Neuronal SIRT1 Activation as a Novel Mechanism Underlying the Prevention of Alzheimer Disease Amyloid Neuropathology by Calorie Restriction*5

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Nicotinamide adenine dinucleotide (NAD)+-dependent sirtuins have been identified to be key regulators in the lifespan extending effects of calorie restriction (CR) in a number of species. In this study we report for the first time that promotion of the NAD+-dependent sirtuin, SIRT1-mediated deacetylation activity, may be a mechanism by which CR influences Alzheimer disease (AD)-type amyloid neuropathology. Most importantly, we report that the predicted attenuation of β-amyloid content in the brain during CR can be reproduced in mouse neurons in vitro by manipulating cellular SIRT1 expression/activity through mechanisms involving the regulation of the serine/threonine Rho kinase ROCK1, known in part for its role in the inhibition of the non-amyloidogenic α-secretase processing of the amyloid precursor protein. Conversely, we found that the expression of constitutively active ROCK1 in vitro cultures significantly prevented SIRT1-mediated response, suggesting that α-secretase activity is required for SIRT1-mediated prevention of AD-type amyloid neuropathology. Consistently we found that the expression of exogenous human (h) SIRT1 in the brain of hSIRT1 transgenics also resulted in decreased ROCK1 expression and elevated α-secretase activity in vivo. These results demonstrate for the first time a role for SIRT1 activation in the brain as a novel mechanism through which CR may influence AD amyloid neuropathology. The study provides a potentially novel pharmacological strategy for AD prevention and/or treatment.

Sirtuins are a family of NAD+-dependent histone/protein deacetylases that are highly conserved in their catalytic domains and are distributed across all kingdoms of life (1–4). These enzymes utilize NAD+ as a substrate to catalyze deacetylation of specific acetylated-protein substrates (1, 5). Sirtuins can deacetylate a variety of substrates and are, therefore, involved in a broad range of physiological functions, including control of gene expression, metabolism, and aging (6). Accumulating evidence implicates sirtuins in calorie restriction (CR)-mediated health effects including increased organism longevity in yeast, worms, flies, and mammals (1–4, 6). Mammalian genomes encode seven distinct sirtuins (SIRT1–SIRT7). SIRT1 is induced by CR in several tissues and has been implicated in various effects such as stress resistance, reduced apoptosis, and metabolic changes associated with CR (1). SIRT1 is also expressed in the developing and the adult mammalian brain (7). Based on these considerations and on the evidence that CR prevents AD-type amyloid neuropathology in animal models (8, 9), we sought to test the hypothesis that CR may reduce AD-type amyloid neuropathology through mechanisms involving promotion of SIRT1. The relevance of CR treatment in experimental models of AD to human pathology is supported by recent epidemiological evidence suggesting that humans who maintain a low calorie diet have a reduced risk of developing AD (10–12).

Abnormal Aβ deposition within the brain is a hallmark of AD neuropathology. Accumulation of aggregated Aβ is hypothesized to initiate a pathological cascade resulting in onset and progression of AD (13). Aβ species with different amino and carboxyl termini are generated from amyloid precursor protein (APP) through sequential proteolysis by β- and γ-secretases in vivo.

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4 The abbreviations used are: CR, calorie restriction; AD, Alzheimer disease; AL, ad libitum; Aβ, β-amyloid; NAD+, nicotinamide adenine dinucleotide; NAM, nicotinamide; ROCK, serine/threonine Rho kinase; APP, amyloid precursor protein; WT, wild-type; sAPPα, soluble APP; DN, dominant negative; CA, constitutively active; H4-k16Ac, acetylated histone 4 lysine 16 residue; Ad, adenosin; CHO, Chinese hamster ovary; m.o.i., multiplicity of infection; h, human; NeuN, neuronal nuclear-specific.
Role of Neuronal SIRT1 in CR Prevention of Aβ Neuropathology

amyloidogenic processing pathways (14). A 1–40 amino acid form of Aβ (Aβ1–40) is the major secreted product of these cleavages, whereas the minor 1–42 amino acid form of Aβ (Aβ1–42) that contains two additional residues at its carboxyl terminus has been suggested as the initiating molecule in the pathogenesis of AD (13). In the non-amyloidogenic pathway, APP is cleaved within the Aβ domain by a third α-secretase, precluding generation and deposition of intact amyloidogenic Aβ peptides in the brain (14).

