RNA Interference Silencing of the Adaptor Molecules ShcC and Fe65 Differentially Affect Amyloid Precursor Protein Processing and Aβ Generation*  

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The amyloid precursor protein (APP) and its pathogenic by-product amyloid-β protein (Aβ) play central roles in Alzheimer disease (AD) neuropathogenesis. APP can be cleaved by β-secretase (BACE) and α-secretase to produce APP-C99 and APP-C83. These C-terminal fragments can then be cleaved by γ-secretase to produce Aβ and p3, respectively. p3 has been reported to promote apoptosis, and Aβ is the key component of senile plaques in AD brain. APP adaptor proteins with phosphotyrosine-binding domains, including ShcA (SHC1), ShcC (SHC3), and Fe65 (APBB1), can bind to and interact with the conserved YENPTY motif in the APP-C terminus. Here we have described for the first time the effects of RNA interference (RNAi) silencing of ShcA, ShcC, and Fe65 on APP processing and Aβ production. RNAi silencing of ShcC led to reductions in the levels of APP-C-terminal fragments (APP-CTFs) and Aβ in H4 human neuroglioma cells stably overexpressing full-length APP (H4-FL-APP cells) but not in those expressing APP-C99 (H4-APP-C99 cells). RNAi silencing of ShcC also led to reductions in BACE levels in H4-FL-APP cells. In contrast, RNAi silencing of the homologue ShcA had no effect on APP processing or Aβ levels. RNAi silencing of Fe65 increased APP-CTF levels, although also decreasing Aβ levels in H4-FL-APP cells. These findings suggest that pharmacologically blocking interaction of APP with ShcC and Fe65 may provide novel therapeutic strategies against AD.

The current treatments for Alzheimer disease (AD), the most prevalent cause of dementia in the elderly, provide only modest, temporary, and palliative benefits (1). This is because current treatments do not target disease progression and, particularly, cerebral accumulation of the amyloid-β protein (Aβ), the key component of senile plaques in AD neuropathology. Aβ is generated via serial proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase (BACE) and γ-secretase. Specifically, full-length (FL) APP is first hydrolyzed by BACE to generate a 99-residue membrane-associated C-terminal fragment (CTF) (APP-C99) (2–5). APP-C99 is further cleaved to release an ~4-kDa peptide, Aβ, and the amyloid precursor protein intracellular domain. This cleavage is achieved by an unusual form of proteolysis in which the protein is cleaved within the transmembrane domain (at residue +40 or +42) by γ-secretase (6–8). The majority of APP is cleaved by α-secretase in the middle of the Aβ region of APP, precluding Aβ generation and leading to the release of a large ectodomain (α-APPs), leaving behind a carboxyl-terminal fragment of 83 amino acids (APP-C83) in the membrane. Although proteolysis of APP-C99 by γ-secretase produces Aβ, proteolysis of APP-C83 by γ-secretase produces p3, an amino-terminally truncated form of Aβ (9–11), which has been shown to induce apoptosis mediated by activation of c-Jun N-terminal kinase, caspase 8, and caspase 3 (12). The cleavage of the APP cytoplasmic tail by γ-secretase generates the amyloid precursor protein intracellular domain, which contains the strongly conserved YENPTY motif, which is also present in the cytodomains of several tyrosine-kinase receptors and in nonreceptor tyrosine kinase. The YENPTY sequence is a consensus motif for the YENPTY motif, which is also present in the cytodomains of several tyrosine-kinase receptors and in nonreceptor tyrosine kinase. The YENPTY sequence is a consensus motif for the binding of adaptor proteins that possess a phosphotyrosine-binding domain present in several APP adaptor proteins, such as the X11, Fe65, Shc, and JIP families (13). We have previously reported that RNA interference (RNAi) silencing of X11 in H4 human neuroglioma cells increases levels of the APP-CTFs and lowers Aβ levels by attenuating γ-secretase-mediated APP cleavage (14).

