Selective Degeneration of Entorhinal-CA1 Synapses in Alzheimer's Disease via Activation of DAPK1

Shu Shu,^{1,5} Houze Zhu,^{1,5} Na Tang,^{1,5} Wenting Chen,^{1,5} Xinyan Li,^{1,5} Hao Li,^{1,5} Lei Pei,^{2,5} Dan Liu,^{3,5} Yangling Mu,^{1,5} Qing Tian,^{4,5} Ling-Qiang Zhu,^{4,5} and Youming Lu^{1,5}

¹Department of Physiology, School of Basic Medicine and Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China,

²Department of Neurobiology, School of Basic Medicine and Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China,

³Department of Genetics, School of Basic Medicine and Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China,

⁴Department of Pathophysiology, School of Basic Medicine and Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China, and

⁵Institute for Brain Research, Collaborative Innovation Center for Brain Science, Huazhong University of Science and Technology, Wuhan 430030, China

Excitatory pyramidal neurons in the entorhinal cortical layer II region (ECII_{PN}) form functional excitatory synapses with CA1 parvalbumin inhibitory neurons (CA1_{PV}) and undergo selective degeneration in the early stages of Alzheimer's disease (AD). Here, we show that death-associated protein kinase 1 (DAPK1) is selectively activated in ECII_{PN} of AD mice. Inhibition of DAPK1 by deleting a catalytic domain or a death domain of DAPK1 rescues the ECII_{PN}-CA1_{PV} synaptic loss and improves spatial learning and memory in AD mice. This study demonstrates that activation of DAPK1 in ECII_{PN} contributes to a memory loss in AD and hence warrants a promising target for the treatment of AD.

Key words: Alzheimers' disease; DAPK1; learning and memory; synaptic degeneration

Significance Statement

Our recent study reported that excitatory pyramidal neurons in the entorhinal cortical layer II region (ECII_{PN}) target to CA1 parvalbumin-type inhibitory neurons (CA1_{PV}) at a direct pathway and are one of the most vulnerable brain cells that are selectively degenerated in the early stage of Alzheimer's disease (AD). Our present study shows that death-associated protein kinase 1 (DAPK1) is selectively activated in ECII_{PN} of AD mice. Inhibition of DAPK1 by deleting a catalytic domain or a death domain of DAPK1 rescues the ECII_{PN}-CA1_{PV} synaptic loss and improves spatial learning and memory in the early stage of AD. These data not only demonstrate a crucial molecular event for synaptic degeneration but also provide a therapeutic target for the treatment of AD.

Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly, affecting >10 million people in China and >35

million people worldwide. The deposition of senile plaques that primarily consist of amyloid- β (A β) peptide is a major pathological hallmark in the brains of AD patients and has long been considered to be associated with a progressive loss of central neurons in certain regions of the brain (Tanzi et al., 1987; Goate et al., 1991; Jack et al., 2010; Duffy et al., 2015). However, recent studies have shown that impairments in learning and memory, the early clinical signs of AD, are caused by synaptic dysfunction rather than neuronal cell loss. For example, in AD patients, cognitive decline is closely associated with a reduction in the number of presynaptic glutamatergic terminals (Gomez-Isla et al., 1996; Kammenetz et al., 2003; Oddo et al., 2003). Tg2576-APPswe mice (AD mice), which carry a transgene encoding the 695 amino acid isoform of the human A β precursor protein with the Swedish mutation and exhibit plaque pathologies similar to those in AD patients, show decays in synaptic transmission and impairments in spatial learning and memory in an age-dependent manner (Chapman et al., 1999; Jacobsen et al., 2006; Scheff et al., 2007).

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Correspondence should be addressed to either Dr. Ling-Qiang Zhu or Dr. Youming Lu, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. E-mail: zhulq@hust.edu.cn or lym@hust.edu.cn.

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However, which of the many synapses in the brain undergo selective degeneration during the early stages of AD and whether this selective degeneration contributes directly to the loss of spatial learning and memory are still unknown.

Excitatory pyramidal neurons in the entorhinal cortex (EC, EC_{PN}), which primarily target to the hippocampus, are the most vulnerable brain cells in the early stages of AD (Hsia et al., 1999; Yassa, 2014; Yang et al., 2016). The EC_{PN} are largely distributed in the EC layer II (ECII_{PN}) and III (ECIII_{PN}) regions. These neurons innervate excitatory pyramidal neurons and parvalbumin (PV) inhibitory GABA-containing neurons in the CA1 hippocampus (CA1_{PV}) and are associated with spatial and temporal associative memories (Kitamura et al., 2014; Yang et al., 2016). Our recent study showed that amyloid deposition occurs in the brain of AD mice at 8 months of age, whereas synaptic transmission between ECII_{PN} and CA1_{PV} undergoes degeneration at 6 months of age (Yang et al., 2016). This finding indicates that synaptic degeneration is not associated with the presence of amyloid plaques. Yet, the molecular mechanisms underlying degeneration of ECII_{PN} synapses remain unknown. Our present studies demonstrate that death-associated protein kinase 1 (DAPK1) became activated selectively in the ECII_{PN} of AD mice. We show that activation of DAPK1 is responsible for a selective degeneration of ECII_{PN}-CA1_{PV} synapses and that inhibition of DAPK1 is therapeutically effective for the intervention of spatial learning and memory declines in AD.