CR extends the life span in a wide variety of animals and has for decades been the only lifestyle-related regimen known to promote longevity and prevent morbidity in mammals (1–4, 6). The recent determination that the yeast sirtuin Sir2 is required for CR-induced increases in lifespan in this organism suggests that sirtuins may play key roles in mediating CR-related health effects in other organisms (1–4, 6). The latter hypothesis is supported by a number of recent studies showing that increasing sirtuin gene dosage promotes lifespan increases in worms and flies (1, 6). In addition, studies in mammals show that sirtuins (and SIRT1 in particular) regulate stress resistance, adipogenesis/adipolysis, and metabolic responses to fasting (6). The sirtuin deacetylation reaction consumes NAD^[+] with a deacetylated lysine product (1). Furthermore, NAM inhibits yeast Sir2 and SIRT1 at 50–150 μM concentrations (15). The recycling of NAM back to NAD^[+] by the enzyme nicotinamide phosphoribosyltransferase may be crucial for maintenance of cellular NAD^[+], and for maintaining SIRT1 functions (1, 6). Indeed, NAD^[+] levels in liver were recently shown to increase with fasting (16), and changes in the NAD^[+] /NAM ratio in vivo may critically influence cellular responses to CR and modulate SIRT1 activities in mammals (17).

In this study we first examined if CR treatment influences SIRT1 expression/activity and the NAD^[+] /NAM ratio in the brain and then systematically explored the causal link between SIRT1-mediated deacetylation activity and Aβ peptide generation in vitro. We report for the first time that promotion of NAD^[+] -dependent SIRT1 deacetylase activity may be a mechanism by which a CR dietary regimen may prevent experimental AD amyloid neuropathology. Most importantly, we report that a mechanism through which SIRT1 in part prevents Aβ peptide generation is through promotion of the non-amyloidogenic processing of APP by means of inhibition of ROCK1 expression. These findings point to SIRT1 activation and enhancement of the NAD^[+] /nicotinamide (NAM) ratio in the brain as potentially attractive pharmacological strategies for AD prevention and/or treatment.

EXPERIMENTAL PROCEDURES

Tg2576 Mice and Diets—In this study 4-month-old female Tg2576 mice (18) (Taconic, Inc., Germantown, NY) were randomly assigned to CR or ad libitum (AL) dietary regimens. CR was achieved by feeding Tg2576 mice 70% of the calories consumed by the pair-controlled AL animals, as previously reported (8). At 10 months of age, the mice were sacrificed, and brain specimens were harvested as previously reported (8). All animal studies were conducted following protocols approved by the Mount Sinai School of Medicine Institutional Animal Use Committee.

Measurement of NAD^[+] and NAM—A weighed aliquot of frozen pulverized tissue allocated for biochemical studies (cerebellum) was extracted in 800 μL of HClO₄ (1.1 m) containing 6 nmol of [18O]NAD^[+] (91% isotopic enrichment) and 2.5 nmol of [18O]NAM (96% isotopic enrichment). After centrifugation for 3 min at room temperature, supernatants were neutralized with KOH and injected onto a high performance liquid chromatography for separation of NAD and NAM from other cell components. NAD^[+] and NAM peaks were collected as determined by retention time of authentic standards. Samples were lyophilized, and matrix-assisted laser desorption ionization-mass spectroscopy (MS) (positive mode) was used to detect NAD^[+] and SIRT1 (positive mode) was used to measure the NAM signal (19). A peak ratio of 664/666 provided quantitation of the nmol of NAD^[+] in a sample as determined from the 6 nmol of [18O]NAD^[+] added to the original sample. Similarly, NAM levels were determined using the ratio 123/125 multiplied by 2.5 nmol of NAM to obtain total nmol of NAM per sample. Both NAD^[+] and NAM mole contents were then divided by the tissue volume (density, 1.0 μl/mg of tissue) to obtain the metabolite concentration in tissue. The methodology employed is based upon a previous approach that measured NAM contents in yeast (19). Corrections were applied for isotopic abundances, and relevant blanks and controls were also run. Each sample group contained at least four animals, and each sample was run in duplicate.

