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Brain Dicer1 is down-regulated in a mouse model of Alzheimer's disease via A β 42-induced repression of nuclear factor erythroid 2-related factor 2

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Running title: Chronic A β exposure reduces Dicer1 via Nrf2-ARE signaling

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Abstract

Background

Oxidative stress critically underlies the neurodegenerative pathogenesis of Alzheimer's disease (AD). Depletion of *Dicer1*, an endoribonuclease central to microRNA maturation, also leads to neurodegeneration. We therefore hypothesized that altered *Dicer1* expression may play a role in AD.

Results

Using immunoblotting and quantitative real-time PCR, we found that *Dicer1* protein and mRNA levels were reduced in the hippocampi of animals of the AD mouse model APP^{swe}/PSEN1^{dE9} compared with littermate controls. SiRNA-mediated *Dicer1* knockdown induced oxidative stress, reduced mitochondrial intermembrane potential, and increased apoptosis in cultured neurons. A β 42 exposure decreased *Dicer1* and also down-regulated the oxidative stress-induced transcriptional regulator nuclear factor erythroid 2-related factor 2 (Nrf2). Conversely, Nrf2 overexpression increased *Dicer1* mRNA and protein levels and reverted the A β 42-induced *Dicer1* reduction. To further investigate *Dicer1* regulation, we cloned *Dicer1* promoter variants harboring the Nrf2-binding site, the antioxidant response elements (ARE), into a luciferase reporter and found that simultaneous transfection of Nrf2-expressing plasmid increased luciferase expression from these promoter constructs. ChIP assays indicated that Nrf2 directly interacted with the ARE motifs in the *Dicer1* promoter. Furthermore, *Dicer1*

overexpression in cultured neurons reverted A β 42-induced neurite deficits. Of note, injection of Dicer1-expressing adenovirus into the hippocampus of the AD mice significantly improved spatial learning.

Conclusions

These findings indicate that Dicer1 expression is reduced in the AD brain and that chronic A β exposure decreases Dicer1 levels in neurons via Nrf2–ARE signaling. Our results uncover a significant role for Dicer1 in AD and highlight that Dicer1 expression responds to oxidative stress in the brain.

Keywords

Dicer1, Alzheimer's disease, hippocampus, APP^{swe}/PSEN1^{dE9} mice, Nrf2, antioxidant response element, oxidative stress, neurodegeneration, gene regulation, neurotoxicity

Background

Alzheimer's disease (AD) is an inexorable neurodegenerative disorder, characterized with extracellular amyloid plaque deposition, intracellular neurofibrillary tangle formation, extensive neuronal loss, and cognitive dysfunction [1, 2]. The pathogenesis of AD involves the key component of oxidative stress which is dependent on the balance between reactive species and anti-oxidation systems [3]. Accumulation of reactive oxygen/nitrogen species generates oxidative stress and produces ensued cytotoxicity [4] as well as does ferroptosis due to iron accumulation in AD brain [5, 6]. Amyloid peptide inactivates membrane channel and transporter including sodium/calcium exchanger, calcium ATPase, glutamate and glucose transporter--these hazardous effects involve the roles of reactive oxygen species and lipid peroxidation[7-9]. Anti-oxidation systems are used to cope with oxidative injury, in which the regulation of the key enzymes or proteins, used for synthesizing glutathione or recycling peroxiredoxins, revolve around a transcriptional factor, nuclear factor erythroid 2-related factor 2 (Nrf2) [10, 11]. Nrf2 is a basic leucine zipper protein that regulates expression of a multitude of antioxidant proteins in response to oxidative injuries. Under quiescent situation, Nrf2 is retained in cytoplasm by Keap1, but upon oxidative stimulation, it is released from the binding and translocated into nucleus, triggering the transcription of antioxidant genes [12].

Human Dicer1 is an enzyme with multidomains consisting of an amino-terminal domain with helicase activity, a DUF383 domain with unknown function, a PAF (Piwi-Argonaute-Zwille) domain, two RNase III domains, and a carboxyl-terminal domain for double-stranded RNA binding [13]. In addition to traditional roles such as in generating

small regulatory RNAs including miRNAs or siRNAs [14], Dicer1 also participates in maintaining heterochromatin structure [15, 16], involving in inflammation [17, 18], and breaking chromosomal DNA in *Caenorhabditis elegans* undergoing apoptosis [19]. Degeneration of retinal pigment epithelial cell (RPE), a characteristic of advanced dry age-related macular degeneration (AMD), is associated with Dicer1 depletion through which AluRNA, a specific substrate of Dicer1 degradation, accumulates and results in inflammasome activation and MyD88 signaling which lead to ensued cell death [20, 21]. In the central nervous system, Dicer1 is widely expressed in neurons and glia. Conditional loss of Dicer1 in neuron or glia produces different phenotypes and differentially impacts the brain function--the inducible impairments depend on developmental stages [22-25]. RPE degeneration induced by Dicer1 depletion involves inflammasome activation which likely accompanies with oxidative stress which is an etiological factor in neurodegeneration occurring in AD. As such, we exploited the roles of Dicer1 in AD and further explored whether Dicer1 expression was regulated by Nrf2, a transcription factor balancing redox level. We further investigated potential therapy by overexpressing Dicer1 in the hippocampus of APP^{swe}/PSEN1^{dE9} (APP/PS1) mice.

Materials and methods

Materials

The following primary antibodies were used in this study: Dicer1 (Sigma, St Louis, MO, USA, cat# WH0023405M1, research resource identifier, RRID: AB_1841286), caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, cat# sc-271759, RRID: AB_10709891), Keap 1 (Proteintech, Suzhou, China, cat# 60027-1-Ig, RRID: AB_2132623), and rabbit polyclonal anti-Nrf2 (Proteintech, cat# 16396-1-AP, RRID: AB_2782956). Activated caspase 3 (Beyotime Biotechnology, Haimen, China, cat# AC033), β III-tubulin (Beyotime Biotechnology, cat# AT809), Histone 3 (Beyotime Biotechnology, cat# AF0009), Vimentin (Beyotime Biotechnology, cat# AF1975), GAPDH (Bioworld, Nanjing, China, cat# MB001), NeuN (Beyotime Biotechnology, cat# AF1072). The following secondary antibodies were also used in this study: goat anti-mouse horseradish peroxidase conjugated IgG (Boster Biological Technology, Co. Ltd, Wuhan, China), goat anti-Rabbit horseradish peroxidase conjugated IgG (Boster Biological Technology). The following reagents or cell lines were used in this study: a human A β 42 peptide (GenScript, Nanjing, China, cat# RP10017), the Dicer1 siRNA duplex and the negative control (NC) siRNA duplex (Genepharma, Suzhou, China), MTS reagent (CellTiter 96 AQueous One Solution, Promega, Beijing, China, cat# 3580), pfu High fidelity enzyme (Qiagen, Beijing, China, cat# KP202), pJet1.2 vector (Thermo Fisher Scientific, Carlsbad, CA, USA, cat# K1231), pGL6-basic (Beyotime Biotechnology, Haimen, China, cat# D2105), pRL-TK Vector (Beyotime Biotechnology, Inc, cat# D2762), a human pCMV-Nrf2 (Sino Biological, Beijing, China, cat# HG17384-U), a mouse pCMV-Nrf2 (Sino Biological, Beijing, China, cat# MG56971-UT), or an empty

pCMV3 vector (Sino Biological, Beijing, China, cat# D2602), mitochondrial extraction kit (Beyotime Biotechnology, cat# C3601), 2',7'-Dichlorodihydrofluorescein diacetate (Thermo fisher, Shanghai, China, cat# 2938), JC-1 probe solution (Sigma, St Louis, MO, USA, cat# CS0760), Neuro-2a (N2A)(ATCC, Manassas, VA cat# CCL-131, RRID:CVCL_0470), SK-N-BE(2) (ATCC Cat# CRL-2271, RRID:CVCL_0528).

Animal

All mice were fed water and food *ad libitum* in a temperature- and humidity-controlled animal facility with an automatic illumination on a 12-h on/off cycle in Wenzhou Medical University. APP^{swe}/PSEN1^{dE9} mice (Jackson Laboratory, stock number 004462) express a K595N/M586L *Swedish* mutations and a mutant human *presenilin1* with deletion of exon9 under the control of mouse prion promoter. The mice were multiplied and genotyped according to the guidance by Jackson Laboratory. Both genders of 4- and 11-month-old transgenic mice were used and WT littermates were used as controls. Behavioral experiments were performed during the daytime and at the same time period in each day.

Intrahippocampal injection of adenovirus expressing Dicer1

The adenovirus (Ad) incorporating sequences of either Ad-pCMV-EGFP or Ad-pCMV-Dicer1:T2A:EGFP in which the transcription of Dicer1 and EGFP is driven by independent promoter were packaged and generated by Cyagen Biosciences Inc. (Guangzhou, China). Before stereotactic injection, animals were anaesthetized by

intraperitoneal injection of ketamine/xylazine (0.1/0.05 mg/g body weight) and mounted on a stereotactic frame (KOPF, KD Scientific). The virus were injected into bilateral hippocampus with 2µl of viral titre (1.2×10^9 vg/mL) in each hemisphere using a 10-µl Hamilton syringe (Hamilton Medical, Reno, NV, USA) connected to a 30-gauge micropipette, at an injection rate of 0.2 µl/min. The injection coordinates were anteriorposterior, -2 mm, mediolateral, $\pm$ 2mm, dorsoventral, -2mm from bregma. The person in charge of injection wore protective equipment and the injections were conducted in a biological safety cabinet in a biological safety level II lab setting. Autoclave of disposals was used to prevent contamination. The injections were conducted in 3.5-/4-month-old mice and behavior test was performed during 17-23 days after injection in which learning curve was acquired during 17-22 days and probe trial conducted at the 23rd day after injection. Halves of mice were used for immunohistochemistry and halves of them used for western blot.

Morris Water Maze

Water maze tests were performed using three groups of mice, WT mice (with intrahippocampal injection of Ad-EGFP) and APP/PS1 mice (intrahippocampal injection of Ad-EGFP or Ad-Dicer1-T2A:EGFP). The water maze (1.2-m diameter) was filled with water (24°C) and made opaque by the addition of nontoxic white paint. The water maze was surrounded by a black curtain (placed 80 cm away) that held three salient visual cues. Initially, mice were randomly trained in four quadrants of the water maze, and were allowed a maximum of 60 s to find a hidden platform (10-cm diameter, 1 cm

under the water surface). Mice were trained for 6 days (4 trials per day, 10-min interval between trial). On day 7, mice were given a 60-s probe test (scanning the platform) to test their spatial memory. SLY-WMS Morris Water Maze System (Beijing Sunny Instruments Co. Ltd) consisting of a frame grabber, a video camera, a water pool, and an analysis software was used to monitor the animal's swimming pattern, distance, speed, and the amount of time spent in each of the four quadrants.

Cell culture

Newly born mice were used for culturing dissociated hippocampal and cortical neurons (CNs and HNs, respectively). Hippocampal or cortical tissue was mechanically dissociated after digestion with 0.25% trypsin (Beyotime, Haimen, China) and 0.1% DNaseI (Takara, Dalian, China) at 37°C for 15 min. DMEM/F12 containing 10% fetal bovine serum (Gibco, Billings, MA, USA) was then added, and the cells were centrifuged at 120 g for 3 min and the supernatants were then plated on poly-D-lysine-coated 8 cm-culture dishes in neurobasal medium supplemented with 2% B27 (Invitrogen, Carlsbad, CA, USA) and 10 μ M cytosine arabinoside (Sigma-Aldrich, Saint Louis, MO, USA). After 24 hours, the culture medium was replaced with fresh neurobasal medium supplemented with 2% B27 and the neuronal cultures were used for experiments after three days *in vitro culture* (DIV 3). Before transfection, the medium was replaced with DMEM/F12 containing 0.1% bovine serum albumin. After 24 h in culture, neurons were used for transfection, A β 42 oligomer treatment or adenovirus infection. N2A, SK-N-BE(2)

or HEK 293T cells was cultured in Dulbecco's modified Eagle's Medium(DMEM) containing 10% Fetal bovine serum(FBS).

