Brain Dicer1 Is Down-Regulated in a Mouse Model of Alzheimer’s Disease Via Aβ42-Induced Repression of Nuclear Factor Erythroid 2-Related Factor 2

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Abstract
Dicer1 is a microRNA-processing enzyme which plays critical roles in neuronal survival and neuritogenesis. Dicer1 deletion induces neurodegeneration or degeneration in retinal pigment epithelium, which is associated with oxidative stress. Oxidative stress is thought to be central in the pathogenesis of Alzheimer’s disease (AD). Therefore, we hypothesize that Dicer1 may play roles in AD. Using immunoblotting and quantitative real-time PCR, Dicer1 protein and mRNA were reduced in the hippocampi of the AD mouse model APPswe/PSEN1dE9 compared with littermate controls. SiRNA-mediated Dicer1 knockdown induced oxidative stress and apoptosis and reduced mitochondrial membrane potential in cultured neurons. Chronic Aβ42 exposure decreased Dicer1 and nuclear factor erythroid 2-related factor 2 (Nrf2) which were reversed by N-acetyl-cystein. Nrf2 overexpression increased Dicer1 mRNA and protein and reverted the Aβ42-induced Dicer1 reduction. We further 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 Aβ42-induced neurite deficits. Notably, injection of Dicer1-expressing adenovirus into the hippocampus of the mice significantly improved spatial learning. Altogether, we found novel roles of Dicer1 in AD and a novel regulatory pathway for Dicer1. These results suggest that Dicer1 is a target in AD therapy, especially at the early stage of this disorder.

Keywords Dicer1 • Alzheimer’s disease • Hippocampus • APPswe/PSEN1dE9 mice • Nrf2 • Antioxidant response element • Oxidative stress • Neurodegeneration • Gene regulation • Neurotoxicity

Abbreviations
AD Alzheimer’s disease
APP/PS1 APPswe/PSEN1dE9
AMD Age-related macular degeneration
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
NLR protein Nucleotide-binding domain and leucine-rich repeat containing (NLR) protein
NLRP3 NLR family pyrin domain containing 3
ROS Reactive oxygen species
DG Dentate gyrus
CA1 CA1 hippocampus
CA3 CA3 hippocampus

Introduction
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 antioxidant systems [3]. Accumulation of reactive oxygen/nitrogen species generates oxidative stress and produces ensuing cytotoxicity [4]; for example, ferroptosis due to iron accumulation causes neurodegeneration 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]. Antioxidation systems are used to cope with oxidative injury, in which the regulation of the key enzymes or proteins, used for synthesizing glutathione or recycling peroxidorexins, 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]. Apart from generating small regulatory RNAs including microRNAs or siRNAs [14], Dicer1 also participates in maintaining heterochromatin structure [15, 16], 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 and subsequent accumulation of AluRNA, a substrate of Dicer1 degradation, resulting in inflammasome activation which likely accompanies with oxidative stress which is an etiological factor in neurodegeneration 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 APPswe/PSEN1dE9 (APP/PS1) mice.

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. Since the CA1, CA3, and dentate gyrus brain regions have distinct function in memory formation, we examined Dicer1 expression in these three regions. The staining of Dicer1 was reduced in the CA1, CA3, and dentate gyrus (DG) of APP/PS1 mice compared with WT littermate, at the age of 4 months and 11 months (Fig. 1a). The protein was also significantly reduced in the homogenates from the hippocampus of 4-month APP/PS1 mice compared with 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) (Fig. 1b and c). Consistent with reduced protein, Dicer1 mRNA was also reduced in the hippocampus of the AD mice compared with WT at the age of 4 months (p = 0.0335), and at the age of 11 months as well (p = 0.0162) (Fig. 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 with WT littermate (p = 0.022, n = 6 for each genotype, 3 female and 3 male) (Fig. 1d) and Nrf2 mRNA expression was also reduced in the hippocampus of APP/PS1 mice compared with WT littermate (p = 0.0069, n = 6 for each genotype, 3 female and 3 male) (Fig. 1e).