Neocortical SIRT1 Immunocytochemical Localization—Neocortical tissue sections (50 μm) adjacent to those used for assessment of amyloid plaque pathology in CR Tg2576 mice were washed with Tris-buffered saline (20 mM, pH 7.4) before incubation with 0.2% Triton X-100 for 30 min for permeabilization. Endogenous peroxidase activity was quenched by incubating the sections with 0.3% hydrogen peroxide for 5 min. After a rinse with Tris-buffered saline (TBS), the sections were incubated with blocking buffer (5% goat serum in TBS) for 120 min. The sections were then incubated with primary antibodies SMI-32 (1:2000 dilution, Sternberger Monoclonals Inc., Lutherville, MD) and anti-SIRT1 IgG (1:100 dilution, Upstate Biotechnology, Inc., Lake Placid, NY) in blocking buffer at 4 °C overnight. The sections were washed 3 times with Tris-buffered saline and then incubated with a mixture of anti-mouse IgG Texas Red-conjugated and anti-rabbit IgG peroxidase-conjugated (1:200 dilution) antibodies in blocking buffer for 2 h at 37 °C. The specific labeling of SIRT1 was detected using Tyramide Signal Amplification kit (Molecular ProbesTM) according to the manufacturer’s instructions. The sections were mounted with Vectashield, the sections were observed under either a fluorescent microscope (Zeiss Axioplan 2 Imaging system) or Leica TCS-SP (UV) confocal system, and images were acquired.

Cell Culture and Treatments—Embryonic (E14) neocortical primary neuronal cultures derived from Tg2576 transgenic mice (Tg2576 neurons) were prepared as previously described (20). Chinese hamster ovary (CHO) cells expressing human APP carrying the K670N,M671L Swedish mutation (APPsw) (a gift from Dr. Robakis) as in Tg2576 neurons were grown in McCoy’s 5A medium supplemented with 10% fetal bovine
serum, 1% streptomycin/penicillin (Invitrogen), and 400 μg/ml G418 (Invitrogen). In a cell culture study, CHO-APPswe cells or Tg2576 neurons were seeded at 4 × 10⁶ cells/cm² and cultured at 37 °C in the presence of 5% CO₂. For adenoviral infection or pharmacological studies, Tg2576 neuronal (5 days old) or CHO-APPswe (24 h after plating), cultures were infected with wild-type (WT) SIRT1 (16), dominant negative (DN) SIRT1 (21), or LacZ control adenoviruses at doses defined as multiplicity of infection (m.o.i.) or co-treatment with NAD⁺. NAD⁺ (75 mM, Sigma) was freshly prepared and dissolved in water before use. For the constitutively active (CA) ROCK1 study, CHO-APPswe cells were transfected with pCAG empty vector or CA ROCK1 cDNA followed by infection with Ad-WT SIRT1 of Ad-lacZ 6 h later. The transfection was carried out using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions and as previously described (22). Conditioned medium was collected at indicated time points for Aβ detection. Cell viability was assessed by LDH assay.

Aβ Enzyme-linked Immunosorbtent Assay—The quantitative assessment of Aβ peptides in brain and cultured cells was performed as previously described (23).

**Western Blot Analysis**—Aliquots of frozen pulverized neocortical tissue (circular and parietal cortex) were lysed in radioimmuno precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitors. Lysates were then subjected to Western blot analysis with the indicated antibody as previously reported (8). The following antibodies were used in this study: polyclonal anti-SIRT1 antibody (1:3000), polyclonal anti-histone H4 (Ac16) (1:2000, Serotec, Raleigh, NC), polyclonal anti-amyloid precursor protein carboxy-terminal (751–770) antibody (anti-O443, 1:5000 dilution, Calbiochem), monoclonal 22C11 antibody (1:1000, Chemicon International, Temecula, CA), monoclonal 6E10 antibody (1:1000, Senetek, St. Louis, MO), rabbit polyclonal antibody against ROCK1 (1:5000; Chemicon International), and polyclonal β-actin antibody (1:3000, Sigma).

**Secretase Activity Assays**—α-, β-, and γ-secretase activities were assessed using commercially available kits (R&D Systems, Minneapolis, MN) as previously described (24, 25).

**SIRT1 Transgenic Mice**—For the generation of the hSIRT1 transgenic mice, a 2.3-kilobase cDNA fragment containing the entire coding region of the hSIRT1 (a gift from Dr. Sauve) was inserted into a unique HindIII-NotI restriction site located within the second exon of the rat neuron-specific enolase gene, as previously described in this laboratory (20). A 7.3-kilobase Sall fragment containing the hSIRT1 was purified and micro-injected into 1-cell mouse eggs as previously described (20). Founders were identified by PCR-based genotyping, and generation of individual F1 lines was obtained by mating founders with strain-matched C57B6-SJL WT mice for analysis of SIRT1 and ROCK1 expression and α-secretase activity.

**Statistical Analysis**—All values are expressed as the means ± S.E. Differences between means were analyzed using a two-tailed Student t test. In all analyses the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using the Prism Stat program (GraphPad Software, Inc., San Diego, CA).