ShcA (encoded by gene SHC1) and ShcC (encoded by gene SHC3), the phosphotyrosine-binding domain-containing adaptor proteins that signal to cellular differentiation and survival pathways, are other types of APP adaptor proteins that also bind to and interact with the YENPTY motif of APP (15, 16).

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Activation of Shc signal transduction pathways in vitro has been reported to trigger APP-Shc interaction in glial cells but not in neurons (15). To date, the effects of reduced expression of ShcA and ShcC on APP processing and Aβ production, the key components of AD neuropathogenesis, have not been assessed. For this purpose, we established RNAi for ShcA and ShcC in H4 human neuroglioma cells overexpressing FL-APP (H4-FL-APP cells) and APP-C99 (H4-APP-C99 cells) and evaluated the effects of RNAi-mediated silencing of ShcA and ShcC on APP processing and Aβ production.

Fe65, along with Fe65L1 and Fe65L2, are APP adaptor proteins, which bind to the YENPTY motif of APP via their phosphorytrosine-binding domains (13). Overexpression in Fe65 or Fe65L accelerates secretory processing and maturation of APP and promotes APPs and Aβ secretion in H4-FL-APP cells and in Madin-Darby canine kidney cells (17, 18). However, the effects of RNAi knockdown of APBB1 or Fe65 on APP processing and Aβ production have not been assessed. Therefore, we established RNAi for Fe65 in H4-FL-APP cells and evaluated the effects of RNAi-mediated silencing of Fe65 on APP processing and Aβ production.

EXPERIMENTAL PROCEDURES

Cell Lines—We employed H4 human neuroglioma cells stably transfected to express FL-APP (H4-FL-APP cells) or APP-C99 (H4-APP-C99 cells) in the experiments. Peptide APP-C99 is the product of BACE, which therefore contains α- and γ-but not β-cleavage sites. This cell line provides a valid system to assess whether any effects on APP processing is dependent on BACE-mediated APP processing. The cells were cultured in Dulbecco’s modified Eagle’s medium (high glucose) containing 9% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 200 μg/ml G418.

RNAi and DAPT Treatment—Small interfering RNA (siRNA) duplexes were designed and obtained from Qiagen, Inc. (Valencia, CA) against human SHC1, the gene encoding ShcA (5’AGUUGGAGCGGUAACCUAUU-3’, 5’UUAGGUUACCGUCCACCUU-3’); SHC3, the gene encoding ShcC (5’GCAUUCUGGAAAAGGAGCAA-3’, 5’UUGCUCCUCU-UCCAAAGAUGC-3’); and APBB1, the gene encoding Fe65 (5’AGAUAUGAUUGGUCCAGAA-3’, 5’UUGGAGCAACCUAUUU-3’). Control siRNA (5’UUCUCCGAACGUGU-CAGU-3’, 5’ACGUGACACGUUCGGAA-3’) was also obtained from Qiagen, Inc. siRNAs were transfected into cells by using electroporation (AMAXA, Gaithersburg, MD) as described by Xie et al. (14). Briefly, we mixed 1 million cells, 100 μl of AMAXA electroporation transfection solution, and 10 μl of 20 μM siRNA together and then employed the C-9 program in the AMAXA electroporation device for the cell transfection. The transfected cells were then placed in one of the 6-well plates containing 1.5 ml of cell culture medium. The cells were harvested 48 h after siRNA treatments. DAPT (250 nM, 18 h treatment time), a γ-secretase inhibitor, was employed in the experiments as a positive control.

Cell Lysis and Protein Amount Quantification—Cell pellets were detergent-extracted on ice using immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40) plus protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The lysates were collected, centrifuged at 12,000 revolutions/min for 10 min, and quantified for total proteins by the BCA protein assay kit (Pierce).