Materials and Methods

Animals. All mice used in this study were bred and reared under the same conditions in the University's core animal facility in accordance with institutional guidelines and the Animal Care and Use Committee (Huazhong University of Science and Technology, Wuhan, China). The mice were housed in groups of 3–5 per cage and maintained with a 12 h light-dark cycle, with lights on at 8:00 A.M., at consistent ambient temperature ($22 \pm 1^\circ\text{C}$) and humidity ($50 \pm 5\%$). Tg2576-APP_{sw} mice (the AD mice), which expressed a mutant form of A β precursor protein (APP) (isoform 695) with the Swedish mutation (KM670/671NL), were purchased from The Jackson Laboratory. In the present study, all the AD mice were identified as homozygous. Male mice were used in this study.

Generation of the mutant mice. To generate the AD/ECII_{PN}^{Chr2+} mice, we expressed Chr2-eGFP in the ECII_{PN} of mutant mice that had a loxP-flanked STOP sequence followed by Chr2 (E123A)-eGFP. The Rosa-CAG-Flag-Chr2eGFP-WPRE targeting vector was designed with a CMV-IE enhancer/chicken β -actin/rabbit β -globin hybrid promoter (CAG), an FRT site, a loxP-flanked STOP cassette, a Flag-eGFP sequence, a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE; to enhance the mRNA transcript stability), a poly-A signal, and an attB/att-flanked PGK-FRT-Neo-poly-A cassette. This entire construct was inserted into the Gt(RSA)26Sor locus via electroporation into C57BL/6-derived embryonic stem (ES) cells. The targeted ES cells were selected and injected into C57BL/6 blastocysts, and chimeric animals were then bred to C57BL/6 mice (Chr2^{loxP/loxP}).

Next, we created the rAAV1/2-D28K-Cre virus particles. We designed the rAVE-D28K-Cre vector by inserting a Cre recombinase sequence immediately downstream of the calbindin 1 (or calbindin-D28K, D28K) translational STOP codon via *Apal/KpnI*. The rAVE plasmids were cotransfected with AAV helper1/2 mixers into HEK293 cells to generate rAAV1/2-D28K-Cre virus particles with a high titer ($>5 \times 10^{12}$ genomic particles/ml), as described previously (Tu et al., 2010; Pei et al., 2015; Yang et al., 2016). The virus particles (1.5 μl) were bilaterally injected into the EC superficial layer (anteroposterior 4.8 mm, mediolateral 2.8 mm, dorsoventral 3.5–4.0 mm) in the AD/Chr2^{loxP/loxP} and control/Chr2^{loxP/loxP} mice, thus leading to Chr2-eGFP expression in the ECII_{PN} of the AD or control mice (AD/ECII_{PN}^{Chr2+} and control/ECII_{PN}^{Chr2+} mice).

To generate the AD/ECII_{PN}^{CD-} mice, we first created a mutant strain of mice with selective deletion of the catalytic domain (CD) of DAPK1

(DAPK1^{CDloxP/loxP} mice) by targeting the exon 2 region, as described previously (Pei et al., 2015). The DAPK1^{CDloxP/loxP} mice were crossed with the AD/ECII_{PN}^{Chr2+} mice, thus resulting in AD/ECII_{PN}^{CD-} mice, in which the DAPK1 CD was selectively deleted in the Chr2⁺-ECII_{PN}. To generate the AD/ECII_{PN}^{DD-} mice, we created a mutant strain of mice with a selective deletion of the death domain (DD) of DAPK1 (DAPK1^{DDloxP/loxP} mice). In brief, an FRT-flanked Neo resistance positive selection cassette was inserted downstream of exon 27; one loxP site was introduced upstream of exon 26 and another loxP site was introduced downstream of the Neo cassette. After linearization, the targeting vector was transfected into C57BL/6J embryonic stem cells via electroporation. Six positive clones were identified by Southern blotting with a 5' probe, a 3' probe, and a Neo probe. Two positive clones were injected into BALB/c blastocysts and implanted into pseudopregnant females. Chimeric mice were crossed with C57BL/6J mice to obtain F1 mice carrying the recombined allele containing the floxed DAPK1 allele and the Neo selection cassette. These mice were mated with Flp recombinase-expressing C57BL/6J Flp mice to remove the Neo resistance cassette and to generate a line of Neo-excised DAPK1^{DDloxP/loxP} mice. When these mice were bred with the AD/ECII_{PN}^{Chr2+} mice, the offspring were AD/ECII_{PN}^{DD-} mice, in which the DAPK1 DD was selectively deleted in the Chr2⁺-ECII_{PN}.

