8,9]

Retinoic acid, a derivative of vitamin A (retinol), is a signaling molecule that regulates several biological processes in vertebrates, including development, organogenesis, the immune response, and cell−cell signaling.[10−13] Because humans cannot synthesize vitamin A, it must be absorbed through the diet as
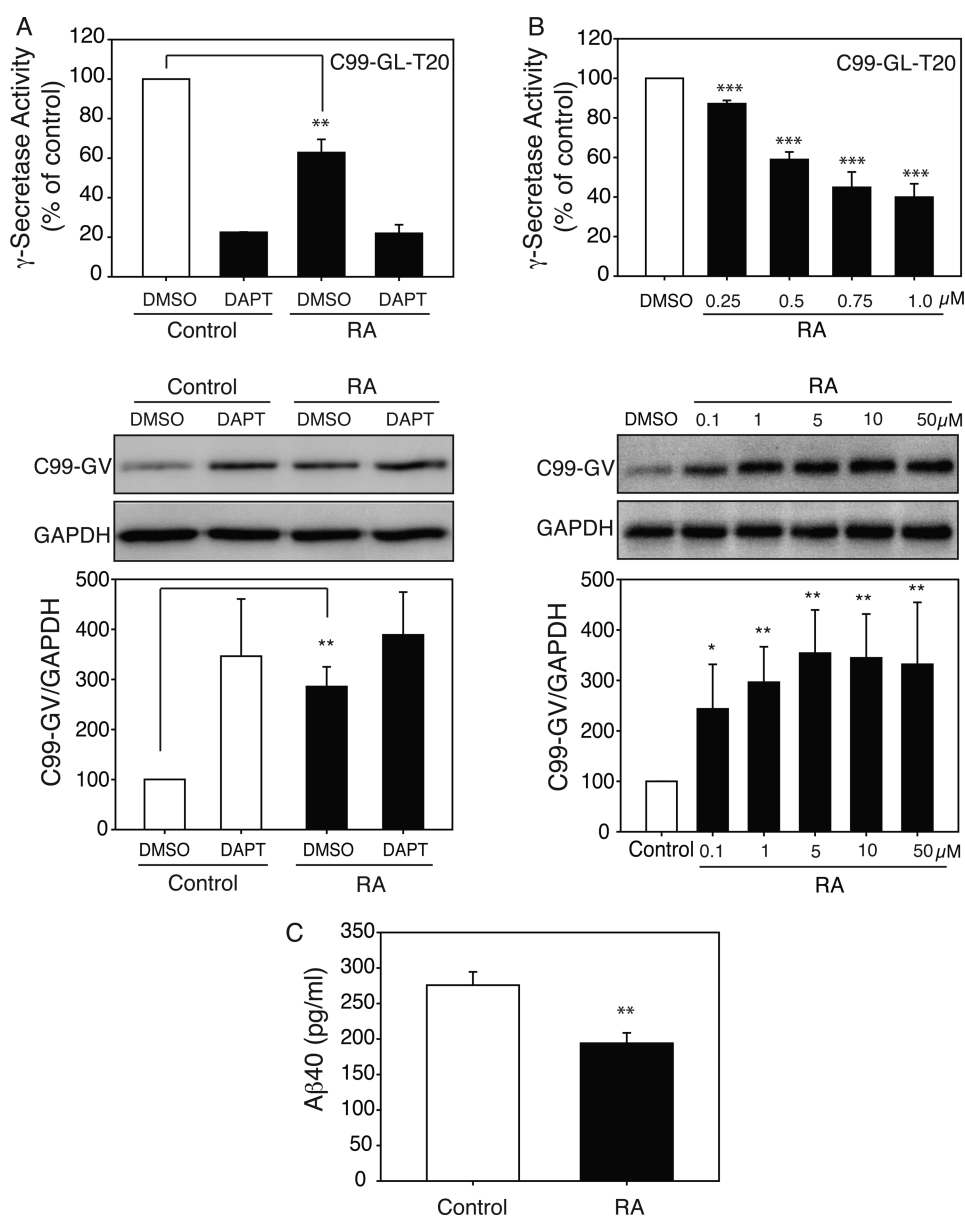
Figure 1. Retinoic acid inhibits γ-secretase activity. (A) T20 cells were treated with either 0.1% DMSO (control, open bar) or 1 μM RA (solid bar) in the presence or absence of a known γ-secretase inhibitor DAPT (5 μM) for 24 h. The resulting γ-secretase activity was subsequently determined using the Steady-Glo luciferase assay reagent (histogram at top). Levels of the γ-secretase substrate C99-GV were determined by Western blotting with an anti-Gal4 antibody (upper panel); GAPDH levels were determined as an internal control (lower panel). The level of C99-GV normalized to that of GAPDH (C99-GV/GAPDH) in DMSO-treated control cells was defined as 100% relative abundance (histogram at bottom). (B) T20 cells were treated with the indicated doses of RA, and γ-secretase activity (histogram at top) and APP-C99-GV levels (middle and histogram at bottom) were determined as described above. A dose-dependent effect of RA was observed. (C) Levels of secreted Aβ40 in conditioned media from T20 cells treated with 0.1% DMSO (open bar) or 1 μM RA (solid bar) for 24 h. Levels were determined using a quantitative Aβ40 ELISA kit. Quantitative results are expressed as the mean ± SD from at least three independent experiments and were analyzed by Student's t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