Western Blot Analysis of APP Processing—Western blot analysis was performed as described by Xie et al. (19). Briefly, 40 μg of total protein of each sample was subjected to SDS-PAGE using 4–20% gradient Tris/glycine gels under reducing conditions (Invitrogen). Next, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) using a semidry blotter (Bio-Rad) and visualized by chemiluminescence (Amersham Biosciences). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies (rabbit polyclonal antibodies against APP, N-cadherin, Fe65, and ShcC) overnight at 4°C. Following this, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, membranes were developed with enhanced chemiluminescence kits (Amersham Biosciences). Densitometric analysis of the bands was performed with the ImageJ software (National Institute of Health, Bethesda, MD). The levels of ShcA and ShcC were normalized to actin levels as an internal reference.

Cell lines and RNAi treatment conditions were set up as follows: 1) H4-FL-APP cells were transfected to express FL-APP (H4-FL-APP cells) or APP-C99 (H4-APP-C99 cells) and evaluated the effects of RNAi-mediated silencing of ShcA and ShcC on APP processing and Aβ production. 2) Fe65, along with Fe65L1 and Fe65L2, are APP adaptor proteins, which bind to the YENPTY motif of APP via their phosphorytrosine-binding domains (13). Overexpression in Fe65 or Fe65L accelerates secretory processing and maturation of APP and promotes APPs and Aβ secretion in H4-FL-APP cells and in Madin-Darby canine kidney cells (17, 18). However, the effects of RNAi knockdown of APBB1 or Fe65 on APP processing and Aβ production have not been assessed. Therefore, we established RNAi for Fe65 in H4-FL-APP cells and evaluated the effects of RNAi-mediated silencing of Fe65 on APP processing and Aβ production.

FIGURE 1. Effects of ShcC RNAi on ShcC levels in H4-FL-APP cells. In H4-FL-APP cells, ShcC siRNA treatment decreased the levels of ShcC. A, RNAi knockdown of ShcC in Western blot analyses. ShcC immunoblots showed reductions in the ShcC levels in the cells treated with ShcC siRNA (columns 4–6) as compared with control siRNA (columns 1–3). There was no significant difference in the amounts of β-actin in the control siRNA- and ShcC siRNA-treated H4-FL-APP cells. B, the ShcC levels assessed by quantifying ShcC in the Western blot. ShcC siRNA treatment (black bar) significantly decreased the ShcC levels as compared with control siRNA treatment (white bar) (*, p < 0.05) normalized to β-actin.

Nonidet P-40 plus protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The lysates were collected, centrifuged at 12,000 revolutions/min for 10 min, and quantified for total proteins by the BCA protein assay kit (Pierce).
and polyclonal antibody PA1–752 at a ratio of 1:1000 (ABR, Golden, CO) was used to detect Fe65 (90 kDa). Polyclonal antibody ab2077 at a ratio of 1:1000 (Abcam, Cambridge, MA) was used to detect BACE (70 kDa). Antibody A8717 at a ratio of 1:1000 (Sigma) was used to visualize FL-APP (110 kDa), APP-C83 (12 kDa), and APP-C99 (10 kDa) in the Western blot analysis. The intensity of signals was analyzed and quantified using an image program (NIH Image version 1.62). We used the levels of β-actin to normalize the levels of ShcA, ShcC, Fe65, FL-APP, APP-C99, and APP-C83 (i.e. determining the ratio of ShcA amount to β-actin amount) to control for loading differences in total protein amounts. We have presented the changes in the protein levels of ShcA, ShcC, Fe65, FL-APP, APP-C99, and APP-C83 in the cells treated with ShcA, ShcC, or Fe65 siRNAs as the percentage of those in the cells treated with control siRNA.

Quantitation of Aβ Using Sandwich Enzyme-linked Immunosorbert Assay—Following the treatment with saline, control, Fe65, ShcA, or ShcC siRNA-conditioned medium were collected, and secreted Aβ was measured by a sandwich enzyme-linked immunosorbert assay as described by Xie et al. (19).