Single-cell Western blots, kinase assays, and coimmunoprecipitation. EC tissues were isolated from the AD/ECII_{PN}^{Chr2+} mice, the AD/ECII_{PN}^{CD-} mice, the AD/ECII_{PN}^{DD-} mice and their respective controls and sliced and digested in buffer containing 10 mM Tris-Cl, pH 7.6, 50 mM NaF, 1 mM Na₃VO₄, 1 mM edetic acid, 1 mM benzamidine, 1 mM PMSF, 1 mg/10 ml papain, and a mixture of aprotinin, leupeptin, and pepstatin A (10 $\mu\text{g}/\text{ml}$ each) for 30 min. The cell suspension of ECII_{PN}^{eGFP+} was automatically isolated using an S3e Cell Sorter (Bio-Rad). The isolated ECII_{PN} were homogenized and diluted with a buffer containing 200 mM Tris-Cl, pH 7.6, 8% SDS, and 40% glycerol. The protein concentration was determined using a BCA kit (Pierce). Final concentrations of 10% β -mercaptoethanol and 0.05% bromophenol blue were added, and the samples were boiled for 10 min in a water bath. The proteins in the extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were scanned by using an Infrared Imaging System (Odyssey, LI-COR). The blots were incubated with antibodies against DAPK1 (1:500, Epitomics, catalog #3798-1). The band densities were quantitatively analyzed by using Kodak Digital Science 1D software (Eastman Kodak).

Immunocomplex kinase assay was used to determine the catalytic activity of DAPK1 in the lysates and myosin light chain (MLC) that was phosphorylated at Ser-18/Thr-20 (pMLC) by activated DAPK1 was blotted with anti-pMLC (1:1000, Millipore). The enzymatic activity of DAPK1 was expressed as the ratio of pMLC to DAPK1 protein under the same conditions. Immunoprecipitation was used to determine the association of DAPK1 with its substrates. The lysates ($\sim 200 \mu\text{g}$ protein) were incubated with nonspecific IgG (2 μg) or polyclonal rabbit anti-DAPK1 antibody (2 μg , Millipore) overnight at 4°C , and this was followed by the addition of 40 μl of Protein G-Sepharose (Sigma) and incubation for 3 h at 4°C . The precipitates were washed four times with lysis buffer, denatured with SDS sample buffer, and separated by 12% SDS-PAGE. The proteins were transferred onto nitrocellulose membranes using a Bio-Rad mini-protein-III wet transfer unit overnight at 4°C . The transferred membranes were then incubated with blocking solution [5% nonfat dried milk dissolved in TBST buffer containing 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature, washed three times, and incubated with anti-goat primary antibody against Tau (1:1000, Santa Cruz Biotechnology), anti-rabbit antibody against Erk (1:1000; Millipore), or anti-DAPK1 (1:1000, Sigma) for 1 h at room temperature. The membranes were washed three times with TBST buffer, incubated with the appropriate secondary antibodies (1:1000 dilution) for 1 h, and washed four times. Signal detection was performed with an enhanced chemiluminescence kit (GE Healthcare). The lanes marked "input" were loaded with 10% of the starting material used for immunoprecipitation.

Electrophysiology. Brain slices (300 μm) were prepared as previously described (Tu et al., 2010; Pei et al., 2015; Yang et al., 2016). The slices were transferred to a holding chamber containing ACSF consisting of 124

mm NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.2 mM MgCl₂·6H₂O, 1.25 mM NaH₂PO₄·2H₂O, 10 mM C₆H₁₂O₆, and 2 mM CaCl₂, pH 7.4 and 305 mOsm. The slices were allowed to recover at 31.5°C for 30 min and then at room temperature for 1 h. Acute slices were transferred to a recording chamber continuously perfused with oxygenated ACSF (2 ml/min) and maintained at room temperature. For whole-cell patch-clamp recordings from the CA1_{PV}, brain slices from the AD mice, in which mCherry was expressed in the CA1_{PV} cells, were visualized via fluorescence IR-DIC using an Axioskop 2FS equipped with Hamamatsu C2400–07E optics. When stable whole-cell recordings were achieved with good access resistance (~ 20 M Ω), basic electrophysiological properties were recorded. All the data were acquired at 10 kHz and filtered with a 2 kHz low-pass filter. The evoked EPSCs were recorded in the slices by delivering blue laser light (5 ms, 405-nm-wavelength laser at power densities ranging from 0.1 to 5 mW/mm²) directly onto the ECII_{PN}, which had normal intrinsic properties in AD/ECII_{PN}^{Chr2+} (resting membrane potentiation = 69.3 ± 2.1 mV; input resistance = 386 ± 21 M Ω , mean $\pm$ SEM, $n = 36$ cells/6 mice), AD/ECII_{PN}^{CD-} (resting membrane potentiation = 68.7 ± 2.0 mV; input resistance = 389 ± 19 M Ω , mean $\pm$ SEM, $n = 33$ cells/5 mice), and AD/ECII_{PN}^{DD-} mice (resting membrane potentiation = 69.9 ± 1.9 mV; input resistance = 401 ± 17 M Ω , mean $\pm$ SEM, $n = 39$ cells/5 mice) when they were at 180 ± 5 d of age, compared with the age-matched controls (resting membrane potentiation = 70.1 ± 1.7 mV; input resistance = 403 ± 13 M Ω , mean $\pm$ SEM, $n = 32$ cells/5 mice).