Plasmid construction and Luciferase reporter assay

With genomic DNA extracted from HEK 293T cells as a template, the region upstream of Dicer1 transcription start site (tss) (Entrez acc.no.:NM_177438.2) were amplified, producing an amplicon containing sequence from -1280 (relative to tss) to +3 (promoter 3), using forward primer CGACGCGTTT**AGATTGCG**CCACTACATT and reverse primer CCCAAGCTTCCCAAGCTTCCCAAGCTTCTTTGTGTCC. Two more regions were amplified spanning -1121 or -896 to +3 by alternating forward primer CGACGCGTAGCCTGGGT**AGACAGAGC**GAACTCT (promoter 2) or CGACGCGTACCT**AGCTGGCT**GGGACCCAGCATTTA (promoter 1), respectively. The bold letters indicated the predicted AREs sites with consensus sequence TGA(C/G)NNNGC. A sequence not containing AREs site was also amplified by changing a forward primer CGACGCGTGGGCAGTCAGAGAGAGAGGAAAGGAAGG spanning -581 to +3 (promoter 0). The region spanning individual ARE sequence was also amplified by using forward primer CGACGCGTAGGAGATCAAGACCATCCTGGG and reverse primer CCCAAGCTTTAGTGGCGCAATCTCAGCT for ARE3 (124 bp). ARE2 (192 bp) was amplified using forward primer CGACGCGTCAGCCTGGGTGACAGAGCGAAAC and reverse primer CCCAAGCTTCTGGTCTGCAAGGCA. ARE1(185 bp) was amplified using forward primer CGGCACGCGTGAGCTGCCTTGTGACTTTGCCTTC and reverse primer

CCCAAGCTTCGTCTTTCAACACTTGATCA . PCR products were amplified by pfu High fidelity enzyme and cloned into a pJet1.2 vector and then subcloned into pGL6-basic containing firefly luciferase coding sequence. The constructs spanning tss were referred to as Dicer 1 promoter 3-Luc, promoter 2-Luc, promoter 1-Luc, and promoter 0-Luc and the constructs containing individual AREs were referred to as ARE3-Luc, ARE2-Luc, and ARE1-Luc. ARPE1 in promoter 1-Luc was mutated with forward primer incorporating mutated base pair in bold letters **GTACCTGGCTGGGACCCAGCATTTA** and reverse primer CCCAAGC-TTCCCAAGCTTCTTTGTGTCC; promoter 1-Luc was used as the template. Using the resultant plasmid as the template, the forward primer incorporating Mlu I restrict site and the reverse primer containing HindIII restriction site were used to generate product, which was subcloned into PGL6-basic, namely, mutant promoter-1. The forward primer was CGACGCGTACCGTACCTGGCTGGGACCCAG CATTTA and the reverse primer was CCCAAAGCTTCCCAAGCTTCCCAAGCTTCTTTGTGTCC with restriction sites underlined.

The HEK293T cells were seeded at a density of 3×10^4 cells/per 48 wells and transfected according to the manufacturer's protocol (Lipofectamine 2000, Invitrogen). The *Renilla* luciferase-containing reporter (pRL-TK Vector) was mixed with individual construct containing firefly luciferase, and a human pCMV-Nrf2 or an empty pCMV3 vector at a ratio of 1:20:20, respectively. The relative luminescence unit (RLU) of luciferase/renilla assay was measured per the manufacturer's protocol (Beyotime Biotechnology, cat#RG027) by a plate reader (SpectraMax M5, Molecular devices, San Jose, CA, USA).

Chromatin immunoprecipitation (CHIP) assay

The CHIP assay kit (Beyotime Biotechnology, cat# P2078) was used to examine the interaction between Nrf2 and Dicer1 promoter. All the procedures were performed according to the manufacturer's instructions. SK-N-BE cells were plated on 10-cm culture plate until ~70% confluency and added with 37% formaldehyde diluted to a 1% final concentration. To crosslink the protein-DNA complexes, the cells were incubated with formaldehyde on a shaker for 10 min at 37 °C. 1X glycine solution at a final dilution was added to quench the formaldehyde, which was rocked at room temperature for 5 min, and then the media were removed. The cells were immediately washed twice and harvested with ice-cold phosphate buffered saline (PBS) containing 1mM phenylmethylsulfonyl fluoride (PMSF), with 2×10^8 cells in each microcentrifuge tube. The harvested cells were subject to centrifuge with 1000 x g at 4 °C, resuspended in SDS (sodium dodecyl sulfate) lysis buffer containing 1 mM PMSF, and incubated on ice for 10 min. Following the lysis, the samples were sonicated to shear chromatin, centrifuged for 5 min at 13,000 x g at 4 °C, and the supernatants were harvested and diluted 10-fold in CHIP dilution buffer containing 1 mM PMSF. After dilution, 15% of each sample was collected to use as a positive input in PCR analysis. The rest of each sample (~ 2 mL) were pre-absorbed with addition of 70 µL protein A+G agarose/salmon sperm DNA, and the mixture were rocked for 30 min at 4 °C, centrifuged for 1 min at 1,000 x g, and the supernatants were collected. After this pre-absorption, rabbit anti-Nrf2 antibody (Proteintech) was used to precipitate DNA-protein complexes; rabbit IgG (Beyotime Biotechnology, cat#A7016) was used as a negative control. To capture the antibody, 60 µL protein A+G agarose/salmon sperm DNA was added, rocked for 60 min

at 4 °C, and centrifuged for 1 min at 1,000 x g. The supernatants were discarded and the pellets were washed sequentially with Low, High Salt, and LiCl Immune Complex Wash buffer, and TE buffer. The pellets were used for PCR amplification and the following specific primers were used in PCR analyses for amplifying fragment containing ARE1 in *Dicer1* promoter: sense, 5'-CACAGACCTGAGCTGGCTGGGAC-3'(-794~-817 relative to tss), antisense, 5'- CAGATTGCGATGTGCGTGAAA-3'(-691~-712 relative to tss). For amplifying the fragment containing ARE2 in the promoter, the primers used were 5'-CAGCCTGGGTGACAGAGCGAAAC-3' (-1069~-1091 relative to tss) for sense, and 5'- CTGGTCTGCAAGGCAGTTTAT-3'(-876~-906 relative to tss) for antisense; for amplifying ARE3-containing fragment in the promoter, the primers were 5'- AGGAGATCATGAGATTGCTGGG-3'(-1250~-1271 relative to tss) for sense, and 5'- TAGTGGCGCAATCTCAGCT-3' (-1097~-1115 relative to tss) for antisense. DNA fragments from each group were amplified by PCR SuperMix(cat# 10572014, Thermo Fisher) with an initial denaturation at 94 ° C for 5 min, followed by 39 cycles (94 ° C for 30 s, 60 ° C for 45 s, and 72 ° C for 20 s). Finally, the reaction was ended with 5-min extension at 72 ° C. The PCR products were assessed by use of 2% agarose electrophoretic gel.

Aβ42 oligomer preparation

The oligomer form of Aβ42 was prepared as described [26]. Briefly, the peptide was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (Sigma-Aldrich) to remove any aggregates, stored in 4°C for 30 min, dried under room temperature, and dissolved in

dimethyl sulfoxide (DMSO, Sigma-Aldrich) to 1 mM. The dissolved peptide was diluted in phenol-free F12 medium to 500 nM and incubated at 4°C for 24 h. The solution was subjected to centrifugation at 14,000 x g for 30 min at 4°C. The supernatant was collected as the oligomer preparation which was used at a final concentration of 100 nM.

Cell viability assay

Neurons were cultured in 96-well plates at a density of 5×10^3 per well in DMEM/F-12 medium supplemented with B-27. After transfected with Dicer1 siRNA duplex (sense, 5'-GCACAUCAAGGUGCUACUATT-3', antisense, 5'-UAGUAGCACCUUGAUGUGCTT-3') or negative control siRNA duplex (sense, 5'-UUCUCCGAACGUGUCACGUTT-3', antisense, 5'-ACGUGACACGUUCGGAGAATT-3') by lipofectamine 2000 for 12 h, the medium was replaced with fresh DMEM/F-12 medium and continued to culture for 36 h. At the end of treatment, each well was added with 10 μ L MTS reagent (500 μ g/mL) for 4 h. The absorption values were read in a plate reader at 490nm (SpectraMax M5, Molecular devices).

Measurement of mitochondrial membrane potential and reactive oxygen species (ROS) activity

Primary cultured cortical neurons at 9×10^5 cells/well and hippocampal neurons at 5×10^5 cells/ well were plated into 6-well plates and cultured until DIV3. The neurons were transfected with Dicer1 duplex siRNAs (50 pM) per well by lipofectamine 2000 for

12 h in DMEM/F12 plus 2% B27. After transfection, the cultured medium was replaced with fresh neurobasal medium supplemented with 2% B27 and continued to culture for 36 h. For mitochondrial membrane potential($\Delta\Psi_m$) assay, the neurons at the end of treatment were stained with JC-1 probe solution at 37 °C for 20 min and observed under fluorescence microscope (DMI8, Leica Biosystems, Wetzlar, Germany). The mitochondria were also extracted by a mitochondrial extraction kit. Neural mitochondrion (30 μ g) from each group was incubated with JC-1 probe solution at 37 °C for 20 min. The reaction product was added into a 96-well plate for fluorescence measurement, which was read with excitation at 490 nm and emission at 520 nm for monomer (green) and with excitation at 530 nm and emission at 590 nm for aggregates (red). The $\Delta\Psi_m$ values were represented by the ratios between aggregate and monomer fluorescence in relative fluorescence units (RFU). For reactive oxygen species (ROS) assay, CNs and HNs were incubated with 10 μ M 2',7'-Dichlorodihydrofluorescein diacetate in neurobasal medium supplemented with 2% B27. After incubation at 37°C for 30 min, the neurons were added into 96-well plate at the density of 4×10^4 per well and subjected to measurement with plate fluorescence reader at 485nm (SpectraMax M5, Molecular devices).

Neurite outgrowth assay

The neuronal cultures were treated by oligomer A β 42 at 100 nM for 24 h and then infected with Ad-EGFP or Ad-Dicer1-T2A:EGFP virus (5×10^7 vg/mL) for 48 h. For determination of neurite, the neurites at least two times longer than the soma diameter

were included in the measurement (Image pro plus, Olympus Optical Co., Ltd, Tokyo, Japan). The measurement of neurite length was conducted in three independent culture preparation and the values were averaged from 150 infected neurons in either group.