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FIGURE 2. Effects of ShcC RNAi on APP processing and Aβ levels in H4-FL-APP cells. In H4-FL-APP cells, ShcC siRNA treatment decreased the levels of APP-C83, APP-C99, and Aβ. A, APP processing in Western blot analyses. APP-CTF immunoblotting showed decreases in the levels of APP-C99 and APP-C83 in the cells treated with ShcC siRNA (columns 3 and 4) as compared with control siRNA (columns 1 and 2). γ-Secretase inhibitor DAPT (column 5), the positive control in the experiment, increased the levels of APP-C99 and APP-C83 as compared with control treatment. FL-APP immunoblotting revealed that there was no significant difference in the levels of FL-APP in the cells treated with control siRNA, ShcC siRNA, or DAPT. There was no significant difference in the amounts of β-actin in the control siRNA-, ShcC siRNA- and DAPT-treated H4-FL-APP cells. B, APP processing assessed by quantifying the ratio of APP-C83 to FL-APP in the Western blot. ShcC siRNA treatment (black bar) significantly decreased the ratio of APP-C83 to FL-APP as compared with control siRNA treatment (white bar) (*, p < 0.05) normalized to β-actin. C, APP processing assessed by quantifying the ratio of APP-C99 to FL-APP in the Western blot. ShcC siRNA treatment (black bar) significantly decreased the ratio of APP-C99 to FL-APP as compared with control siRNA treatment (white bar) (*, p < 0.05) normalized to β-actin. D, effects of ShcC RNAi on Aβ levels in H4-FL-APP cells. ShcC siRNA treatment (black bar) decreased both Aβ40 and Aβ42 levels as compared with control siRNA treatment (white bar) (*, p < 0.05) normalized to total protein amount.

and polyclonal antibody PA1–752 at a ratio of 1:1000 (ABR, Golden, CO) was used to detect Fe65 (90 kDa). Polyclonal antibody ab2077 at a ratio of 1:1000 (Abcam, Cambridge, MA) was used to detect BACE (70 kDa). Antibody A8717 at a ratio of 1:1000 (Sigma) was used to visualize FL-APP (110 kDa), APP-C83 (12 kDa), and APP-C99 (10 kDa) in the Western blot analysis. The intensity of signals was analyzed and quantified using an image program (NIH Image version 1.62). We used the levels of β-actin to normalize the levels of ShcA, ShcC, Fe65, FL-APP, APP-C99, and APP-C83 (i.e. determining the ratio of ShcA amount to β-actin amount) to control for loading differences in total protein amounts. We have presented the changes in the protein levels of ShcA, ShcC, Fe65, FL-APP, APP-C99, and APP-C83 in the cells treated with ShcA, ShcC, or Fe65 siRNAs as the percentage of those in the cells treated with control siRNA.

Quantitation of Aβ Using Sandwich Enzyme-linked Immunosorbert Assay—Following the treatment with saline, control, Fe65, ShcA, or ShcC siRNA-conditioned medium were collected, and secreted Aβ was measured by a sandwich enzyme-linked immunosorbert assay as described by Xie et al. (19).

Specifically, 96-well plates were coated with mouse monoclonal antibodies specific to Aβ40 (2G3) or Aβ42 (21F12). Following blocking with Block Ace, the wells were incubated overnight at 4°C with test samples of conditioned cell culture medium, and then an anti-Aβ (α-Aβ-HR1) conjugated to horse-radish peroxidase was added. Plates were then developed with tetramethylbenzidine reagent, and well absorbance was measured at 450 nm. Aβ levels in test samples were determined by comparison with the signal from unconditioned medium spiked with known quantities of Aβ40 and Aβ42.

Statistics—Analysis of variance with repeated measurements was employed to compare the difference from the control group. p values <0.05 were considered statistically significant.

RESULTS

ShcC RNAi Decreased the Levels of APP-CTFs and Aβ in H4-FL-APP Cells—We first established conditions under which RNAi silencing of ShcC significantly reduced ShcC levels in H4-FL-APP cells. The cells were harvested 48 h after being transfected with either control siRNA or ShcC siRNA and were subjected to Western blot analyses in which antibody ShcC was used to visualize the ShcC levels. Immunoblotting for ShcC revealed a visible reduction in the ShcC levels following ShcC siRNA treatment as compared with control siRNA treatment (Fig. 1A). ShcC siRNA treatment significantly reduced ShcC levels by 41% (normalized to β-actin) as compared with control siRNA treatment (Fig. 1B).