Extracellular single-unit recording. Extracellular single-unit and local field power spectrum recordings were made from the CA1 neurons (anteroposterior -1.7 mm, mediolateral 1.0 mm, and dorsoventral 0.5 – 0.8 mm target to CA1). Mice were connected to the recording equipment via AC-coupled unity-gain operational amplifiers (Plexon). Signals were amplified 4000- to 8000-fold. The spikes and local field potentials were recorded simultaneously and isolated by using a 250 Hz low-pass filter and a 250 Hz high-pass filter and the commercial software OmniPlex (Plexon). Spike sorting was performed off-line using graphical cluster-sorting software (Offline Sorter, Plexon). To estimate the quality of the cluster separation, we calculated the isolation distance and the L-ratio using Plexon SDK. Only the units with an L-ratio < 0.05 and a distance > 15 were included.

To isolate and analyze spike units from individual CA1_{PN} versus the CA1_{PV}, we calculated the valley-to-peak time and the half-width of the spikes. Spikes from CA1_{PN} were identified and distinguished from those of CA1_{IN} according to the duration of the negative spike, the firing pattern (complex spikes), and the low average firing rate. Spikes from CA1_{PV} were validated as described previously (Yang et al., 2016). The average firing rate was expressed as the total number of spikes divided by the total length of the recording period.

Morris Water Maze. A pool 1.5 m in diameter was filled with water that was made opaque with white nontoxic ink and maintained at 25.0°C. Animals were brought to the behavior room (where they were housed for the duration of the training), handled for 1–2 d, and trained, as described previously (Tu et al., 2010; Pei et al., 2015; Yang et al., 2016). The entire protocol lasted 7 d. The first training day consisted of a probe trial followed by a “visible platform” trial, in which the platform was indicated by a red flag. Next, mice were subjected to their first “hidden platform” learning trial, during which they were allowed to rest on the platform for 30 s before being released from one of the pool's starting points (north, south, east, or west). The animals were allowed 60 s to find the platform and were allowed to stay there for 30 s; if an animal did not find the platform within 60 s, it was removed from the water and placed on the platform for 30 s. During days 1–6, four trials were performed corresponding to the four different randomized release points. Therefore, the total training took 6 d followed by a final 24 h probe on the seventh day.

Open-field tests and rotarod tests. Locomotor activity was assessed in clear boxes measuring 100 cm $\times$ 100 cm that were outfitted with photo-beam detectors for monitoring horizontal and vertical activity, as described previously (Tu et al., 2010; Pei et al., 2015; Yang et al., 2016). The data were collected using a PC and were analyzed using the MED Associates Activity Monitor Data Analysis software. Mice were placed in a corner of the open-field apparatus and allowed to move freely. The variables recorded included resting time (seconds), ambulatory time (sec-

onds), vertical/rearing time (seconds), jump time (seconds), stereotypic time (seconds), and average velocity (centimeters per second). The mice were not exposed to the chamber before testing. The data were recorded for each animal during 30 min intervals. For the rotarod test, mice were subjected to a 1 week learning period, after which they were able to perform on an accelerating rotarod. The test was then performed twice per week until the mice were unable to remain on the rotating bar for > 10 s in three consecutive attempts, which was defined as rotarod failure.

Data analysis. All variance values in the text and figure legends are represented as the mean $\pm$ SEM. Parametric tests, including t tests and two-way ANOVAs, were used when assumptions of normality and equal variance (F test) were met; two-way repeated-measures ANOVA was used to analyze the Morris Water Maze data. Differences were considered statistically significant when $p < 0.05$.

Results

Activation of DAPK1 in ECII_{PN} of AD mice

To determine the molecular mechanisms underlying the selective degeneration of ECII_{PN} synapses in AD mice, we focused our studies on DAPK1, an enzyme critical for synaptic losses in central neurons (Tu et al., 2010). To functionally and morphologically identify individual ECII_{PN}, we generated AD/ECII_{PN}^{Chr2+} mice, in which channelrhodopsin-2-E123A (Chr2), a modified version of a light-gated cation channel, and an eGFP were selectively expressed in the vulnerable ECII_{PN} of the AD mice. A mouse model of AD was chosen because these mice carry a transgene coding for the 695 amino acid isoform of the human Alzheimer A β precursor protein with the Swedish mutation and exhibit plaque pathologies similar to those in AD patients.