carotenoids or retinyl esters from plants and animals. In the first step of RA biosynthesis, retinol is oxidized to retinaldehyde by alcohol dehydrogenases (ADHs) or retinol dehydrogenases (RDHs). In the second and final step, retinaldehyde is oxidized to RA by retinaldehyde dehydrogenases (RALDHs).[11] Subsequently, RA either translocates into the nucleus of target tissues to regulate gene expression by binding to nuclear receptors or undergoes degradation in nontarget tissues through further oxidation by cytochrome P450 (CYP26) enzymes.[10,11] The first group of RA receptors (RARα, RARβ, and RARγ) is activated by the most abundant form of RA, all-

trans RA, and the second group of receptors, known as retinoid X receptors (RXRα, RXRβ, and RXRγ), binds to the RA isomer 9-cis RA.[14] The first indication that vitamins may be involved in the development of AD came from the study of Zaman et al., which reported that the plasma concentrations of vitamins A, E, and β-carotene were significantly reduced in AD patients compared with age-matched controls.[15] Previous studies have also reported that vitamin A deprivation results in a loss of hippocampal long-term synaptic plasticity in mouse, which is reversed by dietary replenishment of vitamin A.[16] Dietary retinoid deficiency results in significantly increased Aβ

deposition in rats, and RA inhibits $A\beta$ deposition, and rescues memory deficits in a transgenic mouse model of AD.[17,18] Furthermore, retinaldehyde dehydrogenase-2 (RALDH-2), an enzyme involved in RA biosynthesis, was shown to be down-regulated in the brains of AD patients.[19]

Expression of a number of AD-related genes, including those encoding for APP and presenilins, has been shown to be influenced by RA.[20−22] A number of recent studies have found that RA up-regulates expression of APP-processing enzymes; Holback et al. reported that RA treatment of human neuroblastoma SHSY5Y cells enhanced mRNA and protein levels of the α-secretase ADAM10 and the β-secretase BACE1.[23] Two independent groups reported a significant increase in ADAM10 expression and sAPPα secretion after RA treatment, further suggesting that RA has a stimulatory effect on ADAM10 activity.[22,24] Moreover, intracerebral injection of acitretin, a vitamin A analogue, has been reported to inhibit $A\beta$ generation in the APP/PS1−21 double transgenic mouse,[24] consistent with a similar effect of RA in an AD transgenic mouse.[17] A recent study by Jarvis et al. demonstrated that RARα signaling inhibits $A\beta$ generation in vitro via increased ADAM10 expression.[18] Collectively, these studies suggest that RA renders a protective effect against AD by up-regulating the nonamyloidogenic processing of APP through increased ADAM10 expression.

Although the above studies suggest that the protective effects of RA are primarily mediated through the ADAM10-mediated nonamyloidogenic processing of APP, there are contradicting reports on the effects of RA on the levels of C-terminal APP fragments.[17] Ding et al.[17] demonstrated that RA administration reduced both α- and β-CTF in cortical and hippocampal lysates of APP/PS1 double transgenic AD mice, whereas Tippmann et al.[24] observed that RA treatment caused an increase in α-CTF with a concomitant decrease in β-CTF in SHSY5Y-APP695$_{swe}$ cells. Given that γ-secretase catalyzes the final step in the proteoloytic processing of APP, and that β-CTF is a direct substrate for γ-secretase, here we investigated whether RA regulates APP processing and $A\beta$ generation by acting directly on γ-secretase. We employed several cell-based reporter assays to monitor the effect of RA on γ-secretase-mediated cleavage of APP-C99 and on $A\beta$ generation. We demonstrate here, for the first time, that RA acting via RARα/RXRα directly inhibits γ-secretase-mediated processing of APP-C99, thereby decreasing $A\beta$ generation. Furthermore, we identify ERK kinase as an important mediator of γ-secretase inhibition by RA.