Fig. 1 Reduction of Dicer1, decreased Nrf2 and increased activated caspase 3 in the hippocampus of APP/PS1 mice. a APP/PS1 and WT 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). omitting primary antibody did not produce staining. Bar, 25 μm for all panels. b The supernatants from homogenized hippocampal of APP/PS1 and WT mice 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 with 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 with 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 was normalized to WT (Mann-Whitney U test, *p = 0.0001 in 4 months, **p = 0.0038 in 11 months, n = 6 per genotype). e Activated caspase 3 relative to GAPDH was normalized to WT (Mann-Whitney U test, *p = 0.0098 in 4 months, **p = 0.0008 in 11 months, 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 = 0.0167) (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 = 0.0304) or 11 months (p = 0.0195) (paired t test).
Nrf2 is a master regulator of antioxidation genes regulating redox homeostasis [26]; Dicer1 depletion induces cytotoxicity via oxidative stress [20]. Thus, we simultaneously examined the protein levels of Nrf2 and activated caspase 3 in the homogenates from APP/PS1 hippocampi. Similarly, Nrf2 protein levels in AD mice were significantly reduced compared with WT littermate at the age of 4 months ($p = 0.0001$) and 11 months ($p = 0.0038$), respectively (Fig. 1b and d). In accordance with these findings, Nrf2 mRNA in hippocampus from AD mice was reduced compared with WT littermate at 4 months ($p = 0.0304$) and at 11 months as well ($p = 0.0195$), respectively (Fig. 1g). Activated caspase 3 in the AD mice was significantly increased compared with WT littermate at 4 months ($p = 0.0098$) and 11 months ($p = 0.0008$), respectively (Fig. 1b and e).
Fig. 2 Dicer1 knockdown induced production of reactive oxygen species (ROS), reduced mitochondrial membrane potential, and enhanced apoptosis in primary murine hippocampal neuronal cultures. a The ROS levels in hippocampal neuronal cultures (HNs) under transfection of scrambled siRNA (NC siRNA) and of Dicer1-specific siRNA (Dicer1 siRNA) 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>0.05 for comparison. b Representative images of JC-1 staining in HNs subject to transfection with Dicer1 siRNA (Dicer1 siRNA) and with scrambled siRNA (NC siRNA). The neurons were subject to Dicer1 knockdown and mitochondria (30 μg) were isolated and stained with JC-1. The values of mitochondrial membrane potential (ΔΨm) 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>0.05 for comparison. c Mitochondrial membrane potential (ΔΨm) of HNs subject to transfection with Dicer1 siRNA (Dicer1 siRNA) and with scrambled siRNA (NC siRNA). The neurons were transfected with Dicer1 siRNA or NC siRNA as described in the materials and methods. The absorption values at OD 450 nm in Dicer1 siRNA group were measured and normalized to NC siRNA group. Student’s t test was used to compare the differences, *<p>0.05 for comparison. d Viability of neurons subject to transfection with Dicer1 siRNA (Dicer1 siRNA) and with scrambled siRNA (NC siRNA). The neurons were transfected with Dicer1 siRNA or NC siRNA as described in the materials and methods. The absorption values at OD 450 nm in Dicer1 siRNA group were measured and normalized to NC siRNA group. Student’s t test was used to compare the differences, *<p>0.05 for comparison. e The effects of Dicer1 knockdown by Dicer1 siRNA were normalized to those of transfection with scrambled siRNA (NC siRNA) and with scrambled siRNA (NC siRNA). Red fluorescence represented aggregate in mitochondria at hyperpolarized membrane potentials under the context of compromised mitochondria. Bar 10 μm for all panels. f The optical densities of activated caspase 3 relative to βIII-tubulin from f were normalized to NC siRNA group, and the results were averaged from three independent culture preparation. Student’s t test was used to compare the differences, *<p>0.05 for comparison.

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 chosen as Dicer1 siRNA in the following experiments. As expected, Dicer1 knockdown by the specific siRNA induced ROS production in HNs (<p>0.0001) compared with NC siRNA (Fig. 2a). Similarly, Dicer1 knockdown reduced mitochondrial membrane potential indicated by JC-1 staining in the HNs (<p>0.0399) (Fig. 2b and c). 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) (Fig. 2d). The protein levels of activated caspase 3 under Dicer1 knockdown were also increased in the HNs (<p>0.020) compared with the effects of scrambled siRNA (NC siRNA) (Fig. 2e and g). Compared with transfection with NC siRNA, the Dicer1 siRNA significantly decreased Dicer1 protein in the HNs (<p>0.044) (Fig. 2e and f). 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–h).

Aβ42 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 Aβ and the deletion mutation (dE9) significantly accelerates the shift of the Aβ40/ Aβ42 ratios, thus elevating the levels of Aβ42 [27, 28]. Thus, we investigated the effect of Aβ42 on Dicer1 expression in the CNs. Initially, we tested the time-course effect of Aβ42 on Dicer1 and Nrf2. Aβ42 significantly reduced the protein levels of Dicer1 and Nrf2 after 12–48 h treatment but did not change Nrf2 protein level upon 6-h treatment (Fig. 3a–c). Aβ42 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 Aβ42 oligomer, we examined one of Dicer1 substrates, B1 RNA (Alu sequence), with the cells transfected with vehicle plasmid. The levels of B1 RNA under Dicer1 knockdown were also increased in the HNs (<p>0.020) compared with the effects of scrambled siRNA (NC siRNA) (Fig. 2e and g). Compared with transfection with NC siRNA, the Dicer1 siRNA significantly decreased Dicer1 protein in the HNs (<p>0.044) (Fig. 2e and f). 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–h).