Western blot analysis

The 4- or 11-month-old mice were fasted overnight , and at the next morning, mice were deeply anesthetized by isoflurane and transcardially perfused with ice-cold phosphate buffer (PBS). The hippocampi were isolated and freshly frozen in liquid nitrogen. For analyzing the neurons treated by amyloid peptide, the cytoplasmic and nuclear proteins were extracted by plasma and nuclear protein isolation kit (Cat# P0027, Beyotime Biotechnology), respectively. For analyzing the cells for overexpressing Nrf2 plasmid, SK-N-BE (2) or N2A cells were seeded in 6-well plates at the density of 1×10^6 or 5×10^5 cells/well, respectively. Tissue or cell samples were homogenized in Super RIPA buffer composed of components in mM: 460 Tris–HCl, pH 7.4, 138 NaCl, 1 EDTA, 2.5 NaF, 2.5 Na₃VO₄, 1 phenylmethanesulfonylfluoride, 1 dithiothreitol, supplemented with 0.1% Nonidet P-40, and 1 X protease/ 1 X phosphatase inhibitor cocktail (Sigma, St.Louis, MO). Proteins were resolved on 12% SDS-PAGE gels (Bio-Rad) and transferred to nitrocellulose membranes (ThermoFisher). Membranes were blocked in blocking buffer (Beyotime Biotechnology) and then incubated with primary antibody. Following wash, the membranes were then incubated with secondary antibody, and

developed with chemiluminescence system kit (ThermoFisher). The blots were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The hippocampi were isolated and freshly frozen in liquid nitrogen. SK-N-BE cells or Neuro-2A cells were plated on 6-well plate until ~70% confluency, and transfected with 1.5 µg pCMV3 Nrf2 vector or pCMV empty vector for 48 h in DMEM supplemented with 10% FBS, respectively. Extraction of total RNAs from hippocampi or transfected cells and elimination of genomic DNA were performed by a RNA extraction kit (TIGEN BIOTECH, Co, LTD., Beijing, China, cat# DP424). RNA (200 ng) was used to synthesize the cDNA by a first stand cDNA synthesis kit (cat# R111-02, Vazyme, NanJing, China). Fifty nanograms of cDNA was used as a template to amplifying individual mRNA. The following primers were used in qRT-PCR: *hDicer 1*, sense, CCCGGCTGAGAGAACTTACG, antisense, CTGTAACCTTCGACCAACACCTTTAAA; *hNrf2*, sense, TTCCCGGTCACATCGAGAG, antisense, 5'-TCCTGTTGCATACCGTC TAAATC-3'; *mDicer1*, sense, 5'-GTCAGCCGTCAGAACTCACTC-3', anti-sense, 5'-ACAGTCAAGGCGACATAGCAA-3'; *mNrf2*, sense, 5'-CAGCATAGAGC AGGACATGGAG-3', antisense, 5'- GAACAGCGGTAGTATCAGCCAG-3'; *B1* RNA, sense, 5'-TGCCTTTAATCCCAGCACTT-3', anti-sense, 5'- GCTGCTCACA CAAGGTTGAA-3'; the 18srRNA, sense, 5'-TTCGTATTGCGCCGCTAGA-3', antisense, 5'-CTTTCGCTCTGGTCCGTCTT-3'. The SYBRTM Green (Invitrogen) was used as the dye for the qRT-PCR reaction following the manufacturer's instruction. The

amplification processes were initiated at 50 ° C for 2 min and a secondary step at 95 ° C for 10 min, followed by 40 cycles of PCR reactions (95 ° C for 10 s and 60 ° C for 1 min). Reaction specificity, indicated by the presence of a single amplification peak for each PCR reaction, was examined by use of dissociative curves. $2^{-\Delta\Delta C_t}$ method was used to quantify the reaction, which was normalized to sham treatment or transfection of pCMV3 empty vector. Primary cultured cortical neurons and hippocampal neurons were plated on 6-well plate, and treated with A β 42 oligomer (100 nM) or sham treatment for 48 h in neurobasal medium with 2% B27, respectively. *Dicer1* mRNA levels or *B1* RNA levels were assessed by qRT-PCR as above.

Immunofluorescence

The 4- or 11-month-old WT, APP/PS1 non-fasted mice were deeply anesthetized by isoflurane, killed, and then transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA) in PBS. The isolated brains were postfixed in 4% PFA overnight, then stored in PBS containing 0.01% sodium azide. Brains were mounted in agarose and 10 μ m coronal sections were obtained using a Leica VT1000S Vibratome. Antigen demasking was performed in 0.01M sodium citrate solution (pH 6.0) at 99°C for 40 min. After washing the sections in PBS three times, 5 min each, at room temperature (RT), the sections were blocked using 5% normal donkey serum in PBS containing 0.2% Triton X-100 for 1 h at RT. The sections were incubated with the rabbit anti-NeuN (1:800, Beyotime Biotechnology) or monoclonal anti-Dicer 1 (1:300, Sigma-Aldrich) overnight at 4°C. For mice injected with virus, the sections were incubated with

anti-Dicer 1 (1:300, Sigma-Aldrich) or anti-activated caspase 3 (1:300, Beyotime Biotechnology). After washing 3 times with PBS (3×5 min at RT), sections were incubated with secondary antibodies: Alexa Fluor 594 goat anti-rabbit or Alexa Fluor 488 goat anti-mouse IgG (1:1000, Life Technologies Corporation, Carlsbad, CA, USA) for 1 h at RT, then washed three times with PBS (3×5 min at RT). The sections were then mounted on coverslips (Citotest Labware Manufacturing Co., Ltd, Jiangsu, China) and the images of CA3 hippocampus were obtained with a fluorescent microscope (DMi8, Leica Biosystems).

Experimental design and statistical analysis

During Water Maze test, mice were evenly allocated to each experimental group according to the gender and ages, and the tests were conducted in a blinded manner, that is, the investigator was blinded to the group allocation of the genotype or treatment during the experiment and when assessing all the results. Animal experiment sample size calculator: <http://www.lasec.cuhk.edu.hk/sample-size-calculation.html> was used to determine the sample sizes, assuming 40% difference in the mean and standard deviation of 20% at power 95% and type I error 0.01 [27]. Similarly, determination of neurite outgrowth was conducted in a blinded manner. All the data acquired during experiments were included for analysis and there were no sample size differences between the beginning and end of experiments. All data were presented as means $\pm$ SD unless specified. Single data points were shown as overlaying dot-plot on bar-graph when sample sizes were smaller than 15, otherwise, shown as bar-graph.

For parametric data, Student's *t-test* was used for comparing differences between two groups. In experiments with more than two groups, one-way ANOVA was performed followed by Tukey's *post hoc* test for comparisons among groups. For analysis of two groups of non-parametric data, the Mann-Whitney *U*-test was used for determining the differences. For Morris water maze experiment, two-way ANOVA with repeat measures followed by Tukey's *post hoc* test was used for analyzing the time spent in four quadrants, frequency to crossing platform, swimming speed and distance. Statistical analyses were performed with Graphpad Prism 7.04 software (Graphpad software, Inc. La Jolla, CA, USA). Difference was regarded as significance when $p < 0.05$.

Results

Dicer1 or Nrf2 expression was reduced in the hippocampus of APP/PS1 mice

Dicer1 is reduced in the RPE cells in advanced dry AMD[20] and knockout of Dicer1 induces neurodegeneration [22, 23]. In light of these evidence, we explored Dicer1 expression in the brain of the AD mice, APP/PS1. The staining of Dicer1 was reduced in the CA1, CA3, and dentate gyrus (DG) of APP/PS1 mice compared to WT littermate, at the age of 4 months or 11 months (Figure.1A). The protein was also significantly reduced in the homogenates from the hippocampus of 4-month APP/PS1 mice compared to WT littermate ($p=0.0092$, $n=6$ for each genotype, 3 female and 3 male) and this reduction became severe at the age of 11 months ($p=0.0002$, $n=6$ for each genotype, 3 female and 3 male) (Figures.1B and 1C). Consistent with reduced protein, Dicer1 mRNA was also reduced in the hippocampus compared to WT at the age of 4 months ($p=0.0335$), and at the age of 11 months as well ($p=0.0304$) (Figure.1F). Similarly, Dicer1 was significantly reduced in the homogenates from the parietal lobe of APP/PS1 mice at the age of 6 months when compared to WT littermate ($p=0.022$, $n=6$ for each genotype, 3 female and 3 male,)(Supplemental Figure.1). Nrf2 is a master regulator of anti-oxidation genes regulating redox homeostasis[28]; Dicer1 depletion induces cytotoxicity via oxidative stress[20]. Thus, we simultaneously examined the protein levels of Nrf2 and activated caspase3 in the homogenates from APP/PS1 hippocampi. Similarly, Nrf2 protein levels in AD mice were significantly reduced compared to WT littermate at the age of 4 months ($p=0.0001$) and 11 months ($p=0.0038$), respectively (Figures. 1B and 1D). In accordance with these findings, Nrf2 mRNA in hippocampus from AD mice was reduced compared to WT littermate at 4

months ($p=0.0167$) and at 11 months as well ($p=0.0195$), respectively (Figure. 1G). Activated caspase3 in AD mice was significantly increased compared to WT littermate at 4 months ($p=0.0098$) and 11 months ($p=0.0008$), respectively (Figures. 1B and 1E).

Dicer1 knockdown induced neurotoxicity

Dicer1 depletion induced cytotoxicity involving the roles of ROS and inflammation in the RPE cells [21]. Thus, we explored the relevant effects by knocking down Dicer1 with specific siRNA in the primary murine hippocampal neuronal cultures (HNs). The Dicer1 siRNA used was selected from three siRNAs basing on efficacy (Supplemental Figure. 2A) and siRNA3 was used as Dicer1 siRNA in the following experiments. As expected, Dicer1 knockdown by the specific siRNA induced ROS production in HNs ($p=0.00001$) compared to NC siRNA (Figure. 2A). Similarly, Dicer1 knockdown reduced mitochondrial membrane potential indicated by JC-1 staining in the HNs ($p=0.0399$) (Figures. 2B and 2C). We further explored the effect of Dicer1 knockdown on neuronal survival and the related signaling. Under the condition of Dicer1 knockdown, neuronal survival was compromised in the HNs ($p=0.0012$) (Figure. 2D). The protein levels of activated caspase3 under Dicer1 knockdown were also increased in the HNs ($p=0.020$) compared to the effects of scrambled siRNA (NC siRNA) (Figures. 2E and 2G). Compared to transfection with NC siRNA, the Dicer1 siRNA significantly decreased Dicer1 protein in the HNs ($p=0.044$) (Figures. 2E and 2F). The effects of Dicer1 knockdown on ROS production, mitochondrial integrity and neuronal survival were also

replicated in the primary murine cortical neuronal cultures (Supplemental Figures. 2B-2H).

A β 42 oligomer decreased Dicer1 and Nrf2 in neurons upon chronic exposure

As above, we found that Dicer1 was reduced in APP/PS1 mice; the mice contain robust level of soluble A β and the deletion mutation (dE9) significantly accelerates the shift of the A β 40/ A β 42 ratios and elevates the levels of A β 42 [29, 30]. Thus, we investigated the effect of A β 42 on Dicer1 expression in the CNs. Initially, we tested the time-course effect of A β 42 on Dicer1 or Nrf2, and A β 42 significantly reduced the protein levels of Dicer1 or Nrf2 after 12-48 h treatment but did not change Nrf2 protein level upon 6-h treatment (Figures. 3A-3C). A β 42 oligomer also decreased Dicer1 mRNA in the CNs ($p=0.0277$) or in the HNs ($p=0.0387$) (Supplemental Figure. 3A). To investigate the consequence of Dicer1 reduction by A β 42 oligomer, we examined one of Dicer1 substrates, B1 RNA (Alu-equivalent in rodents) in the CNs ($p=0.0469$) or in the HNs ($p=0.0252$) (Supplemental Figure 3B). We further tested whether reduction of Dicer1 by A β 42 could be rescued by N-acetyl-cysteine (NAC), an antioxidant. As expected, A β 42 treatment for 48 h reduced Dicer1 in CNs compared to sham treatment ($p=0.00001$), which was significantly rescued by NAC ($p=0.032$)(Figures.3D and 3E). Simultaneously, we examined total Nrf2, which was also reduced by A β 42 ($p=0.0005$), the effect rescued by addition of NAC ($p=0.0009$) (Figures. 3D and 3F). Since Nrf2 affects anti-oxidant gene transcription via interacting with AREs in nucleus [31], we further examined the levels of nucleic or cytosolic Nrf2 under A β 42 treatment or those

under simultaneous addition of NAC. A β 42 reduced either nucleic Nrf2 ($p=0.0326$) (Figures. 3G and 3I) or cytosolic Nrf2 ($p=0.0076$) (Figures. 3H and 3J), which was rescued by addition of NAC ($p=0.00001$ for nucleic) or ($p=0.0419$ for cytosolic), respectively (Figures. 3G and 3H).