**RESULTS**

**CR Increases SIRT1 Expression and NAD⁺ Levels in the Brains of ~10-Month-old Tg2576 Mice**—For the in vivo studies, beginning at 4 months of age, Tg2576 transgenic mice, which model AD-type amyloid neuropathology (18), were enrolled in a CR diet regimen providing ~30% fewer daily calories relative to average caloric consumption of age- and gender-matched control AL-fed Tg2576 mice as previously described (8). Consistent with previous evidence, CR treatment resulted in body weight stabilization over the ~6 month study period among CR Tg2576 mice relative to the AL-fed group (supplemental Fig. 1). When mice were sacrificed at ~10 months of age, we found a significant elevation of SIRT1 protein content (p < 0.05) (Fig. 1A) and NAD⁺ content (p < 0.001) (Fig. 1B) as well as a significant reduction in NAM content (p < 0.01) (Fig. 1B) in the brain of CR Tg2576 mice relative to the age- and gender-matched AL-fed control Tg2576 mice (see supplemental Fig. 2 for more information on NAD⁺/NAM assay). The increased SIRT1 protein content and the ~4-fold increased NAD⁺/NAM ratio (p < 0.001) in the brain of CR Tg2576 mice (Fig. 1B) coincided with an apparent marked elevation of SIRT1 deacetylase activity in the brain, as reflected by significantly reduced acetylation levels of the SIRT1 substrate H4-k16Ac (p < 0.05), assessed by Western blot assay using an antibody specific for H4-k16ac residue (Fig. 1C) (26), relative to AL-fed Tg2576 control mice. The observed difference in immunoreactive H4-k16Ac content was not attributable to different H4 contents in the brain of CR relative to AL Tg2576 mice (data not shown). In parallel control studies we found that CR-mediated SIRT1 activation in the brain of Tg2576 coincided with reduced acetylated H3-k9Ac levels in the absence of significant changes in H4-k8Ac relative to control AL-fed treated Tg2576 mice (data not shown). Our evidence is consistent with a previous report that SIRT1 deacetylates histone polypeptides with a preference for H4-k16Ac and H3-k9Ac (26). Based on this evidence and the fact that H4-k16Ac is selectively influenced in the brain of Tg2576 in response to CR (Fig. 1C), we decided to monitor H4-k16Ac signaling as the specific index for SIRT1-mediated deacetylase activity in vitro (see below). The elevation in SIRT1 deacetylase activity in this study coincided with significantly decreased Aβ1–40 and Aβ1–42 peptide contents (Fig. 1D) in the same brain tissue and was accompanied by near complete prevention of AD-type amyloid neuropathology (supplemental Fig. 3, A and B). The evidence revealing CR-mediated attenuation in AD-type neuropathology in 10-month-old CR Tg2576 mice in this study, is consistent with our previous independent study showing similar responses in 12-month-old Tg2576 mice after 9 months of CR treatment (8).

To identify the cellular localization of SIRT1 expression in CR Tg2576 mice, we immunocytochemically surveyed SIRT1 in adjacent contralateral tissue from neocortical sections used to evaluate AD-type amyloid neuropathology. We found that SIRT1 immunoreactivity in CR Tg2576 mice (green immunostaining) was primarily localized within the boundaries of both the outer (OG) and inner (IG) granular layers of the neocortex (Fig. 1E, panel A); sparse SIRT1 immunolabeling was also detected in both outer (OPL) and inner (IPL) pyramidal layers of the parietal neocortex as
Role of Neuronal SIRT1 in CR Prevention of Aβ Neuropathology

FIGURE 1. Calorie restriction increases SIRT1 expression and NAD+ levels in the brain of ~10-month-old Tg2576 mice. A, in this study ~4-month-old female Tg2576 mice were fed AL or caloric CR diets for 6 months. Neocortex tissue lysates (cingulated and parietal cortex) from AL- and CR-treated mice were separated by SDS-PAGE and probed with a rabbit polyclonal antibody against SIRT1; 3T3 cell-nuclear extract controlled for specificity of SIRT1 signal in the brain, whereas β-actin immunoreactivity served as a loading control. B, assessment of NAD+ and NAM contents in the brain of CR- and AL-fed control Tg2576 mice. C, assessment of neocortical SIRT1 activity by Western blots using an antibody specific for acetylated histone 4 lysine 16 residue (H4-K16Ac). D, assessment of neocortical Aβ1–40 and Aβ1–42 peptide concentrations in CR- and AL-fed control Tg2576 mice. E, cellular and regional distribution of neocortical SIRT1 immunoreactivity in the contralateral neocortex used for SIRT1 Western blot analysis; panel a, green and red reflect SIRT1 and SMI-32 immunoreactivities respectively; panels b–c, green and red immunostaining reflect SIRT1 and NeuN immunoreactivities, respectively; panel d, yellow reflects overlapping SIRT1 and NeuN immunoreactivities in CR Tg2576 mouse neocortex. In panel a, the arrows point to SMI-32/SIRT1-positive neurons by double-labeled immunocytochemistry. In panels b–d, arrows point to the same labeled neurons. Bar graphs demonstrate group means ± S.E.; n = 4 animals/group; *, p < 0.05; **, p < 0.01, ***, p < 0.001; 2-tailed t-test, AL-fed versus CR-treated group. OG, outer granular layer; IG, inner granular layer; OPL, outer pyramidal layer; IPL, inner pyramidal layer.