Specifically, we created a conditional line of mutant mice that expressed a double-floxed inverted open reading frame of Chr2-eGFP (*Chr2^{loxP/loxP}* mice; Fig. 1A). We also constructed a Type 1/2 recombinant adeno-associated (rAAV1/2)-D28K-Cre virus vector, in which Cre recombinase was expressed under the control of the D28K promoter (Fig. 1B). Virus particles at high titer were injected directly into the ECII of the AD/*Chr2^{loxP/loxP}* mice, resulting in the Chr2-eGFP expression specifically in the ECII_{PN} (Fig. 1B). ECII_{PN} cells were named as island cells, in which Wfs1 is expressed (Kitamura et al., 2014). Consistent with this previous study, we showed that ECII_{PN} that directly innervate CA1_{PV} were labeled with an antibody against Wfs1 protein (Fig. 1C). Thus, ECII_{PN}^{Chr2+} neurons comprise a group of excitatory island cells.

We next isolated ECII_{PN}^{Chr2+} cells from the brain tissues of the AD/ECII_{PN}^{Chr2+} mice using flow cytometry and cell sorting techniques. To determine the catalytic activity of DAPK1 in the purified ECII_{PN}^{Chr2+} cells, we applied an immunocomplex kinase assay with MLC as an endogenous substrate of DAPK1 (Tu et al., 2010). We precipitated DAPK1 protein complex in the cell lysates of the ECII_{PN}^{Chr2+} cells from the AD/ECII_{PN}^{Chr2+} mice and the controls (the control/ECII_{PN}^{Chr2+} mice) using an antibody against DAPK1. The precipitates were then blotted with antibodies against a pMLC and DAPK1, respectively (Fig. 1D). Our data revealed that the levels of pMLC were increased in the ECII_{PN}^{Chr2+} cells from the AD/ECII_{PN}^{Chr2+} mice after 150 d of age (Fig. 1D), compared with the age-matched control/ECII_{PN}^{Chr2+} mice (Fig. 1E). The pMLC was catalyzed by an activated DAPK1 as MLC kinase (MLCK) that also targets MLC at serine-20 was undetectable in the precipitates from the ECII_{PN}^{Chr2+} cells of the AD/ECII_{PN}^{Chr2+} mice (Fig. 1F). An increase of the pMLC was found in the ECII_{PN}^{Chr2+} cells only, but not in the frontal cortical neurons from the AD/ECII_{PN}^{Chr2+}

Figure 1. DAPK1 is activated specifically in ECII_{PN} of AD mice. **A**, Illustration of the targeting vector used to generate the *ChR2-eGFP^{loxP/loxP}* mice. **B**, Representative image showing the selective expression of ChR2-eGFP in ECII_{PN} of AD/ECII_{PN}^{ChR2+} mice when the rAAV1/2-D28K-Cre virus particles were stereotactically injected into the ECII region. **C**, Representative images indicating that the ECII_{PN}^{ChR2+} are labeled with an antibody against the Wsf1 protein. Similar results were seen in each of the four experiments. **D**, **E**, DAPK1 becomes activated in ECII_{PN} of AD mice at 150 d of age. The ECII_{PN}^{ChR2+} cells were isolated from the brain sections of the AD/ECII_{PN}^{ChR2+} mice (**D**) and the control/ECII_{PN}^{ChR2+} mice (**E**), respectively, when they were at 120, 150, 180, and 210 d of age. The cell lysates were then prepared from the isolated ECII_{PN}^{ChR2+} cells and precipitated with anti-DAPK1. The precipitates were blotted with antibodies against pMLC or DAPK1, as indicated. In bar graphs, the band intensity was normalized to that of the DAPK1 protein from a mouse at 120 d of age (defined as 1.0). Data are mean ± SEM ($n = 5$). * $p < 0.001$ (t tests). **F**, **G**, the pMLC is catalyzed by an activated DAPK1 but not MLCK in the ECII_{PN}^{ChR2+} cells from AD mice. Blots of the precipitates from the ECII_{PN}^{ChR2+} (**F**) and the frontal cortical cells (**G**) extracts of the AD/ECII_{PN}^{ChR2+} mice at 120, 150, 180, and 210 d of age with antibodies against MLCK, pMLC, or DAPK1, as indicated. Similar results were seen in each of the four experiments.

mice (Fig. 1G). Together, these data demonstrate that DAPK1 is activated selectively in ECII_{PN} cells of AD mice during aging.