Nrf2 regulated Dicer1 expression

Nrf2 is a master regulator of transcription of anti-oxidant genes. We hypothesized that Nrf2 may regulate Dicer1 expression. To test this hypothesis, we examined Dicer1 expression in Neuro-2a cells with overexpression of Nrf2, compared to the cells transfected with vehicle plasmid. The levels of *Dicer1* mRNA were increased by Nrf2 overexpression in Neuro-2a cells ($p=0.0016$ for Nrf2 mRNA; $p=0.0482$ for *Dicer1* mRNA) (Figures. 4A and 4B). The protein levels of Dicer1 and Nrf2 were also examined by western blot. Consistent with increased levels of *Dicer1* mRNA induced by Nrf2, overexpression of Nrf2 in Neuro-2a cells significantly increased Dicer1 protein when compared to the cells transfected with vehicle plasmid ($p=0.0004$) (Figures. 4C-4E). We next tested whether reduction of Dicer1 by A β 42 could be rescued by overexpression of Nrf2. As expected, A β 42 decreased Dicer1 ($p=0.0004$) which was rescued by Nrf2 overexpression in Neuro-2a cells ($p=0.0399$) (Figures. 4F-4H). We scanned the promoter of Dicer1 with software (promoter 2.0 prediction server) and found three AREs motif upstream of transcription start site (tss), that is, *Dicer1 promoter* region. We cloned the ARE1-ARE3-containing promoter fragments into PGL6-basic plasmid containing firefly luciferase-coding sequence (-Luc), respectively, and also cloned the

promoters containing different ARE motif into PGL6-basic plasmid, respectively ($p=0.00001$) (Figures. 4I and 4J). Transfection of Nrf2-expressing plasmid (pCMV Nrf2) increased the ratios between *firefly* and *renilla* in constructs containing ARE1($p=0.000219$) or ARE3 motif ($p=0.043$) when compared to the cells transfected with vehicle plasmid, pCMV3 (Figure. 4J). When compared the constructs containing different AREs under transfection with pCMV Nrf2, we found that the ratios of firefly/renilla from ARE1 constructs were significantly from the construct ARE2 ($p=0.000825$), ARE3 ($p=0.0093$), and PGL6-basic ($p=0.00041$), respectively (Figure. 4J). Transfection of pCMV Nrf2 increased firefly/renilla in promoter 1($p=0.00047$), promoter 2 ($p=0.000135$), promoter 3 ($p=0.000114$), and mutant promoter 1 ($p=0.048$) when compared to the cells transfected with pCMV3 (Figure. 4J). When compared different promoter constructs under transfection with pCMV Nrf2, we found that the ratios of firefly/renilla from promoter1 were significantly from promoter 0 ($p=0.000361$) and mutant promoter1($p=0.009$) but not different with promoter 2 ($p=0.194$) or promoter 3 ($p=0.254$) (Figure. 4J). To confirm whether Nrf2 directly interacted with AREs in the *Dicer1* promoter, thereby regulating Dicer1 expression, we used Nrf2 antibody to capture Dicer1 promoter fragments and amplified the precipitated ARE1- (-871~-691 bp, relative to tss), ARE-2 (-1091~-876 bp), or ARE-3 (-1271~-1097 bp)-containing fragments, respectively in SK-N-BE cells (Figure. 4I and 4K). Basing on these data, we projected that Nrf2 regulated Dicer1 transcription via binding with AREs in the promoter in which the interaction between Nrf2 and ARE1 was most important for this regulation. Overexpression of Nrf2 increased Dicer1 was replicated in SK-N-BE cells as well (Supplemental Figure. 4)

Overexpression of Dicer1 recovered neurotoxicity by A β 42 oligomer

Since we have demonstrated that Dicer1 depletion compromised cell survival (Figure 2), we wondered whether overexpression of Dicer1 mitigated A β 42-induced neurotoxicity. The infection rate of the virus reached ~90% in the primary murine hippocampal neuronal cultures (Supplemental Figure 5). A β 42 oligomer treatment reduced the protein levels of Dicer1 compared to sham treatment ($p=0.033$), which was rescued by infection with Ad-Dicer1-T2A:EGFP virus compared to infection with vehicle virus, Ad-EGFP ($p=0.0074$) (Figures. 5A and 5B). A β 42 oligomer treatment increased activated caspase 3 compared to sham treatment ($p=0.0061$), which was partially rescued by infection with Ad-Dicer1-T2A:EGFP virus compared to infection with Ad-EGFP ($p=0.023$) (Figures. 5A and 5C). Under expectation, A β 42 oligomer decreased neuronal survival ($p=0.00067$), which was partially rescued by infection with Ad-Dicer1-T2A:EGFP virus ($p=0.047$) (Figure. 5D). Dicer1 knockdown disrupted neurite in primary cortical neurons ($p=0.011$) (Figures. 5E and 5F), which is reminiscent of the early axonal degeneration in AD. Similarly, A β 42 oligomer treatment trimmed neurite ($p=0.025$), which was significantly recovered by overexpression of Ad-Dicer1-T2A:EGFP virus (Figures. 5G-5J).

Overexpression of Dicer1 improved spatial learning in APP/PS1 mice

We further explored the effect of overexpression of Dicer1 in APP/PS1 mice. Injection of virus induced reliable transfection (Supplemental Figure 6). Consistent with previous observation in Figure. 1A, Dicer1 staining was decreased in CA3 hippocampus

compared to WT littermate at the age of 3.5 months; injection of Ad-Dicer1:T2A:EGFP significantly increased Dicer1 staining in CA3 hippocampus in APP/PS1 mice (Figure. 6A). CA3 hippocampi of APP/PS1 mice contained stronger staining for activated caspase 3 compared to those of WT littermate, which was reduced by injection of Ad-Dicer1:T2A:EGFP virus (Figure. 6B). In parallel experiment, we isolated the whole hippocampi and subjected the homogenates to western blot against Dicer1 and caspase 3. Consistent with the immunofluorescence, there was lower level of Dicer1 in APP/PS1 mice than WT ($p=0.0217$) which was recovered by injection of Ad-Dicer1:T2A:EGFP ($p=0.0093$) (Figures. 6C and 6D); there was higher level of activated caspase 3 in APP/PS1 mice than WT ($p=0.00681$) which was reduced by injection of Ad-Dicer1:T2A:EGFP ($p=0.0067$) (Figures. 6C and 6E). We further investigated whether overexpression of Dicer1 had effect on spatial learning and thus examined behavior with Morris Water Maze. During the training period on days 1-6 after virus injection, the latency to reach platform for APP/PS1 mice was significantly reduced compared to that of WT mice ($p=0.0148$, two-way ANOVA) (Figure. 7A) which is consistent with previous reports that cognitive function is compromised at 3.5-4 months of APP/PS1 mice [32, 33]. This reduction was reversed by injection with Ad-Dicer1:T2A:EGFP virus compared to injection with Ad1-EGFP virus ($p=0.008$) on day 5 ($p=0.0057$), or ($p=0.00001$) on day 6 ($p=0.0025$) (Figure. 7A). Under the condition of combining timepoint and treatment, the latency was significantly reversed by injection with Ad1-Dicer1:T2A:EGFP virus ($p=0.0001$) ($F_{\text{day}} (5, 174) = 36.44$, $p=0.00001$; $F_{\text{treatment}} (2, 174) = 2.045$, $p=0.0241$; $F_{\text{interaction}} (75, 174) = 2.3$, $p=0.0148$, two-way ANOVA) (Figure. 7A). On day 7 after a 60-s probe test for searching platform, we monitored animal's

swimming pattern, distance, speed, and the amount of time spent in each of the four quadrants. Without difference on swimming distance ($p=0.3141$) (Figure. 7D) and swimming speed ($p=0.0948$) (Figure. 7E), APP/PS1 mice spent less time in target quadrant than WT mice ($p=0.0023$) in probe trial test, which was reversed by injection with Ad-Dicer1:T2A:EGFP virus ($p=0.0087$) ($F(2, 42)=5.43$, $p=0.0092$; $F_{\text{quadrant}}(3, 116) = 4.576$, $p=0.1122$; $F_{\text{treatment}}(2, 116) = 0.1032$, $p=1$; $F_{\text{interaction}}(45, 116) = 1.334$, $p=0.0046$, two-way ANOVA) (Figure. 7B). Similarly, the AD mice crossed the platform less frequently than WT mice ($p=0.039$), but the chance was increased by injection with Ad-Dicer1:T2A:EGFP virus ($p=0.043$) (Figure. 7C).

Discussion

In our study, reduction of Dicer1 was detected as early as around 4 months in APP/PS1 mice in which A β deposition begins in the brain at the age of 6 months [29]. Furthermore, knockdown of Dicer1 in cultured neurons induced neurite deficit before soma degeneration, and intrahippocampal injection of Dicer1-expressing virus improved spatial learning. These evidence suggest that reduction of Dicer1 is an early event in AD, possibly before overt A β deposition and targeting Dicer1 may provide a new strategy for AD therapy, with target at the early stage of this disorder.

The expression or activity of Dicer1 is delicately maintained with the regulation at transcriptional, post-transcriptional, and post-translational levels. For instance, SOX4, a transcriptional factor involved in embryonic development and cell fate determination, positively regulates *Dicer1* transcription [34]. Similarly, a transcription factor linked with microphthalmia, melanocyte inducing transcription factor, increases its transcription during melanocyte differentiation [35]. At post-transcriptional level, microRNA such as let-7 miRNA or miR-292 regulates *Dicer1* via targeting its coding region or 3'-UTR, respectively, thereby inhibiting its expression [36, 37]. At post-translation level, phosphorylation of Dicer1 at its RNase IIIb and double-stranded RNA-binding domains promotes its nuclear translocation and inhibits its function [38]--particularly, a phosphomimetic of phospho-serine by replacing serine 1836 with aspartate impairs Dicer1 function and causes postnatal lethality [39]. Distinct with these studies, we delineate a novel regulation of Dicer1, that is, by Keap1-ARE-Nrf2 pathway, which is unreported before. A β 42 reduced Dicer1 protein in neurons at 48 h treatment which was possibly due to reduced nuclear Nrf2 from chronic A β treatment. By contrast, at 6

h treatment, A β 42 reduced Dicer1 to a significant difference but Nrf2 was unchanged which suggested that Dicer1 diminution by acute A β treatment occurred at post-translational level which was independent on Nrf2. In other words, dicer1 depletion induces oxidative stress causing neurotoxicity; chronic A β treatment reduced Dicer1 which involved the roles of reduced Nrf2 whereas acute A β treatment--6 h treatment--reduced Dicer1 which was independent on Nrf2 and possibly took place at post-translational level.