reflected by co-localization of SIRT1 and SMI-32 immunoreactive signal (red immunostaining), which is a specific marker for neocortical pyramidal neurons (Fig. 1E; panel A). Similar neuronal SIRT1 immunoreactive distribution was found in the neocortex of AL-fed Tg2576 (data not shown). Further examination of cellular localization of SIRT1 in the neocortical granular layer in adjacent tissue sections revealed that the SIRT1 immunoreactive signal (Fig. 1E, panel b, green staining) selectively co-localized with neuronal nuclear-specific (NeuN) immunoreactivity (Fig. 1E, panel c, red staining) as determined by overlapping SIRT1 and NeuN staining (Fig. 1E, panel d). The selective neuronal distribution of SIRT1 protein in the neocortex and other brain regions of CR Tg2576 mice suggests that neuronal SIRT1 deacetylase may selectively influence APP processing independently from glial inflammatory-associated Aβ processing and amyloid plaque generation (27). Thus, despite the availability of evidence that exogenous SIRT1 expression in glia may attenuate inflammatory neurotoxicity in vitro (28), CR-mediated SIRT1 responses with respect to AD amyloid neuropathology in the brain could be primarily neuronal based upon the extent to which SIRT1 localizes to these cell types in the brain. Based on this evidence we conducted a systematic series of in vitro studies to evaluate the role of neuronal SIRT1 in CR-mediated attenuation of AD-type Aβ neuropathology in the brain of CR Tg2576 mice. In particular, we investigated if the predicted attenuation in Aβ peptide content by CR in the brain can be achieved by manipulating the expression/activity of SIRT1 in primary embryonic Tg2576 cortico-hippocampal neurons (Tg2576 neurons; E16) in vitro.

SIRT1 Expression Causally Promotes α-Secretase Activity and Attenuates Aβ Peptides Generation in Primary Tg2576 Neuron Cultures and CHO-APPsw Cells—We found that viral expression of WT (16) SIRT1 (10 m.o.i.), leading to an ~3-fold elevation in SIRT1 protein content in the Tg2576 neurons (Fig. 2A and inset), resulted in a significant reduction of endogenous Aβ1–40 and Aβ1–42 peptide contents in the culture medium (p < 0.05 and p < 0.05, respectively) 48 h after infection relative to viral LacZ-infected neu-

5 G. M. Pasinetti, unpublished observation.
Viral expression of the WT SIRT1 in Tg2576 neurons resulted in nuclear localization of SIRT1, as determined by elevated exogenous SIRT1 protein immunoreactive signal (relative to control cultures), which co-localized with nuclear-specific NeuN immunoreactivity (supplemental Fig. 4), similar to what was found in the brain of CR Tg2576 mice. The SIRT1-mediated attenuation of Aβ1–40 and Aβ1–42 peptide contents coincided with a significant elevation in SIRT1 deacetylase activity, as reflected by decreased H4-K16Ac immunoreactivity in the SIRT1 overexpression cell cultures (*p < 0.05) relative to viral LacZ-infected CHO-APPswe control cultures (Fig. 2A and inset). It is apparent from these studies that exogenous SIRT1 expression results in greater attenuation of Aβ1–40 and Aβ1–42 generation in CHO-APPswe relative to Tg2576 neurons. Based on this consideration we continued to investigate how SIRT1 influences Aβ generation and APP proc-
Role of Neuronal SIRT1 in CR Prevention of Aβ Neuropathology

Expression of WT SIRT1 in CHO-APPswc cells resulted in a select elevation of α-(p < 0.05) but not β- or γ-secretase activity (Fig. 2B) and increased content of soluble APPα (sAPPα; 6E10 immunoreactive) (p < 0.05) (Fig. 2C and inset), which is a specific index of elevated α-secretase activity (14). These changes occurred in the absence of detectable changes in total sAPP (22C11 immunoreactive) in the conditioned medium or of full-length APP content (O-443 immunoreactive) (Fig. 2C and inset). In control studies we found that viral SIRT1 expression in wild-type primary embryonic cortico-hippocampal neurons had no influence on the steady level of total sAPP in the condition medium (data not shown). This evidence tentatively excludes that viral SIRT1 expression influenced endogenous APP processing. Thus, it may be possible that SIRT1 may influence Aβ peptide generation through mechanisms involving up-regulation of the non-amyloidogenic pathway of the APP processing. This new evidence is consistent with our previous in vivo observation that CR treatment in Tg2576 mice coincides with promotion of the non-amyloidogenic pathway of APP processing in the brain (8).