Next, we assessed the effects of RNAi-mediated silencing of ShcC on APP processing in H4-FL-APP cells by measuring protein levels of FL-APP, APP-C99, and APP-C83 following ShcC siRNA treatment. 48 h after transfection of ShcC siRNA or control siRNA, the cells were harvested and subjected to Western blot analyses in which antibody A8717 was used to detect FL-APP, APP-C99, and APP-C83. Immunoblot analysis of APP-CTFs revealed decreases in the levels of APP-C99 and APP-C83 in the cells treated with ShcC siRNA as compared with control siRNA (Fig. 2A). As a positive control, the γ-secre-tase inhibitor DAPT was employed to induce the accumulation of APP-C99 and APP-C83. Meanwhile, no significant differences in the FL-APP levels were observed between ShcC siRNA-, control siRNA- and DAPT-treated cells. Quantifica-
Effects of ShcC RNAi on APP processing and Aβ levels in H4-APP-C99 cells—To determine whether RNAi silencing of ShcC can decrease levels of APP-C83, APP-C99, and Aβ, we employed H4-APP-C99 cells. APP-C99 is the product of BACE and harbors α- and γ-cleavage but not β-cleavage sites. 48 h after transfection with either ShcC siRNA or control siRNA in H4-APP-C99 cells, the cells were harvested and subjected to Western blot analyses as described for ShcC in H4-FL-APP cells above. Immunoblotting for APP with antibody A8717 revealed no significant difference in the FL-APP levels in ShcC siRNA- versus control siRNA-treated cells (Fig. 3A). Likewise, ShcC siRNA treatment did not alter levels of APP-C99 or APP-C83 as compared with control siRNA treatment (Fig. 3, A–C).

Next, we measured Aβ levels in the conditioned medium 48 h following treatments with control siRNA or ShcC siRNA, as compared with control siRNA normalized to total protein amount (Fig. 3D). Taken together, these findings suggest that the effects of RNAi silencing of ShcC on APP processing and Aβ generation are at least partially dependent on BACE cleavage of APP.

RNAi Silencing of ShcC Decreased the Levels of BACE in H4-FL-APP Cells—To further determine whether RNAi silencing of ShcC can decrease levels of APP-CTFs and Aβ by reducing levels of BACE, we next assessed the effects of RNAi silencing of ShcC on BACE levels in H4-FL-APP cells. 48 h after transfection of H4-FL-APP cells with ShcC siRNA or control siRNA, the cells were harvested and subjected to Western blot analyses in which antibody ab2077 was used to detect BACE levels. Immunoblotting revealed a visible decrease in the BACE levels in the cells treated with ShcC siRNA as compared with control siRNA (Fig. 4A). Quantification of the Western blots (normalized to β-actin) revealed that ShcC siRNA treatment decreased BACE levels by 38% (p < 0.05) (Fig. 4B) as compared with control siRNA treatment.

RNAi Silencing of ShcC Did Not Affect APP Processing and Aβ Levels in H4-APP-C99 Cells—To determine whether the decreases in the levels of APP-C83, APP-C99, and Aβ following RNAi silencing of ShcC in H4-FL-APP cells were due to alterations in BACE, we employed H4-APP-C99 cells. APP-C99 is the product of BACE and harbors α- and γ-cleavage but not β-cleavage sites. 48 h after transfection with either ShcC siRNA or control siRNA in H4-APP-C99 cells, the cells were harvested and subjected to Western blot analyses as described for ShcC in H4-FL-APP cells above. Immunoblotting for APP with antibody A8717 revealed no significant difference in the FL-APP levels in ShcC siRNA- versus control siRNA-treated cells (Fig. 3A). Likewise, ShcC siRNA treatment did not alter levels of APP-C99 or APP-C83 as compared with control siRNA treatment (Fig. 3, A–C).