Oxidative stress is thought to be central in the pathogenesis of AD, for example, oxidative stress exacerbates insulin resistance in AD brain [40] and disrupts mitochondria [41], resulting in failure to produce sufficient ATP for Na⁺-K⁺-ATPase and Na⁺-Ca⁺⁺ exchanger in order to maintain ion homeostasis in neurons [42-44]. Dicer1 depletion may be an internal sources contributing to oxidative stress by leading to accumulation of Alu RNA [21]. Intrahippocampal injection of Nrf2-expressing lentivirus improves spatial learning in APP/PS1 mice [45] but Nrf2 promotes cancer cell proliferation [46]. As such, manipulation of Nrf2 expression may lead to undesired side effects although it indicates potential benefit in AD brain [45]. By contrast, appropriate expression of Dicer1 may be an alternative strategy to oppose oxidative stress or other pathogenic factors in AD brain.

Dicer1 is ubiquitously expressed in the brain including in neurons, astroglia, and oligodendrocytes [23, 24, 47]. With immunofluorescence, we at least demonstrated that neuronal Dicer1 was decreased in AD hippocampus compared to that in littermate control mice. The glial Dicer1 in astrocytes or in oligodendrocytes was not investigated in this study. Thus, Dicer1 reduction detected from APP/PS1 brain homogenates and

secondary neurodegeneration are the combinatory effects from neurons and glia. Therefore, it is important to determine the percentage of Dicer1 reduction in different cell type in AD brain for therapy purpose.

Dicer1 is central to the processing from pre-microRNAs to mature microRNAs, which regulate neuronal survival and neuritogenesis [48, 49]. Deletion of functional Dicer1 has been found to induce accumulation of Alu RNA and thereby to activate NLRP3 inflammasome and to induce oxidative damages in advanced dry AMD [20, 21]. Conditional ablation of Dicer1 in oligodendrocytes induces neuronal impairment *via* demyelination, oxidative damage, inflammatory gliosis in mice [23]. There is also evidence showing that conditional knockout of Dicer1 in dopaminergic neurons of ventral midbrain leads to neuronal loss through inhibiting microRNAs biosynthesis[50]. Similarly, blocking microRNAs biosynthesis by Dicer1 deletion disrupts morphogenesis in the cortex and in the hippocampus [22]. Apart from Alu RNA accumulation, microRNAs deficiency due to Dicer1 depletion may play unexpected roles in AD neurodegeneration due to mature miRNA deficiency [22, 47].

Conclusions

In this study, we indicated that Dicer1 was reduced in the hippocampus and in the cortex of APP/PS1 mice *in vivo*, and further showed that Dicer1 was reduced by A β 42 oligomer *in vitro*. Dicer1 depletion led to neurite deficit and apoptosis by stress responses including increased ROS production, activated caspase3, and disrupted mitochondrial membrane potential. We further indicated that Nrf2 regulated Dicer1 expression which was linked with Keap1-Nrf2-ARE signaling pathway. Overexpression

of Dicer1 increased neuronal survival and recovered neurite deficit by A β 42 oligomer *in vitro*, and *in vivo*, Dicer1 overexpression in CA3 hippocampus improved spatial learning in APP/PS1 mice. Altogether, we reveal a novel role of Dicer1 in AD and a novel regulation of Dicer1. Our findings suggest Dicer1 may be a target in AD therapy.

Abbreviations

AD, Alzheimer's disease; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; miRNA, microRNA; KEAP1, Kelch-like ECH-associated protein1; RPE, retinal pigment epithelial cell; NLRP, nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing.

Declarations

Ethical approval and consent to participate

All experiments and data reports followed the SfN Policy on Ethics and the study was approved by the Animal Care and Use Committee of Wenzhou Medical University (Approval number# wydw2019-0141). This study did not use human samples or tissues.

Availability of data and materials

The data and materials are available from corresponding author on reasonable request.

Consent for publication

All the authors and funding agencies agree with publication of this study.

Conflict of Interests

All the authors have found no conflict of interests to disclose.

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Authors' contributions

Wang Y and Lian ML conducted all the experiments, collected, and analyzed the data and Wang Y also participated in writing; Zhou J initiated Dicer1 immunoblotting with AD mouse tissues; Wu SZ conceived of and supervised this project, analyzed the data, wrote the manuscript.

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Figures and figure legends

Figure 1. Reduction of Dicer 1, decreased Nrf2 and increased activated caspase-3

in the hippocampus of APP/PS1 mice. (A) APP/PS1 brains were processed into sections which were doubly stained for Dicer1 (green) or NeuN (red). The images were typical staining of CA1, CA3, or DG (n=3 mice per genotype). Bar, 25 μ m for all panels. (B) The supernatants from homogenized hippocampai at the age of 4-/11 months were collected and subject to examination of Dicer1, Nrf2, caspase-3, and activated caspase-3 by western blot. GAPDH was used as a loading control. The protein levels of Dicer1 and Nrf2 in the hippocampal tissues were reduced in APP/PS1 mice (n=6, 3 male and 3 female) compared to WT littermate (n=6, 3 male and 3 female), respectively, at the age of 4 months (Left panel) or at the age of 11 months (right panel). By contrast, the protein levels of activated caspase-3 were increased in APP/PS1 mice (n=6) compared to WT (n=6) at the age of 4-/11 months. (C) The optical densities of Dicer1 relative to GAPDH were normalized to WT at the age of 4 months (**p=0.0092) or 11 months (***p=0.0002) (n=6 per genotype, Mann-Whitney U test). (D) Nrf2 relative to GAPDH were normalized to WT (Mann-Whitney U test, ***p=0.0001 in 4-month, **p=0.0038 in 11-month, n=6 per genotype). (E) Activated caspase 3 relative to GAPDH were normalized to WT (Mann-Whitney U test, **p=0.0098 in 4-month, ***p=0.0008 in 11-month, n=6 per genotype), respectively. (F) Dicer1 mRNA was examined in WT (n=3) and in APP/PS1 mice (n=3) at the age of 4 months (p=0.0335) or 11 months (p=0.0304) (paired t-test). (G) Nrf2 mRNA was examine in WT (n=3) and in APP/PS1 mice (n=3) at the age of 4 months (p=0.0167) or 11 months (p=0.0195) (paired t-test).

Figure 2. knocking down Dicer 1 induced production of reactive oxygen species (ROS), reduced mitochondrial membrane potential, enhanced apoptosis in primary murine hippocampal neuronal cultures. (A) The ROS levels in hippocampal neuronal cultures (HNs) were measured by incubating 2',7'-dichlorodihydrofluorescein diacetate for 20 min at 37 °C. The fluorescence values in relative fluorescence units

(RFU) were acquired in a plate reader at 485 nm and were averaged from four independent culture preparation with duplicate cultures in each preparation.

**** $p=0.00001$. **(B)** Representative images of JC-1 staining in HNs subject to Dicer1 knockdown as above. Red fluorescence represented aggregate in mitochondria at hyperpolarized membrane potentials under healthy condition whereas green fluorescence as monomer in cytoplasm at depolarized membrane potentials under the context of compromised mitochondria. Bar 10 μm for all panels. **(C)** Mitochondrial membrane potential ($\Delta\Psi\text{m}$) of HNs subject to Dicer1 knockdown. The neurons were subject to Dicer1 knockdown and mitochondria (30 μg) were isolated and stained with JC-1. The values of mitochondrial membrane potential ($\Delta\Psi\text{m}$) were indicated by the ratios between aggregated/monomer RFU and averaged from three independent culture preparations. Student's *t-test* was used to compare the differences, * $p=0.0399$. **(D)** Viability of neurons subjected to Dicer1 knockdown. HNs were transfected with Dicer 1 siRNA or NC siRNA as described in the materials and methods. The absorption values at OD 450nm in Dicer1 siRNA group were measured and normalized to NC siRNA group. Student's *t-test* was used to compare the differences, ** $p=0.0012$ for comparison. **(E)** HNs were transfected with Dicer1 siRNA (50 pM) or control siRNA duplex (50 pM) per well and subjected to western blot examination of Dicer1, activated caspase3, caspase 3 and $\beta\text{III-tubulin}$ was used as a loading control. **(F)** The effects of knockdown by Dicer1 siRNA were normalized to those of NC siRNA and averaged from three independent culture preparation. Student's *t-test* was used to compare the differences, * $p=0.044$ for comparison. **(G)** The optical densities of activated caspase-3 relative to $\beta\text{III-tubulin}$ from F were normalized to NC siRNA group, and averaged from

three independent culture preparation. Student's *t-test* was used to compare the differences, * $p=0.02$ for comparison.

Figure 3. Chronic A β 42 oligomer treatment reduced Dicer1 in neurons which was coupled with Nrf2 reduction, the effects rescued by NAC. (A-C) The time-course effect of A β 42 on Dicer1 or on Nrf2. The primary murine cortical neuronal cultures were subjected to vehicle or A β oligomer treatment at indicated time. The cells were harvested for western blot for Dicer1 or Nrf2 and β III-tubulin was used as a loading

control. The relative optical densities of Dicer1 or Nrf2 from triplicate experiments were quantified and indicated in B and C. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s indicated no significance. **(D)** The primary mouse cortical neurons were treated with A β 42 oligomer (100 nM) and antioxidant NAC (2 μ M) for 48 h. The neuronal cultures were harvested for western blot against Dicer 1, Nrf2, and Keap1. β III-tubulin was used as an internal loading control. **(E)** The optical densities of Dicer1/ β III-tubulin from D were normalized to sham treatment and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's *post hoc* test was used to compare the differences. Control vs. A β 42 oligomer treatment, **** $p = 0.00001$; A β 42 oligomer vs. A β 42 oligomer plus NAC treatment (A β +NAC), * $p = 0.032$. **(F)** The optical densities of Nrf2/ β III-tubulin from D were normalized to sham treatment and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's *post hoc* test was used to compare the differences. Control vs. A β 42 oligomer treatment, *** $p = 0.0005$; A β 42 oligomer vs. A β +NAC, *** $p = 0.0009$. **(G)** The neurons were treated with A β 42 oligomer (100 nM) and antioxidant NAC (2 μ M) for 24 h and the nucleic proteins were extracted and blotted against Nrf2, and histone3 served as a loading control and vimentin as a negative control; **(H)** the cytoplasmic proteins were isolated and blotted against Nrf2, and vimentin served as a loading control and histone3 as a negative control. **(I)** The optical densities of nucleic Nrf2 relative to histone 3 averaged from three independent culture preparation in G. One-way ANOVA followed by Tukey's *post hoc* test was used to compare the differences. Control vs. A β 42, * $p = 0.0326$; A β 42 vs. A β +NAC, **** $p = 0.00001$. **(J)** The optical densities of cytosolic Nrf2/vimentin were normalized to sham treatment and averaged from three independent culture preparation

in H. One-way ANOVA followed by Tukey's *post hoc* test was used to compare the differences. Control vs. A β 42 oligomer, *p=0.0076; A β 42 oligomer vs. A β +NAC, *p=0.0419.