In conjunction with these investigations, overexpression of a DN SIRT1 (H355A) (21) was used to provide further insight into the role of SIRT1 in APP processing within neurons. Viral expression of DN SIRT1 (20 m.o.i.) resulted in an ~4-fold elevation in DN SIRT1 protein content in Tg2576 neurons relative to viral LacZ-infected cultures. We found that DN SIRT1 expression in Tg2576 neuron cultures resulted in a significant elevation of Aβ1–40 and Aβ1–42 (p < 0.05 and p < 0.01, respectively) in the conditioned medium relative to viral LacZ control cultures 72 h after infection (Fig. 2D). Importantly, we found that DN SIRT expression in Tg2576 neurons coincided with decreased deacetylase activity, as reflected by increased H4-k16ac immunoreactive signal in the cell cultures (Fig. 2D and inset), supporting the evidence that H4-K16 deacetylation signal in the brain of CR-restricted Tg2576 mice may be a specific index of SIRT1-mediated deacetylase activity. Consistently, we also found that DN SIRT1 expression in Tg2576 neuronal cultures significantly attenuated α-(p < 0.05) but not β- or γ-secretase cleavage activity (Fig. 2E), which coincided with a decreased concentration of sAPPα (p < 0.01) in the absence of changes in total soluble APP in the conditioned medium or full-length APP content relative to control viral LacZ-infected cultures 72 h after treatment (p < 0.05, Fig. 2F and inset). These findings suggest that deacetylation of H4-k16ac in Tg2576 neurons is SIRT1-specific and that SIRT1 deacetylase activity indeed acts to increase α-secretase-mediated non-amyloidogenic pathways of APP processing resulting in the attenuation of Aβ generation. In parallel control studies using Aβ immunoprecipitation-mass spectroscopy studies using 6E10 antibody, we found that, whereas expression of DN SIRT1 in Tg2576 mouse neuron cultures resulted in elevation of Aβ1–40 and Aβ1–42, we also found a selective reduction in Aβ3–16 content (data not shown). Because α-secretase can cleave APP, eventually resulting in the generation of Aβ carboxyl termini fragments ending at the amino acid residue leucine 16 of Aβ, this observation further supports the hypothesis that SIRT1 causally attenuates Aβ generation by selectively promoting α-secretase activity.

NAD+ Treatment Attenuates Aβ Generation in Primary Tg2576 Neuron Cultures—There is evidence that nuclear NAD+ biosynthesis may dramatically influence rodent brain responses to injury through activation of SIRT1 (29). We hypothesized that NAD+, whose content is elevated in the brain of CR Tg2576 mice might influence APP processing through activation of SIRT1. Therefore, we continued to explore pharmacologically the role of NAD+ in SIRT1-mediated effects on APP processing and Aβ peptide generation in Tg2576 mouse neuron cultures. Similar to what we observed in response to viral WT SIRT1 overexpression (Fig. 2), we found that treatment of Tg2576 mouse neuron cultures with NAD+ (Fig. 3A) significantly decreased the content of Aβ1–40 and Aβ1–42 peptides in the conditioned medium in a concentration-dependent manner 24 h after treatment. Consistent with the evidence in the studies with virally mediated expression of WT SIRT1, we also found that treatment of Tg2576 mouse neuron cultures with NAD+ significantly promoted α-(p < 0.05) but not β- or γ-secretase cleavage activity (Fig. 3B), which coincided with dose-dependent increased content of sAPPα in the absence of changes in total soluble APP in the conditioned medium or full-length APP content in the cultured cells relative to vehicle-treated Tg2576 neurons 24 h after treatment (Fig. 3C). Importantly, NAD+-mediated reduction in Aβ1–40 and Aβ1–42 peptide contents (Fig. 3A) coincided with increased SIRT1 deacetylase activity (p < 0.01 and p < 0.05, respectively), as reflected by decreased H4-k16ac immunoreactive content relative to vehicle control cultures (Fig. 3A). Next we continued to determine whether NAD+-mediated reduction of Aβ1–40 and Aβ1–42 peptide levels in Tg2576 neurons is SIRT1-dependent. To address this question Tg2576 neurons were virally infected with DN SIRT1 in the presence or absence of NAD+. As expected, inhibition of SIRT1 activity by DN SIRT1 significantly promoted Aβ1–40 and Aβ1–42 content in the conditioned medium of Tg2576 mouse neurons 72 h after treatment (Fig. 3D). Moreover, we found that the expression of DN SIRT1 resulting in significant inhibition of SIRT1 deacetylase activity (not shown) significantly prevented NAD+-mediated Aβ lowering effects (p < 0.05) relative to viral LacZ-infected NAD+-treated cultures (Fig. 3D). These findings tentatively suggest that NAD+-mediated attenuation of Aβ generation in neurons is mediated by a SIRT1-dependent mechanism.