Next, we measured Aβ levels in the conditioned medium 48 h following treatments with control siRNA or ShcC siRNA, as compared with control siRNA normalized to total protein amount (Fig. 3D). Taken together, these findings suggest that the effects of RNAi silencing of ShcC on APP processing and Aβ generation are at least partially dependent on BACE cleavage of APP.
were harvested and subjected to Western blot analyses in which antibody ShcA was used to detect ShcA levels. ShcA immunoblotting revealed a clear decrease in the ShcA levels in the cells treated with ShcA siRNA as compared with control siRNA or saline (Fig. 5A). Quantification of the Western blots (normalized to β-actin) revealed that ShcA siRNA treatment decreased the ShcA levels by 80% (*, p < 0.05) (Fig. 5B) as compared with control siRNA treatment.

We then assessed the effects of RNAi-mediated silencing of ShcA on APP processing in H4-FL-APP cells. 48 h after transfection with either ShcA siRNA or control siRNA, the cells were harvested and subjected to Western blot analyses as described for ShcC above. Immunoblotting for APP with antibody A8717 revealed no significant difference in the FL-APP levels in ShcA siRNA- versus control siRNA-treated cells (Fig. 6A). Likewise, ShcA siRNA treatment did not alter levels of APP-C99 or APP-C83 as compared with control siRNA treatment (Fig. 6, A–C).

Next, we measured Aβ levels in the conditioned medium. 48 h following treatments with control siRNA or ShcA siRNA, ShcA siRNA treatment did not significantly alter Aβ40 levels with 23 pg/ml/protein (ShcA siRNA) versus 25 pg/ml/protein (control siRNA) or Aβ42 levels 5.9 pg/ml/protein (ShcA siRNA) versus 6.6 pg/ml/protein (control siRNA), as compared with control siRNA normalized to the total protein amount (Fig. 6D). Taken together, these findings indicate that, in contrast to ShcC siRNA, ShcA siRNA treatment did not affect APP processing or Aβ generation in H4-FL-APP cells.

RNAi Silencing of Fe65 Increased APP-CTF Levels and Decreased Aβ Levels in H4-FL-APP Cells—We next assessed the effects of RNAi silencing of Fe65, another APP adaptor molecule, on APP processing and Aβ levels in H4-FL-APP cells. We first established conditions under which Fe65 siRNA treatment reduces Fe65 levels in H4-FL-APP cells. The cells were harvested 48 h after being transfected with either control siRNA or Fe65 siRNA and were subjected to Western blot analyses with antibody PA-751 to measure levels of Fe65. Fe65 immunoblotting revealed decreased levels (63% reduction) of Fe65 following Fe65 siRNA treatment as compared with control siRNA treatment (*, p < 0.05) (Fig. 7).

We then assessed the effects of RNAi-mediated silencing of Fe65 on APP processing in H4-FL-APP cells by measuring levels of FL-APP, APP-C99, and APP-C83 following Fe65 siRNA or control siRNA treatments. 48 h after transfection of Fe65 siRNA or control siRNA, the cells were harvested and subjected to Western blot analyses with antibody A8717 to detect FL-APP, APP-C99, and APP-C83, as described above for ShcA and ShcC. Levels of APP-C99 and APP-C83 were increased in the cells treated with Fe65 siRNA versus control siRNA or saline (Fig. 8A). Meanwhile, no significant differences in the FL-APP levels were observed for Fe65
FIGURE 6. Effects of ShcA RNAi on APP processing and Aβ levels in H4-FL-APP cells. In H4-FL-APP cells, ShcA siRNA treatment did not alter the levels of APP-C99, APP-C83, and Aβ. A, APP processing in Western blot analyses. FL-APP immunoblotting revealed that there was no significant difference in the FL-APP levels in the cells treated with control siRNA or ShcA siRNA. APP-CTF immunoblotting showed no alterations in the levels of APP-C99 and APP-C83 in the cells treated with ShcA siRNA (columns 4–6) as compared with control siRNA (columns 1–3). There was no significant difference in the amounts of β-actin in the control siRNA- and ShcA siRNA-treated H4-FL-APP cells. B, APP processing assessed by quantifying the ratio of APP-C83 to FL-APP in the Western blot. ShcA siRNA treatment (black bar) did not alter the ratio of APP-C83 to FL-APP as compared with control siRNA treatment (white bar) normalized to β-actin. C, APP processing assessed by quantifying the ratio of APP-C99 to FL-APP in the Western blot. ShcA siRNA treatment (black bar) did not alter the ratio of APP-C99 to FL-APP as compared with control siRNA treatment (white bar) normalized to β-actin. D, effects of ShcA RNAi on Aβ levels in H4-FL-APP cells. ShcA siRNA treatment (black bar) did not alter Aβ40 and Aβ42 levels as compared with control siRNA treatment (white bar) normalized to total protein amount.