Nrf2 Regulated Dicer1 Expression

Nrf2 is a master regulator of transcription of antioxidant 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 with 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) (Fig. 4a and b). The protein levels of Dicer1...
Fig. 3 Chronic Aβ42 oligomer treatment reduced Dicer1 and Nrf2 which were rescued by NAC. 

a–c The time-course effect of Aβ42 on Dicer1 and on Nrf2. The primary murine cortical neuronal cultures were subject to vehicle and Aβ oligomer treatment at the indicated time. The cells were harvested for western blot for Dicer1 and Nrf2; βIII-tubulin was used as a loading control. The relative optical densities of Dicer1 and 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–f The primary mouse cortical neurons were subject to vehicle treatment (control), treatment with Aβ42 oligomer (100 nM) (Aβ oligomer) and treatment with Aβ42 oligomer (100 nM) plus antioxidant NAC (2 μM) (Aβ + NAC) for 48 h. The neuronal cultures were harvested for western blot against Dicer1, Nrf2, and Keap1. βIII-tubulin was used as an internal loading control. 

The cytoplasmic proteins were isolated and blotted against Nrf2. Vimentin served as a loading control and histone3 as a negative control for cytoplasmic proteins. 

The optical densities of nucleic Nrf2 relative to histone 3 were 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 < 0.0001. 

The optical densities of cytosolic Nrf2/vimentin 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 < 0.0001; Aβ42 oligomer vs. Aβ + NAC, *p = 0.0032.

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 < 0.0001; Aβ42 oligomer vs. Aβ + NAC, *p = 0.0032.
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 with the cells transfected with vehicle plasmid (\(p = 0.0004\)) (Fig. 4c–e). We next tested whether reduction of Dicer1 by Aβ42 could be rescued by overexpression of Nrf2. As expected, Aβ42 decreased Dicer1 (\(p = 0.0004\)) which was rescued by Nrf2 overexpression in Neuro-2a cells (\(p = 0.0399\)) (Fig. 4f–h). We scanned the promoter of Dicer1 with software (promoter 2.0 prediction server) and found three ARE motifs upstream of transcription start site (tss), that is, Dicer1 promoter region. We cloned the ARE1-ARE3-containing promoter fragments into a luciferase reporter plasmid, PGL6-basic(-Luc), respectively, and also cloned the promoters containing different ARE motif into PGL6-basic plasmid, respectively (\(p < 0.0001\)) (Fig. 4i and j). Transfection of Nrf2-expressing plasmid (pCMV Nrf2) increased the ratios between firefly and Renilla in constructs containing ARE1 (\(p = 0.00219\)) and ARE3 motif (\(p = 0.043\)) when compared with the cells transfected with vehicle plasmid, pCMV3 (Fig. 4j). When compared the constructs containing different AREs under transfection with pCMV Nrf2, we found that the ratios of firefly/Renilla from ARE1 construct were significantly different with the construct ARE2 (\(p = 0.000825\)), ARE3 (\(p = 0.0093\)), and PGL6-basic (\(p = 0.00041\)), respectively (Fig. 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 with the cells transfected with pCMV3 (Fig. 4j). When compared different promoter constructs under transfection with pCMV Nrf2, we found that the ratios of firefly/Renilla from ARE1 construct were significantly different with the construct ARE2 (\(p = 0.000825\)), ARE3 (\(p = 0.0093\)), and PGL6-basic (\(p = 0.00041\)), respectively (Fig. 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 with the cells transfected with pCMV3 (Fig. 4j). When compared different promoter constructs under transfection with pCMV Nrf2, we found that the ratios of firefly/Renilla from promoter 1 were significantly different with promoter 0 (\(p = 0.000361\)) and mutant promoter 1 (\(p = 0.009\)) but not different with promoter 2 (\(p = 0.194\)) or promoter 3 (\(p = 0.254\)) (Fig. 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/-691bp, relative to tss), ARE-2 (-1091/-876bp), or ARE-3(-1271/-1097bp)-containing fragments, respectively, in SK-N-BE cells (Fig. 4i and k). 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. That overexpression of Nrf2 increased Dicer1 was replicated in SK-N-BE cells as well (Supplemental Figure 4).