Figure 4. Nrf2 regulated Dicer1 production via Nrf2-ARE pathway. Neuro-2a cells were transfected with mouse Nrf2-expressing plasmids (pCMV Nrf2, 1.5 μ g) or equal amount of vehicle plasmid (pCMV3) in 6-well plates. Examination of *Nrf2* or *Dicer1* mRNA was performed by qRT-PCR analysis. **(A-B)** Determination of *Nrf2* and *Dicer1* mRNA levels by overexpression of Nrf2 in Neuro-2A cells. Student's *t*-test was used to compare the differences. * $p=0.0016$ for comparison of the levels of *Dicer1* mRNA, * $p=0.0482$ for comparison of the levels of *Nrf2* mRNA. **(C)** Neuro-2a cells were transfected with a murine Nrf2-expressing (pCMV Nrf2) or vehicle plasmid (pCMV3) by use of lipofectamine 2000 for 48 h. At the end of the culture, the cells were harvested

for western blot against Nrf2 and Dicer1. GAPDH was used as a loading control. **(D)** The protein levels of Dicer1/ GAPDH in C were normalized to pCMV3 group and averaged from three independent culture preparation. Student's *t-test* was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, * $p=0.0004$. **(E)** The protein levels of Nrf2/GAPDH in C were normalized to pCMV3 group and averaged from three independent culture preparation. Student's *t-test* was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, *** $p=0.024$. **(F)** Neuro-2a cells were subjected to treatment by vehicle, A β 42 oligomer (100 nM) for 48 h and in parallel experiments, the cells were transfected with pCMV3 or pCMV3 Nrf2 for 48 h and then subjected to A β 42 for 48h. The cells were harvested for determination of Dicer1 and Nrf2 and Tubulin was used as a loading control. **(G)** The protein levels of Dicer1/ Tubulin in F were normalized to pCMV3 group and averaged from three independent culture preparation. Student's *t-test* was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, * $p=0.0004$. **(H)** The protein levels of Nrf2/Tubulin in F were normalized to pCMV3 group and averaged from three independent culture preparation. Student's *t-test* was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, *** $p=0.024$. **(I)** ARE motifs were found by use of the JASPAR database (<http://jaspar.genereg.net/>) within the *Dicer1* promoter. **(J)** The upper panel showed scheme of *Dicer 1* promoter containing three predicted ARE sites (blue square), TA box (pink red), tss indicating transcriptional start site (tss). HEK293 T cells were transfected with individual construct (150 ng), *Renilla* luciferase plasmid (7.5 ng), and pCMV3 or pCMV Nrf2 plasmid expressing human Nrf2 (150 ng). At the end of culture, the cells were harvested and subject to luciferase activity assay. The results were averaged from five independent culture preparation. One-way ANOVA followed

by Tukey's *post hoc* test was used to compare the differences. Comparing co-transfection of pCMV3 Nrf2 with co-transfection of pCMV3 in individual construct, ###
 $p=0.000219$ in ARE1, # $p=0.043$ in ARE3, ### $p=0.00047$ in promoter 1, ### $p=0.000135$ in
promoter2, ### $p=0.000114$ in promoter3, and # $p=0.048$ in mutant promoter 1. Under the
condition of co-transfection of pCMV3 Nrf2, ARE1 vs. pGL6-luc, *** $p=0.00041$; ARE1
vs. ARE2, ** $p=0.000825$; ARE1 vs. ARE3, ** $p=0.0093$; promoter 1 vs. promoter 0,
*** $p=0.000361$; promoter 1 vs. mutant promoter 1, ** $p=0.009$, promoter 1 vs. promoter 2,
 $p=0.194$ (n.s); promoter 1 vs. promoter 3, $p=0.254$ (n.s). (K) CHIP assay of the
interaction between Nrf2 and the ARE motif-containing *Dicer1* promoter in SK-N-BE
cells. Arrows indicated the captured fragments containing ARE-1 or ARE-2 motif. The
optical densities of bands were measured by Image J and the ratios relative to positive
input were shown below the individual bands, respectively.

Figure 5. Overexpression of Dicer 1 reduced Aβ₄₂ oligomer-mediated apoptosis and neurite deficit in primary hippocampal neuronal cultures. (A) The primary murine hippocampal neurons at DIV 3 were treated with Aβ₄₂ oligomer (100 nM) for 24 h or subject to sham treatment in neurobasal medium plus 2% B27. In parallel experiments, the media were replaced with fresh neurobasal medium plus 2% B27 following Aβ₄₂ treatment, and then infected with vehicle virus, Ad-EGFP or Ad-dicer1-T2A:EGFP virus (5 × 10⁷ vg/mL) for 48 h. At the end of treatment, the cultures were harvested and homogenized for western blot against Dicer1, caspase 3, and activated caspase 3. βIII-tubulin was used as a loading control. (B) Dicer1 relative to βIII-tubulin were normalized to sham treatment and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's *post hoc* test was used to compare the differences. Dicer1 was reduced by Aβ₄₂ oligomer treatment compared to sham

treatment (* $p=0.033$) and the reduction of Dicer1 by A β 42 was rescued by infection of Ad-dicer1-T2A:EGFP compared to infection by vehicle virus (** $p=0.0074$). **(C)** Activated caspase3 relative to β III-tubulin in A were normalized to sham treatment and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's *post hoc* test was used to compare the differences. Activated caspase3 was increased by A β 42 oligomer compared to sham treatment (** $p=0.0061$), which was reduced by infection of Ad-dicer1-T2A:EGFP compared to infection by vehicle virus (* $p=0.023$). **(D)** The loss of cell viability of hippocampal neurons treated by A β (** $p=0.00067$) was significantly rescued by infection with Ad-dicer1-T2A:EGFP compared to infection by vehicle virus (* $p=0.047$). The values were averaged from quadruplicate cultures with three independent culture preparation. **(E)** Knockdown of Dicer1 reduced neurite length in primary cortical neuronal cultures. 7×10^4 cortical neurons were transfected with 6-fluorescein amidite (FAM)-labeling Dicer1 siRNA duplex (Dicer1 siRNA FAM) or scrambled siRNA duplex (NC siRNA FAM) for 12 h, respectively. Neurons were observed with fluorescence microscope. The images were representative images of neurons transfected with Dicer1 siRNA FAM or NC siRNA FAM. Bar, 50 μ m. **(F)** Total neurite length per cortical neuron averaged from 17 neurons under either transfection condition from E. Student's *t*-test was used to compare the difference, **** $p<0.0001$. **(G)** Representative hippocampal neurons (150 neurons for each type of treatment) infected by Ad-EGFP virus (Ad-EGFP), A β 42 treatment followed by infection with Ad-EGFP virus (A β Ad-EGFP) and A β 42 treatment followed by infection with Ad-Dicer1-T2A:EGFP virus (A β Ad-Dicer1:T2A:EGFP) were indicated. **(H)** Total neurite length, **(I)** the longest length and **(J)** median length of hippocampal neurons with treatment from G

were calculated and averaged. A β 42 treatment reduced neurite length compared to sham treatment which was rescued by infection with Ad-dicer 1-T2A:EGFP virus.

**** $p < 0.0001$, The neurite length averaged from 150 neurons in each treatment and One-way ANOVA followed by Tukey's *post hoc* test was used to compare the differences.

Figure 6. Overexpression of Dicer1 in APP/PS1hippocampus inhibited activated caspase 3. APP/PS1 mice or WT littermate at the age of 3.5-/4-month were injected with Ad-EGFP or Ad-Dicer1-T2A:EGFP virus (1.2×10^9 vg/mL) in CA3 hippocampi. 17 days after injection, the injected mice were perfused and each brain was sagittally cut into ~60 sections at 10 μ m-thick for each section. The sections every other six sections were chosen for double staining of Dicer1 (red) (**A**) and activated caspase 3 (red) (**B**). The typical images of CA3 hippocampal region were indicated. (**A**) Dicer1 staining (red)

in APP/PS1 CA3 hippocampus was reduced compared to those in WT littermate and infection with Ad-Dicer 1-T2A:EGFP virus dramatically increased Dicer1 staining in CA3 hippocampus. The images were typical of 60 sections from six mice. Bar, 50 μ m. **(B)** The staining of activated caspase 3 (red) in APP/PS1 mice was increased compared to those in WT and this increase in staining was mitigated by infection with Ad-Dicer1-T2A:EGFP virus. The images were typical of 60 sections from six mice. Bar, 30 μ m. **(C)** The CA3 hippocampi were harvested, homogenized, and subjected to centrifugation at 12,000 X g, and the supernatants were used for western blot against Dicer1, caspase3, and activated caspase3, and typical blots were indicated. **(D)** Dicer1 was reduced in APP/PS1 hippocampi compared to WT (* $p=0.0217$), which was increased by infection with Ad-Dicer1-T2A:EGFP virus (** $p=0.0093$). The optical densities averaged from six mice in each group and Kruskal-Wallis test was used to compare the differences. **(E)** Activated caspase 3 relative to GAPDH was increased in APP/PS1 hippocampi compared to WT (** $p=0.00681$), which was reduced by infection with Ad-Dicer1-T2A:EGFP virus (** $p=0.0067$). The optical densities averaged from six mice in each group and Kruskal-Wallis test was used to compare the differences.

Figure 7. Overexpression of Dicer 1 in CA3 hippocampus significantly improved spatial learning in APP/PS1 mice. Ad-Dicer1-T2A:EGFP virus (1.2×10^9 vg/mL) or equal amount of Ad-EGFP virus were injected into CA3 hippocampus in APP/PS1 or WT mice, at the age of 3.5-4 months, and behavior test was conducted at 17-23 days after injection. **(A)** Learning curve of the mice from WT mice injected with Ad-EGFP virus (n=16), APP/PS1 mice injected with Ad-EGFP virus (n=15) or Ad-Dicer1-T2A:EGFP virus (n=14). The differences of latency to reach platform were analyzed by use of two-way ANOVA with repeated measures against day and treatment (virus injected), $p_{\text{day}} = 0.00001$, $p_{\text{treatment}} = 0.0241$, $p_{\text{interaction}} = 0.0148$. Tukey's *post hoc* test was further used to compare the differences between APP/PS1 mice injected with Ad-Dicer1-T2A:EGFP virus and APP/PS1 mice injected with Ad-EGFP virus, $**p = 0.008$ on day 5 and $***p = 0.00001$ on day 6. **(B)** Analysis of probe trial indicated that preference of APP/PS1 mice for the target quadrant was enhanced by Ad-Dicer1-T2A:EGFP virus injection in CA3 hippocampi. Two-way ANOVA with repeated measures against

quadrant and treatment was used to compare the differences, $p_{\text{quadrant}}=0.1122$, $p_{\text{type}}=1$, $p_{\text{interaction}}=0.0046$. Tukey's *post hoc* test was further used to compare the differences among groups, APP/PS1 Ad-Dicer 1-T2A:EGFP (n=16) vs. APP/PS1 Ad-EGFP(n=15), $**p=0.0087$; APP/PS1 Ad-EGFP (n=15) vs. WT Ad-EGFP (n=16), $**p=0.0023$. **(C)** Frequencies of crossing target platform was increased by Dicer1 overexpression. Tukey's *post hoc* test was further used to compare the differences among groups, APP/PS1 Ad-Dicer 1-T2A:EGFP (n=14) vs. APP/PS1 Ad-EGFP (n=15); $*p=0.043$; APP/PS1 Ad-EGFP (n=15) vs. WT Ad-EGFP (n=16), $*p=0.039$. **(D)** Swim distance and **(E)** Swim speed did not show differences among groups (n.s).

Supplemental Figure legends

Figure S1. Reduction of Dicer 1 in 6-month-old APP/PS1 cortex. (A) Representative image of Dicer1 staining in parietal cortex with double staining by anti-Dicer1(green) and anti-NeuN(red). (n=3, per genotype). Bar, 25 μ m for all panels. **(B)** The homogenates of parietal lobe from WT (n=6) and APP/PS1(n=6) were subject to

western blot against Dicer1. GAPDH was used as a loading control. The optical densities of Dicer1 relative to GAPDH averaged from three independent experiments. Mann-Whitney U test was used to compare the differences, $*p=0.022$.