FIGURE 7. Effects of RNAi silencing of Fe65 on Fe65 levels in H4-FL-APP cells. In H4-FL-APP cells, Fe65 siRNA treatment decreased the levels of Fe65. A, Fe65 levels in Western blot analyses. Fe65 immunoblotting showed reductions in the Fe65 levels in the cells treated with Fe65 siRNA (columns 3 and 4) as compared with control siRNA (columns 1 and 2). B, the Fe65 levels assessed by quantifying Fe65 in the Western blot. Fe65 siRNA treatment (black bar) significantly decreased the Fe65 levels as compared with control siRNA treatment (white bar) (*, p < 0.05) normalized to β-actin.

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siRNA- versus control siRNA- and saline-treated cells (Fig. 8A). Fe65 siRNA treatment led to a 252% increase in the ratio of APP-C83 to FL-APP (*, p < 0.05) (Fig. 8B) and a 262% increase in the ratio of APP-C99 to FL-APP (*, p < 0.05) (Fig. 8C) normalized to β-actin, as compared with control siRNA treatment.

Next, we measured Aβ levels in the conditioned medium normalized to the total protein amount. 48 h after treatment with control siRNA or Fe65 siRNA in H4-FL-APP cells, Fe65 siRNA decreased Aβ40 levels (27 pg/ml/protein for Fe65 siRNA versus 50 pg/ml/protein for control siRNA) (*, p < 0.05) (Fig. 8D). Collectively, these data indicate that RNAi silencing of Fe65 affects APP processing and Aβ production in a manner similar to that of γ-secretase inhibitor treatment in H4-FL-APP cells.

DISCUSSION

AD neuropathogenesis is profoundly affected by the balance between Aβ generation and clearance in the brain (20, 21). Aβ is produced via serial proteolysis of APP by two proteases, BACE and γ-secretase (2–5). Cleavage by BACE first generates APP-C99, which is further cleaved by γ-secretase to release Aβ and the β-amyloid precursor protein intracellular domain (6–8). APP is also cleaved by α-secretase to release a large ectodomain (α-APPs) and APP-C83; APP-C83 is sequentially cleaved by γ-secretase to produce p3 and amyloid precursor protein intracellular domain (9–11). Several APP adaptor proteins (13) have previously been shown to affect APP processing and Aβ production in a manner similar to that of γ-secretase inhibitor treatment in H4-FL-APP cells.
pared with control siRNA treatment (columns 4 and 5) or Fe65 siRNA treatment (black bar) significantly increased the ratio of APP-C99 to FL-APP as compared with control siRNA treatment (white bar) (*, p < 0.05) normalized to β-actin. D, effects of RNAi silencing of Fe65 on Aβ levels in H4-FL-APP cells. Fe65 siRNA treatment (black bar) decreased both Aβ40 and Aβ42 levels as compared with control siRNA treatment (white bar) (*, p < 0.05) normalized to total protein amount.

Collectively, our findings demonstrate for the first time that RNAi-mediated knockdown of ShcC (but not ShcA) and Fe65 decreases secretion of Aβ, although by different molecular mechanisms regarding APP processing. These data, together with those of previous studies, imply that pharmacologically blocking interaction of APP with either Fe65 or ShcC and perhaps other APP adaptor proteins may provide a novel means for treating and/or preventing AD by lowering Aβ generation.
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