**Overexpression of Dicer1 Recovered Neurotoxicity by Aβ42 Oligomer**

Since we have demonstrated that Dicer1 depletion compromised cell survival (Fig. 2), we wondered whether overexpression of Dicer1 mitigated Aβ42-induced neurotoxicity. The infection rate of the virus reached ~90% in the primary murine hippocampal neuronal cultures (Supplemental Figure 5). Aβ42 oligomer treatment reduced the protein levels of Dicer1 compared with sham treatment (\(p = 0.033\)), which was rescued by infection with Ad-Dicer1-T2A:EGFP virus compared with infection with vehicle virus, Ad-EGFP (\(p = 0.0074\)) (Fig. 5a and b). Aβ42 oligomer treatment increased activated caspase 3 compared with sham treatment (\(p = 0.0061\)), which was partially rescued by infection with Ad-Dicer1-T2A:EGFP virus compared with infection with Ad-EGFP (\(p = 0.023\)) (Fig. 5a and c). Under expectation, Aβ42 oligomer decreased neuronal survival (\(p = 0.00067\)), which was partially rescued by infection with Ad-Dicer1-T2A:EGFP virus (\(p = 0.047\)) (Fig. 5d). Dicer1 knockdown disrupted neurite in primary cortical neurons (\(p = 0.011\)) (Fig. 5e and f), which is reminiscent of the axonal degeneration in AD. Similarly, Aβ42 oligomer treatment trimmed neurites compared with vehicle treatment (\(p = 0.025\), which was significantly recovered by overexpression of Ad-Dicer1-T2A:EGFP virus (Fig. 5g–j).

**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 Fig. 1a, Dicer1 staining was decreased in CA3 hippocampus compared with 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 (Fig. 6a). CA3 hippocampi of APP/PS1 mice contained stronger staining for activated caspase 3 compared with those in WT littermate, which was reduced by injection of Ad-Dicer1:T2A:EGFP virus (Fig. 6b). In parallel experiment, we isolated the whole hippocampi and subject the homogenates to western blot against Dicer1 and caspase 3. Consistent with the immunofluorescence, lower level of Dicer1 in APP/PS1 mice than WT (\(p = 0.0217\)) which was recovered by injection of Ad-Dicer1:T2A:EGFP (\(p = 0.0093\)) (Fig. 6c and d); 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 (\(p = 0.0067\)) (Fig. 6c and e).

We further investigated whether overexpression of Dicer1 had effect on spatial learning and thus examined behavior with Morris Water Maze. Before examining the effect of virus injection on behavior, we tested spatial memory in WT and APP/PS1 mice without injection. Under expectation, the spatial memory of APP/PS1 mice was compromised compared with WT mice (Supplemental Figure 7). During the training period on days 1–6 after virus injection, the time to reach platform for APP/PS1 mice was significantly longer than WT mice (\(p =

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0.0148, two-way ANOVA) (Fig. 7a) which is consistent with previous reports: cognitive function is compromised in 3.5–4 months of APP/PS1 mice [30, 31]. This reduction was reversed by injection with Ad-Dicer1:T2A:EGFP virus compared with injection with Ad1-EGFP virus (p = 0.008) on day 5 (p = 0.0057), or on day 6 (p = 0.0025) (Fig. 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_{day} (5174) = 36.44, p = 0.00001; F_{treatment} (2174) = 2.045, p = 0.0241; F_{interaction} (75,174) = 2.3, p = 0.0148, two-way ANOVA) (Fig. 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) (Fig. 7d) and swimming speed (p = 0.0948) (Fig. 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_{quadrant} (3116) = 4.576, p = 0.1122; F_{treatment} (2116) = 0.1032, p = 1; F_{interaction} (45,116) = 1.334, p = 0.0046, two-way ANOVA) (Fig. 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) (Fig. 7c).

Discussion

In our study, reduction of Dicer1 was detected as early as around 4 months in APP/PS1 mice in which Aβ deposition begins in the brain at the age of 6 months [27]. Furthermore, knockdown of Dicer1 in cultured neurons induced neurite deficit before soma degeneration, and intrahippocampal injection of Dicer1-expressing virus improved spatial learning. These evidences suggest that reduction of Dicer1 is an early
event in AD, possibly before overt Aβ deposition and targeting Dicer1 may provide a new strategy for AD therapy, with emphasis at the early stage of this disorder. AD inflicts twice as many women as men. X-chromosome instability is regulated by Dicer1 [16] whereby Dicer1 depletion in the brain of AD mice may have implication in X-chromosome instability. To dissect the potential involvement, we are planning to examine the distribution of Dicer1 in cellular compartment, with female AD mice.