■ RESULTS

RA Inhibits γ-Secretase-Mediated Proteolysis of APP-C99 and $A\beta$ Generation. We first examined the role of RA in γ-secretase-mediated proteolysis of APP-C99, by treating T20 cells stably overexpressing tetracycline-inducible APP-C99-Gal4VP16 (C99-GV) and a Gal4-luciferase (Gal4-Luc) reporter with 0.1% DMSO (control) or 1 μM all-trans RA. We report that RA treatment for 24 h significantly decreases (by approximately 40%) cellular γ-secretase activity, compared with controls (Figure 1A). We further corroborated the inhibitory effect of RA by determining the levels of the γ-secretase substrate C99-GV in control and RA-treated cells. C99-GV levels in RA-treated cells were increased by 286% (24 h) compared with those of controls (Figure 1A, middle and lower panels), indicative of reduced cleavage of C99-GV. Furthermore, the effects of RA on γ-secretase activity and C99-GV accumulation were dose-dependent (Figure 1B). In

addition, RA treatment decreased extracellular levels of secreted $A\beta$40 by about 30% (Figure 1C), consistent with its inhibitory effect on γ-secretase activity. Collectively, these results indicate that RA regulates γ-secretase-mediated generation of $A\beta$.

ERK Activation Is Required for RA-Mediated Inhibition of γ-Secretase Activity. Retinoic acid has been shown to regulate a number of signaling kinases, including ERK, JNK, and PKC.[25−27] ERK is a well-known negative regulator of γ-secretase and is activated by RA treatment in a variety of cells, including chicken retinal cells,[28] embryonic stem cells,[29] neuronal cells,[30] and human myeloblastic leukemia cells.[27,31] We therefore hypothesized that RA-mediated suppression of γ-secretase activity may require ERK activation by RA. To test this hypothesis, we treated T20 cells with 0.1% DMSO (control) or 1 μM RA in the presence or absence of the specific and potent MEK/ERK inhibitor PD98059 and determined cellular γ-secretase activity. As previously observed, RA treatment significantly attenuated γ-secretase activity, but this was rescued by cotreatment with PD98059, demonstrating that RA-mediated suppression of γ-secretase activity requires active ERK (Figure 2A). In addition, RA treatment significantly increased the levels of phospho-ERK, which was blocked by PD98059 coapplication (Figure 2B).

RA Inhibits Proteolysis of Full-Length APP in an ERK-Dependent Manner. After establishing that RA inhibits γ-secretase-mediated proteolysis of APP-C99, we proceeded to investigate the effect of RA on the proteolytic processing of full-length APP. APP-GL-T16 cells stably expressing a Gal4/VP16-tagged full-length APP (APP695-GV) construct, a Gal4-Luc reporter were treated with 0.1% DMSO (control) or varying doses of RA for 24 h, and APP proteolysis was determined by measuring AICD-GV-mediated luciferase expression. We found that RA treatment significantly decreased (approximately 35% decrease at 1 μM RA) secretase-mediated proteolysis of APP (Figure 3A). We further investigated the role of ERK in secretase-mediated proteolysis of full-length APP. Control or RA-treated T16 cells were cotreated with either DMSO or PD98059, and APP proteolysis was determined after 24 h. Treatment of cells with PD98059 resulted in a significant increase in APP proteolysis in both control and RA-treated cells (Figure 3B), suggesting that ERK activation inhibits APP proteolysis. These results demonstrate that RA negatively regulates APP proteolysis through an ERK-dependent pathway.

RA Mediated Suppression of γ-Secretase Requires Retinoic Acid Receptors RARα and RXRα. RA signaling is mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which form heterodimers.[11] We next investigated the role of these RA receptors in RA-mediated suppression of γ-secretase activity. The recent report by Jarvis et al. demonstrated that RARα signaling is an important regulator of $A\beta$ levels in neuronal cultures and in the Tg2576 mouse.[18] We therefore investigated whether RARα signaling is also involved in RA-mediated inhibition of γ-secretase. We used lentiviral shRNAs to knockdown RARα and RXRα in T20 cells and determined the effect of RA on γ-secretase activity. Knockdown of both RARα and RXRα significantly mitigated RA-mediated inhibition of γ-secretase activity (Figure 4A), demonstrating that RARα/RXRα signaling plays an important role in RA-mediated regulation of γ-secretase and APP processing. Two independent shRNAs were used for knockdown of both RARα (Figure 4B) and RXRα (Figure 4C), and for each gene, each shRNA resulted in about 65−70% knockdown (Figure 4B,C).