Figure S2. Knocking down Dicer 1 induced production of reactive oxygen species (ROS), reduced mitochondrial membrane potential, enhanced apoptosis in primary murine cortical neuronal cultures. (A) The cortical neuronal cultures were subjected to transfection with Dicer1 siRNA1-3 (50 pM for each) or equal mass of scrambled siRNA (NC siRNA), respectively. After 48 h, the cells were harvested for immunoblotting against Dicer1. The experiments were repeated twice and the siRNA3

was chosen as Dicer1 specific siRNA. **(B)** The ROS levels in cortical neuronal cultures (CNs) were measured by incubating 2',7'-dichlorodihydrofluorescein diacetate for 20 min at 37 °C. The fluorescence values in relative fluorescence units (RFU) were acquired in a plate reader at 485 nm and were averaged from four independent culture preparation with duplicate cultures in each preparation. **** $p=0.00001$. **(C)** Representative images of JC-1 staining in CNs subject to Dicer1 knockdown as above. Red fluorescence represented as red-J aggregate in mitochondria at hyperpolarized membrane potentials under healthy condition whereas green fluorescence as monomer in cytoplasm at depolarized membrane potentials under the context of compromised mitochondria. Bar 10 μm for all panels. **(D)** Mitochondrial membrane potential ($\Delta\Psi\text{m}$) of CNs subject to Dicer1 knockdown. The neurons were subject to Dicer1 knockdown and mitochondria (30 μg) were isolated and stained with JC-1. The values of mitochondrial membrane potential ($\Delta\Psi\text{m}$) were indicated by the ratios between aggregated/monomer RFU and averaged from three independent culture preparations. Student's *t-test* was used to compare the differences, * $p=0.0369$. **(E)** Viability of neurons subjected to Dicer1 knockdown. CNs were transfected with Dicer 1 siRNA or NC siRNA as described in the materials and methods. The absorption values at OD 450nm in Dicer1 siRNA group were measured and normalized to NC siRNA group. Student's *t-test* was used to compare the differences, ** $p=0.0028$ for comparison. **(F)** CNs were transfected with Dicer1 siRNA (50 pM) or control siRNA duplex (50 pM) per well and subjected to western blot examination of Dicer1, activated caspase3, caspase 3 and $\beta\text{III-tubulin}$ was used as a loading control. **(G)** The effects of knockdown by Dicer1 siRNA were normalized to those of NC siRNA and averaged from three

independent culture preparation. Student's *t-test* was used to compare the differences, *** $p=0.0025$ for comparison. **(G)** The optical densities of activated caspase-3 relative to β III-tubulin from F were normalized to NC siRNA group, and averaged from three independent culture preparation. Student's *t-test* was used to compare the differences, *** $p=0.008$ for comparison.

A **B**

Figure S3. Aβ42 oligomer decreased *Dicer1* mRNA and increased B1 RNA in primary neuronal cultures. Primary murine cortical neuronal cultures were treated with Aβ42 oligomer (100 nM) for 48 h and subject to real-time quantitative PCR examination of *Dicer1* mRNA (**A**) and B1 RNA (**B**). Student's *t*-test was used to compare the differences. **p*=0.0277 for comparison of the levels of *Dicer1* mRNA in CNs, **p*=0.0387 for comparison of *Dicer1* RNA in HNs in A. **p*=0.0469 for comparison of the levels of B1 RNA in CNs, **p*=0.0252 for comparison of B1 RNA in HNs. The results were averaged from three independent culture preparation.

Figure S4. Enhancing expression of Nrf2 increased expression of Dicer1 in SK-N-BE cells. (A-B) SK-N-BE cells were left untreated (Blank) or transfected with human Nrf2-expressing plasmids (pCMV Nrf2, 1.5 μ g) or equal amount of vehicle plasmid

(pCMV3) in 6-well plates. Examination of *Nrf2* or *Dicer1* mRNA was performed by qRT-PCR analysis. Determination of *Nrf2* or *Dicer1* mRNA levels by overexpression of Nrf2 in SK-N-BE cells. Student's *t-test* was used to compare the differences. * $p=0.0038$ for comparison of the levels of *Dicer1* mRNA, * $p=0.0014$ for comparison of the levels of *Nrf2* mRNA. **(C)** Forty-eight hours after transfection, the cells were harvested for western blot against Nrf2 and Dicer1. GAPDH was used as a loading control. **(D)** The protein levels of Dicer1 relative to GAPDH in C were normalized to pCMV group and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's *post hoc* test was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, ** $p=0.0021$. **(E)** The protein levels of Nrf2/ GAPDH in C were normalized to pCMV group and averaged from three independent culture preparations. One-way ANOVA, followed by Tukey's *post hoc* test was used to compare the difference, pCMV3 vs. pCMV3 Nrf2, ** $p=0.0017$.

Figure S5. Infection of hippocampal neuronal cultures by adenovirus. The primary mouse hippocampal neurons were culture as described in the materials and methods. At DIV 3, the cells were infected with Ad-pCMV-EGFP or Ad-pCMV-Dicer1:T2A:EGFP (5×10^7 vg/mL) for 60 h. The images were randomly selected and taken under fluorescence microscope with fluorophore filter for DAPI or GFP. Scale bar, 60 μ m.

Figure S6. Infection of CA3 region by intrahippocampal injection of virus. WT or APP/PS1 mice at 4-month were injected with 2 μ L of Ad-pCMV-EGFP or Ad-pCMV-Dicer1:T2A:EGFP virus (1.2×10^9 vg/mL) in each hemisphere as described in the material and methods. After 23 days, the mice were subjected to decapitation, the brain was processed into 10 μ m sections, fixed in 4% paraformaldehyde and stained for DAPI. The images were captured with fluorescence microscope with different fluorophore filter for DAPI and GFP. Scale bar, 80 μ m.

Figures

Figure 1

Reduction of Dicer 1, decreased Nrf2 and increased activated caspase-3 in the hippocampus of APP/PS1 mice. (A) APP/PS1 brains were processed into sections which were doubly stained for Dicer1 (green) or NeuN (red). The images were typical staining of CA1, CA3, or DG (n=3 mice per genotype). Bar, 25 μ m for

all panels. (B) The supernatants from homogenized hippocampi at the age of 4-/11 months were collected and subject to examination of Dicer1, Nrf2, caspase-3, and activated caspase-3 by western blot. GAPDH was used as a loading control. The protein levels of Dicer1 and Nrf2 in the hippocampal tissues were reduced in APP/PS1 mice (n=6, 3 male and 3 female) compared to WT littermate (n=6, 3 male and 3 female), respectively, at the age of 4 months (Left panel) or at the age of 11 months (right panel). By contrast, the protein levels of activated caspase-3 were increased in APP/PS1 mice (n=6) compared to WT (n=6) at the age of 4-/11 months. (C) The optical densities of Dicer1 relative to GAPDH were normalized to WT at the age of 4 months (**p=0.0092) or 11 months (**p=0.0002) (n=6 per genotype, Mann-Whitney U test). (D) Nrf2 relative to GAPDH were normalized to WT (Mann-Whitney U test, ***p=0.0001 in 4-month, **p=0.0038 in 11-month, n=6 per genotype). (E) Activated caspase 3 relative to GAPDH were normalized to WT (Mann-Whitney U test, **p=0.0098 in 4-month, ***p=0.0008 in 11-month, n=6 per genotype), respectively. (F) Dicer1 mRNA was examined in WT (n=3) and in APP/PS1 mice (n=3) at the age of 4 months (p=0.0335) or 11 months (p=0.0304) (paired t-test). (G) Nrf2 mRNA was examined in WT (n=3) and in APP/PS1 mice (n=3) at the age of 4 months (p=0.0167) or 11 months (p=0.0195) (paired t-test).

Figure 2

knocking down Dicer 1 induced production of reactive oxygen species (ROS), reduced mitochondrial membrane potential, enhanced apoptosis in primary murine hippocampal neuronal cultures. (A) The ROS levels in hippocampal neuronal cultures (HNs) were measured by incubating 2',7'-dichlorodihydrofluorescein diacetate for 20 min at 37 oC. The fluorescence values in relative fluorescence units (RFU) were acquired in a plate reader at 485 nm and were averaged from four independent culture preparation with duplicate cultures in each preparation. ****p=0.00001. (B) Representative images of JC-1 staining in HNs subject to Dicer1 knockdown as above. Red fluorescence represented aggregate in mitochondria at hyperpolarized membrane potentials under healthy condition whereas green

fluorescence as monomer in cytoplasm at depolarized membrane potentials under the context of compromised mitochondria. Bar 10 μm for all panels. (C) Mitochondrial membrane potential (Ψm) of HNs subject to Dicer1 knockdown. The neurons were subject to Dicer1 knockdown and mitochondria (30 μg) were isolated and stained with JC-1. The values of mitochondrial membrane potential (Ψm) were indicated by the ratios between aggregated/monomer RFU and averaged from three independent culture preparations. Student's t-test was used to compare the differences, $*p=0.0399$. (D) Viability of neurons subjected to Dicer1 knockdown. HNs were transfected with Dicer 1 siRNA or NC siRNA as described in the materials and methods. The absorption values at OD 450nm in Dicer1 siRNA group were measured and normalized to NC siRNA group. Student's t-test was used to compare the differences, $**p=0.0012$ for comparison. (E) HNs were transfected with Dicer1 siRNA (50 pM) or control siRNA duplex (50 pM) per well and subjected to western blot examination of Dicer1, activated caspase3, caspase 3 and βIII -tubulin was used as a loading control. (F) The effects of knockdown by Dicer1 siRNA were normalized to those of NC siRNA and averaged from three independent culture preparation. Student's t-test was used to compare the differences, $*p=0.044$ for comparison. (G) The optical densities of activated caspase-3 relative to βIII -tubulin from F were normalized to NC siRNA group, and averaged from three independent culture preparation. Student's t-test was used to compare the differences, $*p=0.02$ for comparison.

Figure 3

Chronic A β 42 oligomer treatment reduced Dicer1 in neurons which was coupled with Nrf2 reduction, the effects rescued by NAC. (A-C) The time-course effect of A β 42 on Dicer1 or on Nrf2. The primary murine cortical neuronal cultures were subjected to vehicle or A β oligomer treatment at indicated time. The cells were harvested for western blot for Dicer1 or Nrf2 and β III-tubulin was used as a loading control. The relative optical densities of Dicer1 or Nrf2 from triplicate experiments were quantified and indicated in B and C. **p<0.01, ***p<0.001, ****p<0.0001, n.s. indicated no significance. (D) The primary mouse cortical neurons were treated with A β 42 oligomer (100 nM) and antioxidant NAC (2 μ M) for 48 h. The neuronal cultures were harvested for western blot against Dicer 1, Nrf2, and Keap1. β III-tubulin was used as an internal loading control. (E) The optical densities of Dicer1/ β III-tubulin from D were normalized to sham treatment and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's

post hoc test was used to compare the differences. Control vs. Aβ42 oligomer treatment, ****p=0.00001; Aβ42 oligomer vs. Aβ42 oligomer plus NAC treatment (Aβ+NAC), *p=0.032. (F) The optical densities of Nrf2/βIII-tubulin from D were normalized to sham treatment and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's post hoc test was used to compare the differences. Control vs. Aβ42 oligomer treatment, ***p=0.0005; Aβ42 oligomer vs. Aβ+NAC, ***p=0.0009. (G) The neurons were treated with Aβ42 oligomer (100 nM) and antioxidant NAC (2 μM) for 24 h and the nucleic proteins were extracted and blotted against Nrf2, and histone3 served as a loading control and vimentin as a negative control; (H) the cytoplasmic proteins were isolated and blotted against Nrf2, and vimentin served as a loading control and histone3 as a negative control. (I) The optical densities of nucleic Nrf2 relative to histone 3 averaged from three independent culture preparation in G. One-way ANOVA followed by Tukey's post hoc test was used to compare the differences. Control vs. Aβ42, *p=0.0326; Aβ42 vs. Aβ+NAC, ****p=0.00001. (J) The optical densities of cytosolic Nrf2/vimentin were normalized to sham treatment and averaged from three independent culture preparation in H. One-way ANOVA followed by Tukey's post hoc test was used to compare the differences. Control vs. Aβ42 oligomer, *p=0.0076; Aβ42 oligomer vs. Aβ+NAC, *p=0.0419.