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 [33]. Similarly, a transcriptional factor linked with microphthalmia, melanocyte increases its transcription during melanoocyte differentiation [34]. At post-transcriptional level, microRNA such as let-7 mRNA and miR-292 regulate Dicer1 via targeting its coding region or 3′-UTR, respectively, thereby inhibiting its expression [35, 36]. 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 [37]; particularly, a phosphomimetic of phospho-serine by replacing serine 1836 with aspartate impairs Dicer1 function and causes postnatal lethality [38]. Dicer1 is also subject to cleavage by calpain [39, 40] which is activated in vulnerable regions of AD brain [41]. Distinct with these studies, we delineate a novel way of regulating Dicer1 through Nrf2-ARE pathway, which is unreported before. Thus, the reduction of Dicer1 in the brain of AD mice may be the summation of calpain activation and the reduction of Nrf2-ARE signaling.

In contrast with deleterious roles of Aβ which are widely reported, Aβ peptides also display protective effects. For example, Aβ42 induces neurogenesis from neural stem cells cultured from hippocampus and striatum by activating tyrosine kinase [42] and amyloid peptides fend off bacteria or virus infection which is reminiscent of the role as antimicrobial peptides [43, 44]. In this study, reduction of Dicer1 by Aβ42 suggests a new way of oxidative stress accumulation in AD brain through depletion of Dicer1. Aβ42 reduced Dicer1 protein in neurons at 48-h treatment which was possibly due to reduced nuclear Nrf2 from chronic Aβ treatment. By contrast, at 6-h treatment, Aβ42 reduced Dicer1 to a significant extent (Fig. 4), which are widely reported previously. 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 pCMV Nrf2 with co-transfection of pCMV3 in individual construct, Aβ42 reduced Dicer1 to a significant extent (Fig. 4). 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 pCMV Nrf2 with co-transfection of pCMV3 in individual construct, Aβ42 reduced Dicer1 to a significant extent (Fig. 4).
accumulation of Alu RNA [21]. Intrahippocampal injection of Nrf2-expressing lentivirus improves spatial learning in APP/PS1 mice [50] but Nrf2 promotes cancer cell proliferation [51]. As such, manipulation of Nrf2 expression may lead to undesired side effects although it indicates potential benefit in AD brain [50]. By contrast, appropriate expression of Dicer1 may be an alternative strategy to oppose oxidative stress or other pathogenic factors in AD brain.

Fig. 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) (Aβ) for 24 h and subject to vehicle treatment (control) 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 and 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. βIII-tubulin was used as a loading control. b Dicer1 relative to βIII-tubulin was normalized to vehicle 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 compared with vehicle treatment (*p = 0.033) and the reduction of Dicer1 by Aβ42 was rescued by infection of Ad-dicer1-T2A:EGFP compared with infection by vehicle virus (**p = 0.0074). c Activated caspase 3 relative to βIII-tubulin in A were normalized to vehicle 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 caspase 3 was increased by Aβ42 oligomer compared with vehicle treatment (**p = 0.0061), which was reduced by infection of Ad-dicer1-T2A:EGFP compared with infection by vehicle virus (*p = 0.023). d The loss of cell viability of hippocampal neurons treated by Aβ (***p = 0.00067) was significantly rescued by infection with Ad-dicer1-T2A:EGFP compared with 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 × 10^6 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 and NC siRNA FAM. Bar, 50 μm. f Total neurite length per cortical neuron was averaged from 17 neurons under individual 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), 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 neurite length, and j median neurite length of hippocampal neurons with treatment from g were calculated and averaged. Aβ42 treatment reduced neurite length compared with vehicle treatment; the effect was rescued by infection with Ad-dicer 1-T2A:EGFP virus. ****p < 0.0001. The neurite length was averaged from 150 neurons in each treatment and one-way ANOVA followed by Tukey’s post hoc test was used to compare the differences...
Overexpression of Dicer1 in APP/PS1 hippocampus inhibited activated caspase 3. APP/PS1 mice and WT littermate at the age of 3.5-4-month were subject to CA3 intrahippocampal injection with Ad-EGFP virus (1.2 × 10⁹ vg/mL), named as APP/PS1 Ad-EGFP and WT Ad-EGFP, respectively. APP/PS1 mice were also subject to injection with Ad-Dicer1-T2A:EGFP virus (1.2 × 10⁹ vg/mL), named as APP/PS1 Ad-Dicer1:T2A:EGFP. Seventeen days after injection, the injected mice were perfused and each brain was sagittally cut into ~ 60 sections at 10 μm 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 with those in WT littermate. Infection with Ad-Dicer1-T2A:EGFP virus dramatically increased Dicer1 staining in CA3 hippocampus. The images were typical of 60 sections from six mice. Bar, 50 μm. b The staining of activated caspase 3 (red) in APP/PS1 mice was increased compared with 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 μm. c The CA3 hippocampi were harvested, homogenized, and subject to centrifugation at 12,000×g, and the supernatants were used for western blot against Dicer1, caspase 3, and activated caspase 3. Typical blots were indicated. d Dicer1 was reduced in APP/PS1 hippocampi compared with WT (*p = 0.0217), which was increased by infection with Ad-Dicer1-T2A:EGFP virus (**p = 0.0093). The optical densities were 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 with WT (**p = 0.00681), which was reduced by infection with Ad-Dicer1-T2A:EGFP virus (**p = 0.0067). The optical densities were averaged from six mice in each group and Kruskal-Wallis test was used to compare the differences.
Dicer1 is ubiquitously expressed in the brain including in neurons, astroglia, and oligodendrocytes [23, 24, 52]. 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 process pre-microRNAs to mature microRNAs, which regulate neuronal survival and neuritogenesis [53, 54]. Deletion of functional Dicer1 has been found to induce accumulation of Alu RNA and thereby activating NLRP3 inflammasome and inducing oxidative damages in advanced dry AMD [20, 21]. Conditional ablation of Dicer1 in oligodendrocytes induces neuronal impairment via demyelination, oxidative damage, and inflammatory gliosis in mice [23]. Evidence shows that conditional knock-out of Dicer1 in dopaminergic neurons of ventral midbrain leads to neuronal loss through inhibiting microRNAs biosynthesis [55]. 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, 52].