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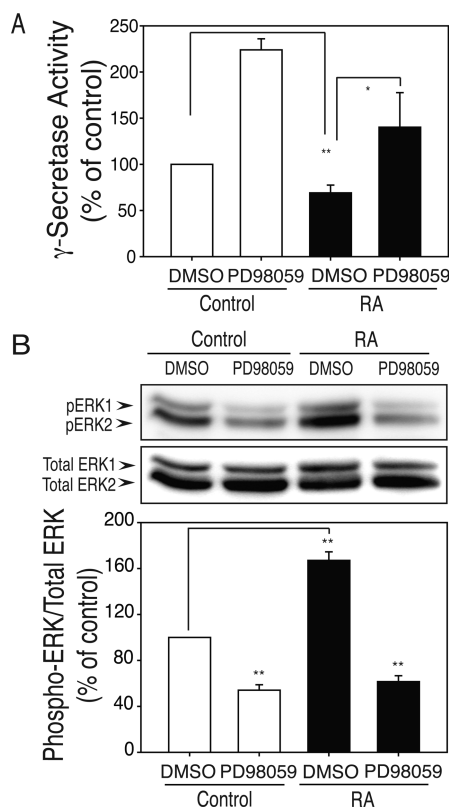dx.doi.org/10.1021/cn400039s | ACS Chem. Neurosci. 2013, 4, 1093−1100

Figure 2. Retinoic acid suppresses γ-secretase activity through activation of ERK. (A) T20 cells were treated with 0.1% DMSO (control) or 1 μM RA in the presence or absence of the ERK inhibitor PD98059 (10 μM) for 24 h, and γ-secretase activity was determined using the Steady-Glo luciferase assay reagent. The luciferase signal of DMSO-treated control cells was normalized to the protein content of the clarified lysate and defined as 100% relative γ-secretase activity (histogram). (B) The clarified lysates utilized in panel A were analyzed by Western blotting with anti-phospho-ERK (upper panel) and anti-total ERK (lower panel) antibodies. Levels of phosphorylated ERK normalized to total ERK in DMSO-treated control cells were defined as 100% ERK activation (histogram). Quantitative results are expressed as the mean ± SD from three independent experiments and were analyzed by Student's t test. ∗ p < 0.05; ∗∗ p < 0.01.

■ DISCUSSION

Retinoid-based drugs have been suggested as potential therapeutics for the treatment of AD.[32,33] RA inhibits Aβ deposition in murine models of AD,[17,22] and there are several mechanisms that may underlie the neuroprotective role of RA. These include enhancing ADAM10-mediated nonamyloidogenic processing of APP by increasing ADAM10 expression and regulating the expression of other AD-related genes, including APP, PS1, and BACE1. In the current study, we have established that RA regulates Aβ generation by directly inhibiting γ-secretase-mediated cleavage of APP-C99. RA treatment significantly decreased γ-secretase-dependent luciferase expression in cells overexpressing either APP-C99 (the immediate substrate of γ-secretase) or full-length APP. We also determined the molecular mechanism underlying the inhibitory effect of RA on γ-secretase activity.

A number of recent studies from various groups, including ours, have demonstrated differential roles of mitogen-activated protein kinases (MAPKs) in regulating γ-secretase-mediated Aβ production. While c-Jun-N-terminal kinase (JNK) positively regulates γ-secretase, another MAPK family member, ERK, has
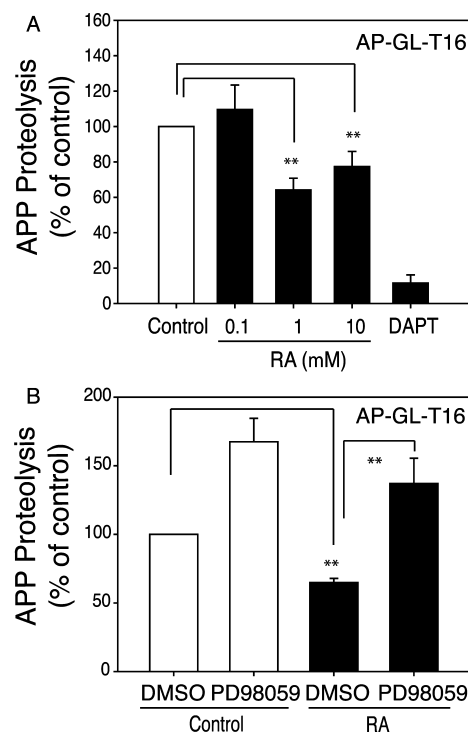