Figure 4

Nrf2 regulated Dicer1 production via Nrf2-ARE pathway. Neuro-2a cells were transfected with mouse Nrf2-expressing plasmids (pCMV Nrf2, 1.5 µg) or equal amount of vehicle plasmid (pCMV3) in 6-well plates. Examination of Nrf2 or Dicer1 mRNA was performed by qRT-PCR analysis. (A-B) Determination of Nrf2 and Dicer1 mRNA levels by overexpression of Nrf2 in Neuro-2A cells. Student's t-test was used to compare the differences. * $p=0.0016$ for comparison of the levels of Dicer1 mRNA, * $p=0.0482$ for comparison of the levels of Nrf2 mRNA. (C) Neuro-2a cells were transfected with a murine Nrf2-expressing (pCMV Nrf2) or vehicle plasmid (pCMV3) by use of lipofectamine 2000 for 48 h. At the end of the culture, the cells were harvested for western blot against Nrf2 and Dicer1. GAPDH was used as a loading control. (D) The protein levels of Dicer1/ GAPDH in C were normalized to pCMV3 group and averaged from three independent culture preparation. Student's t-test was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, * $p=0.0004$. (E) The protein levels of Nrf2/GAPDH in C were normalized to pCMV3 group and averaged from three independent culture preparation. Student's t-test was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, *** $p=0.024$. (F) Neuro-2a cells were subjected to treatment by vehicle, A β 42 oligomer (100 nM) for 48 h and in parallel experiments, the cells were transfected with pCMV3 or pCMV3 Nrf2 for 48 h and then subjected to A β 42 for 48h. The cells were harvested for determination of Dicer1 and Nrf2 and Tubulin was used as a loading control. (G) The protein levels of Dicer1/ Tubulin in F were normalized to pCMV3 group and averaged from three independent culture preparation. Student's t-test was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, * $p=0.0004$. (H) The protein levels of Nrf2/Tubulin in F were normalized to pCMV3 group and averaged from three independent culture preparation. Student's t-test was used to compare the differences, pCMV3 vs. pCMV3 Nrf2, *** $p=0.024$. (I) ARE motifs were found by use of the JASPAR database (<http://jaspar.genereg.net/>) within the Dicer1 promoter. (J) The upper panel showed scheme of Dicer 1 promoter containing three predicted ARE sites (blue square), TA box (pink red), tss indicating transcriptional start site (tss). HEK293 T cells were transfected with individual construct (150 ng), Renilla luciferase plasmid (7.5 ng), and pCMV3 or pCMV Nrf2 plasmid expressing human Nrf2 (150 ng). At the end of culture, the cells were harvested and subject to luciferase activity assay. The results were averaged from five independent culture preparation. One-way ANOVA followed by Tukey's post hoc test was used to compare the differences. Comparing co-transfection of pCMV3 Nrf2 with co-transfection of pCMV3 in individual construct, ### $p=0.000219$ in ARE1, # $p=0.043$ in ARE3, ### $p=0.00047$ in promoter 1, ### $p=0.000135$ in promoter2, ### $p=0.000114$ in promoter3, and # $p=0.048$ in mutant promoter 1. Under the condition of co-transfection of pCMV3 Nrf2, ARE1 vs. pGL6-luc, *** $p=0.00041$; ARE1 vs. ARE2, ** $p=0.000825$; ARE1 vs. ARE3, ** $p=0.0093$; promoter 1 vs. promoter 0, *** $p=0.000361$; promoter 1 vs. mutant promoter 1, ** $p=0.009$, promoter 1 vs. promoter 2, $p=0.194$ (n.s); promoter 1 vs. promoter 3, $p=0.254$ (n.s). (K) CHIP assay of the interaction between Nrf2 and the ARE motif-containing Dicer1 promoter in SK-N-BE cells. Arrows indicated the captured fragments containing ARE-1 or ARE-2 motif. The optical densities of bands were measured by Image J and the ratios relative to positive input were shown below the individual bands, respectively.

Figure 5

Overexpression of Dicer 1 reduced A β 42 oligomer-mediated apoptosis and neurite deficit in primary hippocampal neuronal cultures. (A) The primary murine hippocampal neurons at DIV 3 were treated with A β 42 oligomer (100 nM) for 24 h or subject to sham treatment in neurobasal medium plus 2% B27. In parallel experiments, the media were replaced with fresh neurobasal medium plus 2% B27 following A β 42 treatment, and then infected with vehicle virus, Ad-EGFP or Ad-dicer1-T2A:EGFP virus (5×10^7 vg/mL) for 48 h. At the end of treatment, the cultures were harvested and homogenized for western blot against Dicer1, caspase 3, and activated caspase 3. β III-tubulin was used as a loading control. (B) Dicer1 relative to β III-tubulin were normalized to sham treatment and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's post hoc test was used to compare the differences. Dicer1 was reduced by A β 42 oligomer treatment compared to sham treatment (*p=0.033) and the reduction of Dicer1 by A β 42 was rescued by infection of Ad-dicer1-T2A:EGFP compared to infection by vehicle virus (**p=0.0074). (C) Activated caspase3 relative to β III-tubulin in A were normalized to sham treatment and averaged from three independent culture preparation. One-way ANOVA followed by Tukey's post hoc test was used to compare the differences. Activated caspase3 was increased by A β 42 oligomer compared to sham treatment (**p=0.0061), which was reduced by infection of Ad-dicer1-T2A:EGFP compared to infection by vehicle virus (*p=0.023). (D) The loss of cell viability of hippocampal neurons treated by A β (**p=0.00067) was significantly rescued by infection with Ad-dicer1-T2A:EGFP compared to infection by vehicle virus (*p=0.047). The values were averaged from quadruplicate cultures with three

independent culture preparation. (E) Knockdown of Dicer1 reduced neurite length in primary cortical neuronal cultures. 7×10⁴ cortical neurons were transfected with 6-fluorescein amidite (FAM)-labeling Dicer1 siRNA duplex (Dicer1 siRNA FAM) or scrambled siRNA duplex (NC siRNA FAM) for 12 h, respectively. Neurons were observed with fluorescence microscope. The images were representative images of neurons transfected with Dicer1 siRNA FAM or NC siRNA FAM. Bar, 50 μm. (F) Total neurite length per cortical neuron averaged from 17 neurons under either transfection condition from E. Student's t-test was used to compare the difference, **** p<0.0001. (G) Representative hippocampal neurons (150 neurons for each type of treatment) infected by Ad-EGFP virus (Ad-EGFP), Aβ42 treatment followed by infection with Ad-EGFP virus (Aβ Ad-EGFP) and Aβ42 treatment followed by infection with Ad-Dicer1-T2A:EGFP virus (Aβ Ad-Dicer1:T2A:EGFP) were indicated. (H) Total neurite length, (I) the longest length and (J) median length of hippocampal neurons with treatment from G were calculated and averaged. Aβ42 treatment reduced neurite length compared to sham treatment which was rescued by infection with Ad-dicer 1-T2A:EGFP virus. ****p<0.0001, The neurite length averaged from 150 neurons in each treatment and One-way ANOVA followed by Tukey's post hoc test was used to compare the differences.

Figure 6

Overexpression of Dicer1 in APP/PS1hippocampus inhibited activated caspase 3. APP/PS1 mice or WT littermate at the age of 3.5-/4-month were injected with Ad-EGFP or Ad-Dicer1-T2A:EGFP virus (1.2 X10⁹ vg/mL) in CA3 hippocampi. 17 days after injection, the injected mice were perfused and each brain was sagittally cut into ~60 sections at 10 μ m-thick for ach section. The sections every other six sections were chosen for double staining of Dicer1 (red) (A) and activated caspase 3 (red) (B). The typical images of CA3 hippocampal region were indicated. (A) Dicer1 staining (red) in APP/PS1 CA3 hippocampus was reduced compared to those in WT littermate and infection with Ad-Dicer 1-T2A:EGFP virus dramatically increased Dicer1 staining in CA3 hippocampus. The images were typical of 60 sections from six mice. Bar, 50 μ m. (B) The staining of activated caspase 3 (red) in APP/PS1mice was increased compared to those in WT and this increase in staining was mitigated by infection with Ad-Dicer1-T2A:EGFP virus. The images were typical of 60 sections from six mice. Bar, 30 μ m. (C) The CA3 hippocampi were harvested, homogenized, and subjected to centrifugation at 12,000 X g, and the supernatants were used for western blot against Dicer1, caspase3, and activated caspase3, and typical blots were indicated. (D) Dicer1 was reduced in APP/PS1 hippocampi compared to WT (*p=0.0217), which was increased by infection with Ad-Dicer1-T2A:EGFP virus (**p=0.0093). The optical densities averaged from six mice in each group and Kruskal-Wallis test was used to compare the differences. (E) Activated caspase 3 relative to GAPDH was increased in APP/PS1 hippocampi compared to WT (**p=0.00681), which was reduced by infection with Ad-Dicer1-T2A:EGFP virus (**p=0.0067). The optical densities averaged from six mice in each group and Kruskal-Wallis test was used to compare the differences.

Figure 7

Overexpression of Dicer 1 in CA3 hippocampus significantly improved spatial learning in APP/PS1 mice. Ad-Dicer1-T2A:EGFP virus (1.2×10^9 vg/mL) or equal amount of Ad-EGFP virus were injected into CA3 hippocampus in APP/PS1 or WT mice, at the age of 3.5-4 months, and behavior test was conducted at 17-23 days after injection. (A) Learning curve of the mice from WT mice injected with Ad-EGFP virus (n=16), APP/PS1 mice injected with Ad-EGFP virus (n=15) or Ad-Dicer1-T2A:EGFP virus (n=14). The differences of latency to reach platform were analyzed by use of two-way ANOVA with repeated measures against day and treatment (virus injected), $p_{\text{day}} = 0.00001$, $p_{\text{treatment}} = 0.0241$, $p_{\text{interaction}} = 0.0148$. Tukey's post hoc test was further used to compare the differences between APP/PS1 mice injected with Ad-Dicer1-T2A:EGFP virus and APP/PS1 mice injected with Ad-EGFP virus, $**p = 0.008$ on day 5 and $***p = 0.00001$ on day 6. (B) Analysis of probe trial indicated that preference of APP/PS1 mice for the target quadrant was enhanced by Ad-Dicer 1-T2A:EGFP virus injection in CA3 hippocampi. Two-way ANOVA with repeated measures against quadrant and treatment was used to compare the differences, $p_{\text{quadrant}} = 0.1122$, $p_{\text{type}} = 1$, $p_{\text{interaction}} = 0.0046$. Tukey's post hoc test was further used to compare the differences among groups, APP/PS1 Ad-Dicer 1-T2A:EGFP (n=16) vs. APP/PS1 Ad-EGFP (n=15), $**p = 0.0087$; APP/PS1 Ad-EGFP (n=15) vs. WT Ad-EGFP (n=16), $**p = 0.0023$. (C) Frequencies of crossing target platform was increased by Dicer1 overexpression. Tukey's post hoc test was further used to compare the differences among groups, APP/PS1 Ad-Dicer 1-T2A:EGFP (n=14) vs. APP/PS1 Ad-EGFP (n=15); $*p = 0.043$; APP/PS1 Ad-EGFP (n=15) vs. WT Ad-EGFP (n=16), $*p = 0.039$. (D) Swim distance and (E) Swim speed did not show differences among groups (n.s).