Genetic inhibition of DAPK1 in ECII_{PN} of AD mice

We next determined whether activation of DAPK1 in the ECII_{PN} of AD mice contributes to impairments in synaptic transmission along the ECII_{PN}-CA1_{PV} pathway. We developed two independent approaches to inhibiting DAPK1 function in the ECII_{PN} of AD mice. First, we generated a mutant strain of mice (*DAPK1^{CDloxP/loxP}*) in which a double-floxed inverted open reading frame of DAPK1 with a CD deletion was expressed (Fig. 2A,B). When these mice were bred with AD/ECII_{PN}^{ChR2+} mice, which Cre recombinase was expressed in the ECII_{PN}^{ChR2+} cells (for details, see Fig. 1B), the CD of DAPK1 (*DAPK1^{CD}*) was selectively deleted in the ECII_{PN}^{ChR2+} of the offspring (AD/ECII_{PN}^{CD} mice; Fig. 2C–E). To verify the suc-

cessful generation of AD/ECII_{PN}^{CD} mice, we performed two independent lines of the studies. First, we stained the sections from the AD/ECII_{PN}^{CD} mice with antibody against D28K protein and showed that the DAPK1^{CD} mutant protein in the ECII_{PN}^{ChR2+} cells was recognized by anti-D28K (Fig. 2D). Second, we isolated the ECII_{PN}^{ChR2+} cells from the AD/ECII_{PN}^{CD} mice using flow cytometry and cell sorting techniques. The cell lysates were prepared from the isolated ECII_{PN}^{ChR2+} cells and precipitated with anti-DAPK1. The precipitates were blotted with antibodies against pMLC and DAPK1 proteins, as indicated (Fig. 2E). Our data revealed a complete loss of DAPK1 enzymatic activity in the ECII_{PN}^{ChR2+} cells of the AD/ECII_{PN}^{CD} mice (Fig. 2E).

Next, we generated the mutant mice (*DAPK1^{DDloxP/loxP}* mice) in which a double-floxed inverted open reading frame of DAPK1 lacking a DD was expressed (Fig. 2F,G). When these mice were

Figure 2. Genetic inhibition of DAPK1 in *ECII_{PN}* of AD mice. **A**, Constructs for the generation of *DAPK1^{CDloxP/loxP}* mice. **B**, Representative PCR showing the expression of the mutant transcripts in the *DAPK1^{CDloxP/loxP}* mice. **C**, The *Tg3* mice were generated by crossing the *ChR2^{loxP/loxP}* mice with the AD mice and the *DAPK1^{CDloxP/loxP}* mice and were stereotactically injected with the rAAV1/2-D28K-Cre virus particles into the *ECII_{PN}* region, resulting in the *AD/ECII_{PN}^{CD}* mice. **D**, Representative images showing the *ECII_{PN}* from the *AD/ECII_{PN}^{CD}* mice that expressed the *DAPK1^{CD}* mutant protein (green, ChR2) and that were stained with an antibody against the D28K protein (pink). Similar results were seen in each of the five experiments. **E**, Blots of the *ECII_{PN}* extracts from the *AD/ECII_{PN}^{CD}* mice using antibodies against pMLC or DAPK1, as indicated. The band intensity was normalized to that of the DAPK1 protein from mice at 90 d of age (defined as 1.0). Data are mean $\pm$ SEM ($n = 5$). **F**, Constructs for the generation of the *DAPK1^{DDloxP/loxP}* mice. **G**, **H**, Representative images showing the expression of mutant transcripts (**G**) and protein (**H**) from the *DAPK1^{DDloxP/loxP}* mice. **H**, An antibody against the N-terminal region of DAPK1 (N-DAPK1) recognizes the wild-type (~ 160 kDa) and mutant proteins (~ 140 kDa). **I**, Deletion of the DAPK1 DD does not affect the catalytic activity of DAPK1. Blots of the *ECII_{PN}* extracts from the *AD/ECII_{PN}^{DD}* mice using antibodies against pMLC or N-DAPK1, as indicated. The band intensity was normalized to that of the N-DAPK1 protein from mice at 90 d of age (defined as 1.0). Data are mean $\pm$ SEM ($n = 5$). **J**, Deletion of the DAPK1 DD inhibits the association of DAPK1 protein with its substrates. The *ECII_{PN}* cell lysates from the *AD/ECII_{PN}^{DD+}* (DD^+) or the *AD/ECII_{PN}^{DD-}* (DD^-) mice were precipitated with nonspecific IgG or antibodies against DAPK1. The precipitates were then blotted with antibodies against N-DAPK1, Tau protein, or ERK protein, as indicated. Input: 10 μ g of protein from the *ECII_{PN}* cell lysates without precipitation was loaded. Similar results were seen in each of the four experiments.