Figure 3. Secretase-mediated proteolysis of full-length APP is attenuated by retinoic acid through an ERK-dependent pathway. (A) T16 cells were treated with 0.1% DMSO (open bar) or the indicated doses of RA (solid bar) for 24 h. Cells treated with the γ-secretase inhibitor DAPT were included as controls. Luciferase reporter expression due to secretase-mediated APP proteolysis in treated cells was determined using the Steady-Glo luciferase assay reagent. The luciferase signal of DMSO-treated control cells was normalized to the protein content of the clarified lysate and defined as 100% relative APP proteolysis. (B) T16 cells were treated with DMSO or RA in the presence or absence of an ERK inhibitor, PD98059 (10 μM), for 24 h. Luciferase expression was determined and normalized as described above. Quantitative results are expressed as the mean ± SD from three independent experiments and were analyzed by Student's t test. ∗ p < 0.05; ∗∗ p < 0.01.

been shown to be a negative regulator of γ-secretase activity and Aβ generation.[34−37] We recently demonstrated that ERK-mediated phosphorylation of the γ-secretase components nicastrin (NCT) and presenilin (PS1) results in a dramatic reduction in the proteolytic activity of this protease, accompanied by a marked decrease in Aβ generation.[37] Interestingly, several earlier studies reported that RA activates ERK, consistent with our current finding that RA significantly increases ERK phosphorylation in a dose-dependent manner, without affecting ERK expression (data not shown). We also demonstrated that RA inhibits γ-secretase activity via ERK phosphorylation, because treatment with the ERK inhibitor PD98059 prevents the inhibitory effect. These results are in accordance with those of previous studies, which demonstrated negative regulation of γ-secretase by ERK activation. Our findings thus demonstrate that RA could render protective effect against AD through directly inhibiting γ-secretase activity in an ERK-dependent manner.