In conclusion, we are the first to report that Dicer1 was reduced in the brain of AD animal model. In addition, we indicated that Aβ reduced Dicer1 via repression of Nrf2-ARE signaling and overexpression of Dicer1 in CA3 hippocampus improved spatial learning in the AD mice. However, we are unware that Dicer1 reduction in the brain of AD mice plays a pathogenic role or is only a consequence of AD. To further clarify the roles of Dicer1 in AD, it is important to...
confirm this finding with postmortem tissue from AD patients. We project that this study may open new avenues for investigating potential pathognomonicsof and pathogenesis in AD. Altogether, we reveal a novel role of Dicer1 in AD and a novel regulatory pathway for Dicer1. Our findings suggest Dicer1 may be a target in AD therapy.

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), Keap1 (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# 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 antimouse 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 Aβ42 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# 3480), pfu High fidelity enzyme (Qiagen, Beijing, China, cat# K1205), 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 Scientific, Shanghai, China, cat# 2938), JC-1 probe solution (Sigma, St Louis, MO, USA, cat# CS0760), Neuro-2a (N2A) (ATCC, Manassas, VA, cat# CL-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. APPswe/PSEN1dE9 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 as described [27]. 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.

Experimental Design

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 and treatment during the experiment and when assessing all the results. Animal experiment sample size calculator: http://www.lasec.cuhk.edu.hk/samplersize-calculator.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. 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 the end of experiments. The information including the numbers of animal used, animal allocation to different experiments, and the type of virus injected was indicated in the following table.

| Animals without treatment | Age of 4 months | Age of 11 months | (n) |
|---------------------------|-----------------|-----------------|-----|
|                           | WT              | APP/PS1         |     |
| Female                    | 6               | 6               | 6   |
| Male                      | 6               | 6               | 6   |
| Total                     | 12              | 12              | 12  |
| Applications              |                 |                 |     |
| Western blotting          | 6               | 6               | 6   |
To save the number of mice to be used, half of the mice after behavior test were subject to immunohistochemistry and half of them were used for western blot. Immunohistochemistry and western blot were used to examine the effect of injection of Dicer1-expressing adenovirus on the expression of caspase 3/activated caspase 3 in the AD mice.

**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 (subscript “p” means plasmid). The virus were packaged and generated by Cyagen Biosciences Inc. (Guangzhou, China). Before stereotactic injection, animals were anesthetized 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 2 μl of viral titer (1.2 × 10⁹ 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, ±2 mm, dorsoventral, −2 mm 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.