crossed with the *AD/ECII_{PN}^{ChR2+}* mice, the DD of DAPK1 was selectively deleted (*DAPK1^{DD-}*) in the *ECII_{PN}^{ChR2+}* of the offspring (*AD/ECII_{PN}^{DD-}* mice). The successful expression of *DAPK1^{DD-}* mutant protein was confirmed by blotting the cell lysates from the *ECII_{PN}^{ChR2+}* cells of the *AD/ECII_{PN}^{DD-}* mice with antibody against N-terminal fragment of DAPK1 (N-DAPK1; Fig. 2H). We next examined the enzymatic activity of *DAPK1^{DD-}* mu-

tant protein. We isolated the *ECII_{PN}^{ChR2+}* cells from the *AD/ECII_{PN}^{DD-}* mice using flow cytometry and cell sorting techniques. The cell lysates were prepared from the isolated *ECII_{PN}^{ChR2+}* cells and precipitated with anti-DAPK1. The precipitates were blotted with antibodies against pMLC, tubulin, Tau, and ERK proteins, as indicated (Fig. 2I,J). Our data revealed that the expression of *DAPK1^{DD-}* did not alter the catalytic activity of DAPK1 (Fig. 2I),

Figure 3. Inhibition of DAPK1 protects against the ECII_{PN}-CA1_{PV} synaptic decay in AD mice. **A**, Illustration (top) represents the recording configurations. The mean amplitudes of the evoked NMDA receptor EPSCs in the CA1_{PV} at a holding potential of 60 mV are plotted versus the light intensity increments (0.5 mV intervals) of stimulation of ECII_{PN}^{ChR2+}. Data are mean ± SEM ($n = 25$ recordings/5 mice/group). * $p < 0.001$ (t tests). Representative recordings above the graph are the average of 12 sweeps of the evoked EPSCs at the low (a) and high (b) stimulus intensities. **B**, Representative images show the ChR2-eGFP-labeled terminals (green) of the ECII_{PN} in the CA1 hippocampus stained with anti-PV antibody (pink). **C**, Bar graphs represent the numbers (terminals/0.2 cm²) of the ChR2-eGFP-labeled terminals in the stratum lacunosum-moleculare region and the PV-labeled spines (spines/10 μm dendritic branches) in the stratum lacunosum-moleculare region. Data are mean ± SEM ($n = 5$ mice/group). * $p < 0.001$ (t tests). **D–F**, A working model showing that inhibition of DAPK1 restores excitatory and inhibitory balance in the CA1 circuits of AD mice. Under the physiological conditions (**D**), ECII_{PN} form direct excitatory synapses with CA1_{PV} and balance the excitatory/inhibitory synaptic transmission in CA1 circuits. During the disease progression of AD (**E**), ECII_{PN}-CA1_{PV} synapses are degenerated. This degeneration disables the excitatory and inhibitory balance as a consequence of a loss of inhibitory inputs from CA1_{PV} to CA1_{PN}. Inhibition of DAPK1 in the ECII_{PN} (**F**) effectively intervenes in the degeneration of ECII_{PN}-CA1_{PV} synapses and restores the excitatory and inhibitory synaptic balance in AD mice. **G, H**, Genetic inhibition of DAPK1 restores the balance between excitation and inhibition in CA1_{PV} (**G**) and CA1_{PN} (**H**) cells of AD mice at 180 d of age. Representative recordings of spike units of CA1 neurons in freely moving mice. The spikes in CA1_{PV} (**G**) versus CA1_{PN} (**H**) were isolated based on the valley-to-peak time and the half-width of the spikes. The averaged frequencies of action potential firings of CA1_{PV} (**G**) and CA1_{PN} (**H**) in freely moving mice at 180 d of age were summarized in bar graphs. Data are mean ± SEM ($n = 11$ mice per group). * $p < 0.001$ (t tests).

Figure 4. Inhibition of DAPK1 in the ECII_{PN} improves spatial learning and memory in AD mice. **A–C**, The latency (**A**, **B**) and swim length (**C**) to reach a hidden platform are plotted against the blocks of trials. **A**, Representative path tracings were taken during the training tests on day 6. Data are mean $\pm$ SEM ($n = 11$ mice per group, $F_{(5,60)} = 2.71$). $*p < 0.001$ (ANOVA). **D–G**, The percentage of time spent in search of a hidden platform in each quadrant during the probe trial of mice at 90 (**D**, **E**), 120 (**F**), and 180 (**G**) days old of age. **D**, Representative hot spot of path tracings taken during the probe trials on day 8. Data are mean $\pm$ SEM ($n = 11$ mice per group, $F_{(5,60)} = 3.97$). $*p < 0.001$. **H**, Deletion of DAPK1 DD in the ECII_{PN} of AD mice improves spatial information acquisition. The latency and swim length to reach a hidden platform are plotted against the blocks of trials. Data are mean $\pm$ SEM ($n = 11$ mice per group, $F_{(5,60)} = 4.13$). $*p < 0.001$. **I**, Deletion of DAPK1 DD in the ECII_{PN} of AD mice improves spatial memory. The percentage of time spent in search of a hidden platform in each quadrant during the probe trial. Data are mean $\pm$ SEM ($n = 11$ mice per group, $F_{(5,60)} = 3.11$). $*p < 0.001$.

but this deletion blocked the binding of DAPK1 to DAPK1 substrates, such as the Tau and ERK proteins (Fig. 2J).