Retinoic acid receptors (RARs) and retinoid-X receptors (RXRs) are important mediators of RA signaling, and Jarvis et al. recently reported that RARα signaling is involved in regulating Aβ generation.[18] Our results demonstrate that RARα and RXRα receptors are required for RA-mediated suppression
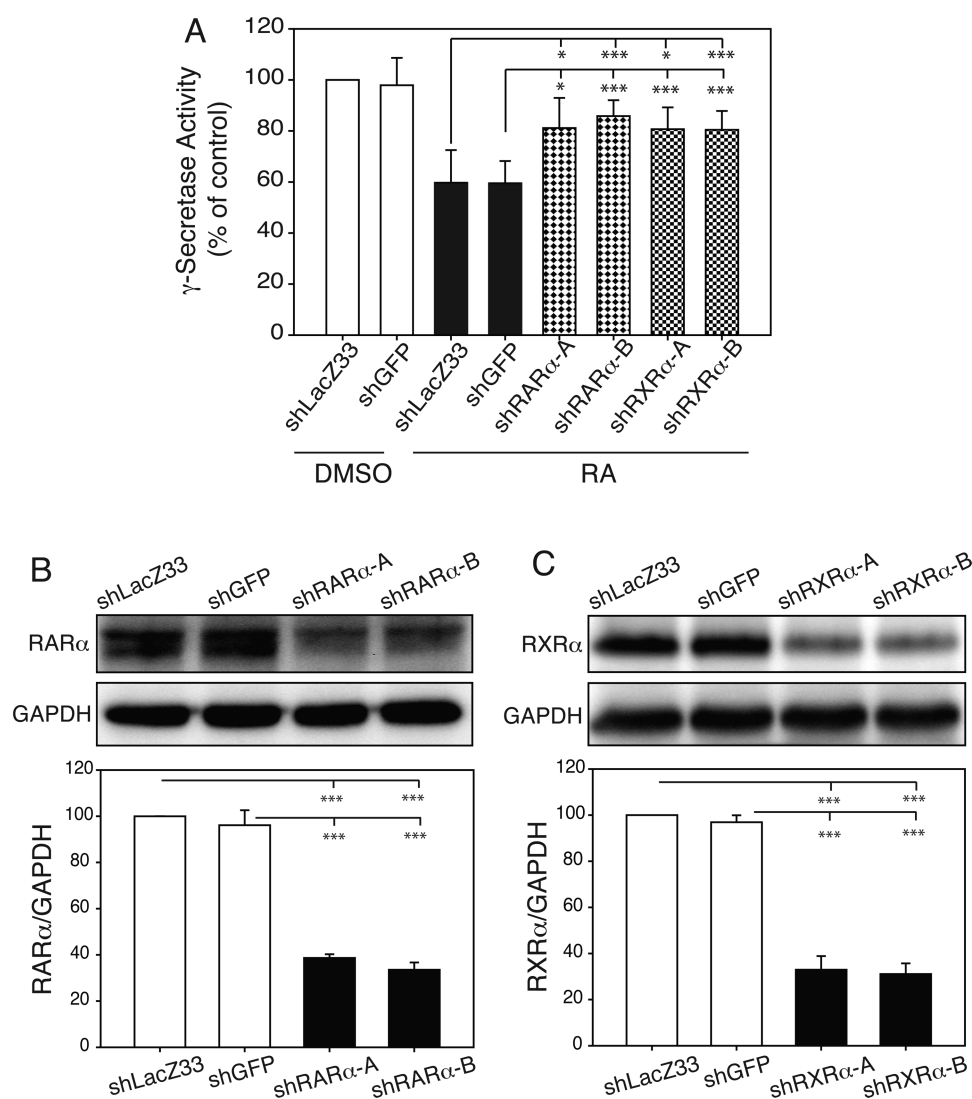
Figure 4. RARα and RXRα mediate the inhibitory effect of RA on γ-secretase. (A) T20 cells were infected with lentiviruses expressing control shRNAs (shLacZ33 and shGFP), shRNAs targeting RARα (shRARα-A or shRARα-B), or shRNAs targeting RXRα (shRXRα-A or shRXRα-B). Following the replacement of media after 8 h with fresh DMEM containing 10% FBS, the cells were incubated for a further 24 h. After 24 h, the cells were treated with either 0.1% DMSO (control, open bar) or 1 μM RA (solid bar) for another 24 h, and γ-secretase activity was determined using Steady-Glo luciferase assay reagent. Knockdown of either RARα (diamond bars) or RXRα (checkered bar) prevented the inhibitory effect of RA on γ-secretase activity. (B,C) Lentiviral shRNA-mediated knockdown of RARα and RXRα, respectively. Two independent shRNAs were used for the knockdown of both RARα and RXRα; each shRNA resulted in about 65−70% knockdown, whereas control shRNAs had no effect on RARα or RXRα proteins. Quantitative results are expressed as the mean ± SD from three independent experiments and were analyzed by Student's t test. ∗∗ p < 0.01; ∗∗∗p < 0.001.

of γ-secretase activity. Our study provides the first direct evidence that, in addition to up-regulating ADAM10-mediated nonamyloidogenic processing of APP and regulating the expression of other AD-related genes, RA also regulates Aβ generation by directly inhibiting the final step of APP processing catalyzed by γ-secretase. Our findings are consistent with a recent report demonstrating that a RARα agonist can reverse Aβ-mediated inhibition of RA synthesis, thereby alleviating cognitive deficits in the Tg2576 AD mouse.[38] Future development of retinoid-derived anti-AD drugs will necessitate detailed analysis of the roles of other retinoids and their derivatives in regulating Aβ generation through γ-secretase-dependent or -independent mechanisms. RA also modulates expression of an Aβ-degrading enzyme, the insulin-degrading enzyme (IDE), in human neuroblastoma cells[39] and stimulates neprilysin (NEP, another Aβ-degrading enzyme) activity in the

osteogenic cell line MBA-15.[40] Given that Aβ-elicited neurotoxicity can result in RA depletion in the AD brain, RA-related RARα/RXRα agonists may exert synergistic neuroprotective effects that alleviate AD pathology, by simultaneously restoring the expression of IDE and NEP and suppressing γ-secretase activity. Our results not only provide further supporting evidence for the protective role of RA in AD but also provide a mechanistic explanation as to how RA-elicited signaling regulates Aβ generation.