**Morris Water Maze**

Water maze tests were performed using three groups of mice: WT mice with intrahippocampal injection of Ad-EGFP, namely, WT Ad-EGFP, APP/PS1 mice with intrahippocampal injection of Ad-EGFP, namely, APP/PS1 Ad-EGFP, and APP/PS1 mice with intrahippocampal injection of Ad-Dicer1−T2A:EGFP, namely, APP/PS1 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 and cortical tissue were 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 μM cytosine arabinoside (Sigma-Aldrich, Saint Louis, MO, USA). After 24 h, the culture medium was replaced with fresh neurobasal medium supplemented with 2% B27 and the neuronal cultures were used for experiments after 3 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, Aβ42 oligomer treatment, and adenovirus infection. N2A, SK-N-BE(2), and HEK 293T cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS).
Plasmid Construction and Dual-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 and produced an amplimer containing sequence from −1280 to +3 (relative to tss) (promoter 3), using forward primer CGACGCCTTGGAG ATTGGCGCCTACTATT and reverse primer CCCAAGCTTCCCAAGCTTCTTGTGTC; Two more regions were amplified by alternating forward primer CGACGCCTAGGTTGACAGGACGGAAATCTT and promoter 2 spanning −1121/+3 bp and CGACGCCTACCTGGTTGACAGGACGGAAATCTT producing promoter 2 spanning −896/+3 bp, respectively. The bold letters indicated the predicted AREs sites with consensus sequence TGA(C/G)NNG. A sequence not containing ARE site was also amplified by changing a forward primer to CGACGCCTATGGCAGTACGACTACATT and reverse primer CCCAAGCTTCTGGTCTGCAAGGCA for ARE3 (124 bp). ARE2 (192 bp) was amplified using forward primer CGACGCCTAGGTTGACAGGACGGAAATCTT and reverse primer CCCAAGCTTCTGGTCTGCAAGGCA for and reverse primer CCCAAGCTTCTGGTCTGCAAGGCA. ARE1 (185 bp) was amplified using forward primer CGCCACGCCTAGGTTGACAGGACGGAAATCTT and reverse primer CCCAAGCTTCTGGTCTGCAAGGCA for ARE3-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. ARE1 in promoter 1-Luc was mutated with forward primer incorporating mutated base pair in bold letters GTACCTGGGCTGGACCCAGCATTTA and reverse primer CCCAAGCTTCCCAAGCTTCTTGTGTC; during the PCR produre, 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 CGACGCCTAGGTTGACAGGACGGAAATCTT and the reverse primer was CCCAAGCTTCCCAAGCTTCTTGTGTC with restriction sites underlined.

The HEK293T cells were seeded at a density of 3 × 10⁴ cells/well in 48-well plates and transfected according to the manufacturer’s protocol (Lipofectamine 2000, Invitrogen). The Renilla luciferase-containing reporter (pRL-TK Vector) was used for normalizing transfection efficiency. During transfection, pRL-TK Vector was mixed with individual construct containing firefly luciferase, and a human pCMV-Nrf2 at a ratio of 1:20:20; co-transfection of an empty pCMV3 vector with dual-luciferase plasmids was used as a control. The relative luminescence unit (RLU) of luciferase/renilla assay was measured per the manufacturer’s protocol (Beyotime Biotechnology, cat#PG027) by a plate reader (SpectraMax M5, Molecular devices, San Jose, CA, USA).

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (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. 1× glycin 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 1 mM phenylmethylsulfonyl fluoride (PMSF), with 2 × 10⁵ cells in each microcentrifuge tube. The harvested cells were subject to centrifuge with 1000 × 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 × 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 1000 × 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 1000 × g. The supernatants were discarded and the pellets were washed sequentially with low salt buffer (50 mM Tris-Cl, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 0.5% NP40, 20 mM NaCl), high salt buffer (50 mM Tris-Cl, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 1% NP40, 500 mM NaCl), LiCl Immune complex wash buffer, and final wash with 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′-CACAGACCTGAGCCTGGTCG GAC-3′ (-794/-817bp relative to tss), antisense, 5′-CAGA...
TTGCATGTGCTGAAAGAA-3′(-691/-712bp). For amplifying the fragment containing ARE2 in the promoter, the primers used were 5′-CAGCCTGGTGACAGCGAAAC-3′ (-1069/-1091bp) for sense, and 5′-CTGGTCTGCAAAGGCAGTAT-3′(-876/-906bp) for antisense; for amplifying ARE3-containing fragment in the promoter, the primers were 5′-AGGAGATCATGAAATTGCTGGG-3′(-1250/-1271bp) for sense, and 5′-TAGTGCCCAATCTCAGCT-3′(-1097/-1115bp) for antisense. DNA fragments from each group were amplified by PCR SuperMix (cat# 10572014, Thermo Fisher Scientific) 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. The primers were used at 50 pmol which were designed with SnapGene software (GSL Biotech LLC, San Diego, CA, USA). The procedures were shown in the following scheme:
Aβ42 Oligomer Preparation

The oligomer form of Aβ42 was prepared as described [56]. 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 subject to centrifugation at 14,000 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 x 10^3 per well in DMEM/F-12 medium supplemented with B-27. After transfection with Dicer1 siRNA duplex (sense, 5’-GCACAUCAGGGCUACUAATT-3’, antisense, 5’-UAGUAAGCCUAGUGGCTTT-3’) or negative control siRNA duplex (sense, 5’-UUCUCCGAACGUTT-3’, antisense, 5’-ACGUAGACA CGUUCGGAGAATT-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, a 20-μL aliquot of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt (MTS) was added to each well. Following incubation at 37 °C for 2 h, the formazan product of blue color was detected at 490 nM (Spectra M5, Molecular Devices, Sunnyvale, CA). The plates were also examined by phase-contrast microscopy to visually confirm the results.