SIRT1-mediated Promotion of Non-amyloidogenic Processing of APP Is ROCK1-dependent—Recent evidence suggests that the inhibition of Rho kinase ROCK1 activity promotes non-amyloidogenic processing of APP (22). Based on this evidence, we hypothesized that a mechanism by which SIRT1 activation may influence non-amyloidogenic APP processing in response to CR is through inhibition of ROCK1 expression. Consistent with this hypothesis, we found a significant reduction in ROCK1 protein content in the brain of 10-month-old CR Tg2576 mice relative to age- and gender-matched AL-fed control Tg2576 mice (p < 0.05) (Fig. 4A). More importantly, we noted that a similar reduction in ROCK1 expression could be reproduced in CHO-APPswc cells 48 h after viral WT SIRT1 (10 m.o.i.) infection relative to viral LacZ-infected cells (Fig. 4B). Conversely, in control studies we found that viral expression of DN SIRT1 (20 m.o.i.) significantly increased ROCK1 protein
content in Tg2576 mouse neurons 48 h after viral DN SIRT1 infection (p < 0.05) relative to LacZ viral-infected Tg2576 neurons (Fig. 4C). These findings strongly suggest that SIRT1 activation may be involved in the mechanisms associated with control of ROCK1 expression.

To further explore the role of ROCK1 in SIRT1-mediated promotion of α-secretase activity reflected by the elevation of sAPPα content, we transfected CHO-APPswc cells with a cDNA encoding CA ROCK1 (22), or pCAG control vector in combination with viral WT SIRT1, or control LacZ infection. As expected, we found that WT SIRT1 (10 m.o.i.) expression resulted in significant elevation in sAPPα (p < 0.05) (Fig. 4D) in the conditioned medium of CHO-APPswc cells 48 h after treatment relative to control LacZ/pCAG-expressing CHO-APPswc cells. In parallel studies we also found that expression of CA ROCK1 significantly reduced sAPPα content in the conditioned medium 48 h after treatment (p < 0.05) relative to control LacZ/pCAG-expressing CHO-APPswc cells (Fig. 4D). Most excitingly, we found that CA ROCK1 expression in CHO-APPswc significantly prevented WT SIRT1-mediated elevation of sAPPα (Fig. 4E) relative to control LacZ/pCAG-expressing CHO-APPswc cells in the absence of detectable changes in total sAPP in the conditioned medium (Fig. 4D, inset) or full-length cellular APP content (Fig. 4D).

In parallel cultures we found that CA ROCK1 expression in CHO-APPswc significantly prevented WT SIRT1-mediated lowering of α-secretase activity relative to control LacZ/pCAG-expressing CHO-APPswc cells (data not shown). This evidence demonstrates that ROCK1 is involved in SIRT1-mediated promotion of non-amyloidogenic α-secretase cleavage of APP processing and that α-secretase activity is required for the action of SIRT1 mediated Aβ lowering activity.