■ METHODS

Cell Culture and Cell Lines. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1 mg/mL penicillin and streptomycin. T-REx293 cells were purchased from Invitrogen and cultured in DMEM supplemented with 10% FBS and 5

μg/mL blasticidin. Stable transfection of cell lines C99-GL-T20 (T20) and APP-GL-T16 (T16) was performed as described previously.[36,41−43] Briefly, C99-GL-T20 cells were stably transfected with a tetracycline-inducible APP-C99-Gal4/VP16 expression vector and a Gal4-luciferase reporter gene construct. APP-GL-T16 cells were stably transfected with a tetracycline-inducible APP695-Gal4/VP16 and a Gal4-luciferase reporter gene construct. T20 and T16 cells were maintained in DMEM supplemented with 10% FBS, 200 μg/mL hygromycin, 5 μg/mL blasticidin, and 250 μg/mL zeocin (DMEM-HZB). Cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

Reagents. BCA protein assay reagent kit, SuperSignal West Dura extended duration substrate, and SuperSignal West Pico chemiluminescent substrate were purchased from Pierce Chemical (Rockford, IL). Mouse anti-GAL4, rabbit anti-GAPDH, rabbit anti-RARα, rabbit anti-RXRα, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were from Cell Signaling Technology. HRP-conjugated anti-mouse IgG and ECL Western blotting detection reagent were from GE Healthcare. Steady-Glo luciferase assay reagent was purchased from Promega. Human Aβ40 colorimetric ELISA kit (Cat. No. KHB3482), Lipofectamine 2000 transfection reagent, FBS, and DMEM were purchased from Invitrogen. All other reagents were of reagent grade or above and were obtained from standard suppliers.

Lentivirus-Mediated Knockdown of RARα and RXRα. Lentiviral short hairpin RNA (shRNA) clones targeting human RARα and RXRα or control sequences (LacZ33 and GFP) were purchased from the National RNAi Core Facility, Academia Sinica, Taiwan. The clone ID numbers for the shRNA plasmids were as follows: TRCN0000072233 (shLacZ33); TRCN0000072181 (shGFP); TRCN0000020370 (shRARα-A); TRCN0000020371 (shRARα-B); TRCN0000021614 (shRXRα-A); TRCN0000021618 (shRXRα-B). To generate shRNA-carrying lentiviral particles, 293T cells were cotransfected with shRNA packaging plasmid (pCMV-ΔR8.91) and envelope plasmid pMDG using the calcium phosphate precipitation method. After 24 h of transfection, the medium was replaced with DMEM containing 5% FBS. The viral particles in the culture media were harvested 48 h later and stored at −80 °C until use. For knockdown of RARα or RXRα, T20 cells were plated onto 12-well plates and incubated overnight; shRNA-expressing lentiviruses (MOI = 1) were then added to the cells. After 8 h incubation at 37 °C, the media was replaced with fresh media, and the cells were incubated for another 48 h. The levels of RARα and RXRα were determined in total cell lysates.

Cell-Based γ-Secretase Assay. The quantitative measurement of γ-secretase activity in T20 and T16 stable cell lines was performed as described previously.[36,42,43] To examine the effect of RA on γ-secretase activity, T20 and T16 cells were washed with PBS, trypsinized, resuspended in DMEM, plated (2 × 10⁴ cells/well) onto 96-well plates, and incubated overnight. The cells were then treated with 0.1% DMSO (control) or various concentrations (0.1−10 μM) of all-*trans* RA with or without 1 μg/mL tetracycline, and incubated at 37 °C for 24 h. Cells treated with 1 μM DAPT (a γ-secretase inhibitor) were included as controls. After 24 h, an equal volume of Steady-Glo luciferase assay reagent was added to each well, and the resulting luciferase signals were measured immediately using a luminescence plate reader. For some experiments, cells were plated onto 6 well plates (4 × 10⁵ cells/well) and treated with RA for 24 h. Cells were washed with 1× PBS before being harvested with 1× PBS containing 20 mM EDTA. Following rinse in 1× PBS, cell pellets were lysed in 100 μL of 1× passive lysis buffer (PLB, Promega). Cell debris was removed by centrifugation at 13 200g for 5 min, and 20 μL of lysate was mixed with 20 μL of Steady-Glo luciferase assay reagent in 96-well LumiNunc microplates (Nunc, Rochester, NY). A VictorLight microplate luminometer was used to measure the luminescence emitted by individual microwells (PerkinElmer). Luminescence was normalized to the protein content of the lysate. The normalized luciferase signal emitted by control (DMSO-treated) cells in tetracycline-free culture media was defined as one fold of activation.