Measurement of Mitochondrial Membrane Potential and Reactive Oxygen Species Activity

Primary cultured cortical neurons at 9 x 10^5 cells/well and hippocampal neurons at 5 x 10^5 cells/well were plated into 6-well plates and cultured until DIV3. The neurons were transfected with Dicer1 siRNA duplexes at a final concentration of 100 nM for 24 h and then infected with Ad-EGFP or Ad-Dicer1-T2A:EGFP virus (5 x 10^7 vg/mL) for 48 h. For determination of neurite, the neurites with the length two times more 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.

Neurite Outgrowth Assay

The neuronal cultures were treated by oligomer Aβ42 at 100 nM for 24 h and then infected with Ad-EGFP or Ad-Dicer1-T2A:EGFP virus (5 x 10^7 vg/mL) for 48 h. For determination of neurite, the neurites with the length two times more 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) and then incubated with primary antibody. 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 reagent composed of components in mM:460 Tris-HCl, pH 7.4138 NaCl, 1 EDTA, 2.5 NaF, 2.5 Na3VO4, 1 phenylmethanesulfonylfluoride, 1 dithiothreitol, supplemented with 0.1% Nonidet P-40, and 1× protease/1× phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Proteins were resolved on 12% SDS-PAGE gels (Bio-Rad) and transferred to nitrocellulose membranes (Thermo Fisher Scientific). 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 (Thermo Fisher Scientific). The blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).
Quantitative Real-Time Polymerase Chain Reaction

The hippocampi were isolated and freshly frozen in liquid nitrogen. SK-N-BE cells and Neuro-2A cells were plated on 6-well plate until ~70% confluency and transfected with 1.5 μg pCMV3 Nrf2 vector for 48 h in DMEM supplemented with 10% FBS; transfection of equal amount of pCMV empty vector was used as a control. Extraction of total RNAs from hippocampi and transfected cells and elimination of genomic DNA were performed by a RNA extraction kit (TIGEN BIOTECH, Co, LTD., Beijing, China, cat# DP424). RNA was dissolved in RNase-free extraction kit (TIGEN BIOTECH, Co, LTD., Beijing, China, cat# DP424). RNA was dissolved in RNase-free ddH2O and the concentration was measured by use of a nanodrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific). 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 quantitative real-time PCR as above.

- hDicer1 forward: 5′-TTCGTATTGCGCCGCTAGA-3′, sense, 5′-CGCTCTGGTCCGTCTT-3′; hDicer1, sense, 5′-GTCAGCCGTCAGACCTCACTC-3′, anti-sense, 5′-ACAGTCAAGGCGACATGCAA-3′; mDicer1, sense, 5′-ACAGTCAAGGCGACATGCAA-3′, anti-sense, 5′-ACAGTCAAGGCGACATGCAA-3′; mNrf2, sense, 5′-TGACCATAAGGCGACATGCAA-3′, antisense, 5′-ACAGTCAAGGCGACATGCAA-3′; B1 RNA, sense, 5′-TGACCATAAGGCGACATGCAA-3′, anti-sense, 5′-TGACCATAAGGCGACATGCAA-3′; 18srRNA, sense, 5′-TGCTCTGACTACAGGTTGAA-3′; anti-sense, 5′-TGCTCTGACTACAGGTTGAA-3′; B1: mDicer1, sense, 5′-ACAGTCAAGGCGACATGCAA-3′, antisense, 5′-ACAGTCAAGGCGACATGCAA-3′; mNrf2, sense, 5′-TGACCATAAGGCGACATGCAA-3′, anti-sense, 5′-TGACCATAAGGCGACATGCAA-3′; 18srRNA, sense, 5′-TGCTCTGACTACAGGTTGAA-3′; anti-sense, 5′-TGCTCTGACTACAGGTTGAA-3′; B1.

All data were presented as means ± 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, 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.

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, and wrote the manuscript.

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Availability of Data and Materials  The data and materials are available from corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of Interest  The authors declare that they have no conflict of interest.

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.

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