FIGURE 3. NAD+ treatment attenuates Aβ generation in Tg2576 mouse cortico-hippocampal neuron cultures in a dose-dependent fashion. A, NAD+ treatments for 24 h resulted in dose-dependent inhibition of Aβ concentration in Tg2576 mouse neurons. B, fluorimetric assessment of α-, β-, and γ-secretase activities. C, assessment of changes in sAPPα concentration expressed as percent of total sAPP immunoreactivity and full-length APP concentrations relative to β-actin immunoreactive signal in the same specimen as in A. In A–C, results are expressed as the percent of vehicle treatment in the control group; values represent the means ± S.E. of determinations made in three separate culture preparations; n = 3 per culture. *, p < 0.05; **, p < 0.01 versus vehicle control group. D, role of SIRT1 in NAD+-mediated attenuation of Aβ generation. Tg2576 mouse neurons were infected with DN SIRT1 adenovirus and co-cultured with 1 mM NAD+. Aβ1–40 and Aβ1–42 concentration in the conditioned medium were assessed by enzyme-linked immunosorbent assay 72 h post-infection (20 m.o.i.). In D, results are expressed as a percentage of vehicle treatment in the control group; values represent means ± S.E. of determinations made in three separate culture preparations; n = 3 per culture. *, p < 0.05 versus vehicle control group. **, p < 0.05 versus NAD+ treatment-only group.
To further confirm the role of SIRT1 in the promotion of ROCK1-mediated α-secretase activity in vivo we generated transgenic mice constitutively expressing human hSIRT1 under control of a neuron-specific enolase promoter (20). Consistent with the evidence in vitro, we found that an ∼2-fold elevation of hSIRT1 expression in the brain of ∼3-month-old hSIRT1 transgenics (p < 0.05) (Fig. 4E) resulted in a significant diminution of ROCK1 protein content (p < 0.05) (Fig. 4F) in the same brain tissue, coincidental with a commensurate elevation of α-secretase activity (p < 0.05) (Fig. 4G) relative to gender-matched WT littermate controls. This evidence further demonstrates that SIRT1 expression in the brain may causally attenuate ROCK1 expression resulting in induction of α-secretase activity.

**DISCUSSION**

The result of this study for the first time links SIRT1 expression/activity and increased NAD+/NAM ratios in the brain tissue of CR-treated Tg2576 mice to prevention of AD-type Aβ neuropathology. Furthermore, we demonstrate that predicted CR-related changes in brain Aβ peptide content can be reproduced by manipulating expression of SIRT1 in embryonic Tg2576 mouse neurons in vitro. Similarly, pharmacological activation of SIRT1 with NAD+ promoted α-secretase activity and attenuated generation of Aβ peptides in vitro. These virally or pharmacologically mediated SIRT1 responses were reversed by DN SIRT1 expression, further supporting the hypothesis that SIRT1-mediated deacetylase activity promotes the α-secretase non-amyloidogenic pathway of APP processing.
Role of Neuronal SIRT1 in CR Prevention of Aβ Neuropathology

attenuating Aβ generation. This study suggests that SIRT1 controls many of the same molecular pathways influenced by CR in brain tissue. Most importantly, we found that a mechanism by which SIRT1 activation promotes α-secretase is in part through SIRT1-mediated down-regulation of ROCK1 expression (Scheme 1).

In particular, we report that the expression SIRT1 in vitro or in the brain of hSIRT1 transgenic mice results in the promotion of α-secretase activity, coincidental with attenuation of ROCK1 expression. This evidence supports the hypothesis that SIRT1 is involved in the α-secretase-mediated cleavage of APP through the regulation of ROCK1 expression. Moreover, we found that the expression of constitutive active ROCK1 in vitro also significantly prevented SIRT1-mediated α-secretase cleavage of APP. This evidence further suggests that α-secretase is required for SIRT1-mediated prevention of AD-type amyloid neuropathology.

SIRT1 deacetylase modulates a number of transcription factors such as FOXO, NF-κB, and p53 (30, 31), all of which are key regulators of transcriptional control of death/survival genes, among other functions. Our findings suggest that SIRT1 activation might cause changes in transcriptional profiles that promote non-amyloidogenic processing of APP as a consequence of inhibition of ROCK1 expression by SIRT1 (Scheme 1). We cannot, however, exclude the possibility that in addition to the promotion of the non-amyloidogenic processing of the APP, SIRT1 may also beneficially influence AD-type neuropathology through mechanisms involving Aβ clearance from the brain. Consistent with this possibility we previously found that CR may influence the expression of insulin degrading enzyme, which is involved in Aβ clearance, in the brain of CR Tg2576 mice (8). Further studies are necessary to clarify the mechanisms through which SIRT1 deacetylase influences ROCK1 transcription and other potential gene targets involved in the regulation of Aβ metabolism in the brain, e.g. insulin degrading enzyme.

Collectively, our findings strongly suggest a role for SIRT1 activation and enhancement of the NAD+/NAM ratio as potential mechanisms by which CR may beneficially influence AD neuropathology. With regard to potential therapeutic applications, SIRT1 activators and molecules targeting the NAD+ biosynthetic pathway appear to offer attractive novel pharmacological directions for future AD treatment.

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Role of Neuronal SIRT1 in CR Prevention of Aβ Neuropathology

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