For determining the role of the RA receptors RARα and RXRα in RA-mediated inhibition of γ-secretase activity, T20 cells were plated (0.5 × 10⁴ cells/well) onto 96-well plates and cultured overnight. shRNA-encoding lentiviruses were then added to the cells (MOI = 1), and the cells were cultured for 8 h; at this time, the media was replaced with fresh. After a further 24 h of incubation, the cells were treated with 0.1% DMSO or RA in the presence or absence of 1 μg/mL tetracycline; the cells were then incubated at 37 °C for a further 24 h. After the incubation period, luciferase activity was determined as described above.

To determine the efficiency of shRNA-encoding lentiviruses, T20 cells were plated (2 × 10⁴ cells/well) onto 12 well plates and cultured overnight. Lentiviruses were then added to the cells (MOI = 1), and the cells were incubated for 8 h. The media was replaced with fresh, and the cells were incubated for a further 48 h. After this time, the cells were washed with 1× PBS before being harvested with 1× PBS containing 20 mM EDTA and lysed in 100 μL of 1× PLB. The levels of RARα and RXRα in total cell lysates were determined using SDS−PAGE and immunoblotting with RARα- and RXRα-specific antibodies. These levels were compared with those of GAPDH to determine the knockdown efficiency of gene-targeting shRNA lentiviruses.

SDS−Polyacrylamide Gel Electrophoresis and Immunoblot Analysis. These procedures have been described in detail previously.[35,36,43] Briefly, clarified cell lysates containing equivalent amount of proteins were mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-HCL, pH 6.8, 4% SDS, 20% glycerol, and 5% β-mercaptoethanol) and boiled at 100 °C for 10 min. Denatured proteins were resolved on Tris-glycine polyacrylamide gels (10−12%) and transferred to poly(vinylidene difluoride) (PVDF) membranes by electroblotting (Immobilon-P; Millipore Corporation, Billerica, MA). Membranes were incubated in blocking solution (5% nonfat dry milk and 0.1% Tween-20 in PBS (PBST)) for 1 h, washed three times with PBST, and incubated with appropriately diluted primary antibodies at 4 °C overnight. Membranes were washed three times with PBST to remove unbound primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies in PBST for 1 h at room temperature. Following extensive washing with PBST, antibody-reactive protein bands were visualized by chemiluminescence using SuperSignal West Dura or Pico reagents (Pierce Chemical). To determine levels of phospho-ERK (pERK), TBS containing 0.1% Tween-20 (TBST) was substituted for PBST. The antibodies used were as follows: mouse anti-Gal4, 1:1000; rabbit anti-GAPDH, 1:1000; rabbit anti-phospho-ERK, 1:1000; rabbit anti-ERK, 1:1000; rabbit anti-RARα, 1:1000; rabbit anti-RXRα, 1: 1000; horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences), 1:10 000; and horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz), 1:1000.

Aβ ELISA. Detection of Aβ in conditioned media has been described in detail previously.[35,36,43] Briefly, to determine the effect of RA on γ-secretase-dependent Aβ production, T20 cells were treated with 0.1% DMSO (control) or 1 μM RA, and incubated at 37 °C for 48 h. The conditioned media were harvested, clarified by centrifugation, supplemented with Complete protease inhibitor cocktail (Roche Applied Science), and stored at −80 °C until ready for use. The levels of secreted Aβ40 in the conditioned media were determined using a quantitative human Aβ40 sandwich ELISA kit (Invitrogen) according to the manufacturer's instructions. The conditioned media of T20 cells without tetracycline treatment were included as blanks.

Quantitative Densitometry and Statistical Analysis. Quantitative analysis of immunoblots was conducted with Image J (NIH) software, by determining the relative intensity of the immunoreactive bands after acquisition of the blot image with ChemiGenus2 (Syngene). Statistical analyses were performed using one-tailed Student's t test. A value of $p \leq 0.05$ was considered significant.

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Author Contributions

A.K., B.-J.W., S.-M.L., and Y.-F.L. conceived and designed the experiments. A.K. and B.-J.W. performed the experiments. A.K., B.-J.W., M.-Y.C., and Y.-F.L. analyzed the data. W.-M.H contributed reagents, materials, and analysis tools. A.K., S.-M.L., and Y.-F.L. wrote the paper. A.K. and B.-J.W. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

$A\beta$, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; C99-CTF, 99-residue C-terminal fragment of APP; AICD, amyloid precursor protein intracellular domain; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; NCT, nicastrin; PS, presenilin; PBS, phosphate-buffered saline; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay

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