Protection against ECII_{PN} synaptic decay in AD mice

To determine whether the genetic inhibition of DAPK1 might avert the ECII_{PN} synaptic decay in AD mice, we performed whole-cell patch-clamp recordings with the CA1_{PV} (Fig. 3A) from the AD/ECII_{PN}^{CD-} and AD/ECII_{PN}^{DD-} mice at 180 $\pm$ 5 d of

age as well as age-matched controls (AD/ECII_{PN}^{ChR2+}, the control/ECII_{PN}^{DD-}, and the control/ECII_{PN}^{ChR2+} mice). EPSCs were evoked by delivering blue laser light directly to the ECII_{PN}^{ChR2+} in brain slices. To exclude disturbances from other projections (i.e., the ECII_{PN}-DG-CA3-CA1_{PV} indirect pathway), we cut off the connections of the dentate gyrus to the CA3 cells in the slices (Fig. 3A) and recorded NMDA-mediated EPSCs at a holding potential of 60 mV. The recordings were performed in the pres-

Figure 5. AD mice are normal in learning-unrelated behavioral tests. **A**, Bar graphs represent that distance moved, rearing counts, and grooming time in the open field tests are identical among groups (mean $\pm$ SEM, $n = 9$ mice per group). **B**, Bar graphs represent that the performance for foot stay and fault on rotarod tests is comparable between genotypes (mean $\pm$ SEM, $n = 8$ mice per group). **C**, Mice are normal in elevated plus maze tests. Bar graphs represent that time in open arms is identical between groups (mean $\pm$ SEM, $n = 9$ mice per group). **D**, Mice are normal in the forced swimming tests. The percentage of time immobile per minute over the whole 5 min trial is identical between groups (mean $\pm$ SEM, $n = 9$ mice per group).

ence of 20 μ M CNQX and 20 μ M bicuculline to block both AMPA receptors and Type A GABA_A receptor-mediated responses. Under this circumstance, we were able to record monosynaptic transmission between ECII_{PN} and CA1_{PV} cells in the slices. We then analyzed the mean amplitudes of the evoked EPSCs with the increases of the stimulus intensities (Fig. 3A). We demonstrated that the genetic inhibition of DAPK1 specifically in the ECII_{PN} did not alter synaptic transmission in the control mice (the peak values of the mean amplitudes were 99 $\pm$ 13 pA in the control/ECII_{PN}^{ChR2+/+} mice vs 92 $\pm$ 15 pA in the control/ECII_{PN}^{CD-/-} mice), but it was effective in reversing the ECII_{PN}-CA1_{PV} synaptic decline in the AD mice (peak values of the mean amplitudes were 65 $\pm$ 8 pA in the AD/ECII_{PN}^{ChR2+/+} mice vs 93 $\pm$ 9 pA in the AD/ECII_{PN}^{CD-/-} mice).

Next, we examined the structure of ECII_{PN}-CA1_{PV} synapses in the AD/ECII_{PN}^{CD-/-} mice (Fig. 3B). At presynaptic sites, the excitatory terminals in the stratum lacunosum-moleculare, an afferent axon terminal zone of the ECII_{PN}^{ChR2+/+} in the CA1 hippocampus, were analyzed (Fig. 3B). The density of ChR2-eGFP-labeled terminals (50 μ m $\times$ 200 μ m) in the AD mice at 180 $\pm$ 5 d of age was 29.1 $\pm$ 3.2% lower than that of the age-matched controls (Fig. 3C). This reduction in the number of excitatory synaptic terminals in the stratum lacunosum-moleculare region of the AD mice was completely rescued in both the AD/ECII_{PN}^{CD-/-} and AD/ECII_{PN}^{DD-/-} mice (Fig. 3C). At postsynaptic sites, we stained the sections with an antibody against the PV protein (Fig. 3B), which revealed that the dendritic branch spines (50 μ m segment) from the antibody-labeled CA1_{PV} cells (50 seg-

ments per animal) in the stratum lacunosum-moleculare region of the AD mice decreased by 35.1 $\pm$ 3.9% compared with those in the age-matched controls (5.3 $\pm$ 0.63 vs 3.3 $\pm$ 0.36, mean $\pm$ SEM, $n = 50$ dendritic branches/5 mice/group, $p < 0.001$; Fig. 3C). The inhibition of DAPK1 completely rescued the synaptic loss of the CA1_{PV} in the AD mice (5.1 $\pm$ 0.59 in the AD/ECII_{PN}^{CD-/-} mice, 4.9 $\pm$ 0.62 in the AD/ECII_{PN}^{DD-/-} mice vs 5.2 $\pm$ 0.58 in the controls, mean $\pm$ SEM, $n = 50$ dendritic branches/5 mice/group, $p > 0.05$; Fig. 3C).

Impairments of synaptic transmission along the ECII_{PN}-CA1_{PV} excitatory pathway, which primarily target the dendrites of CA1 excitatory pyramidal neurons (CA1_{PN}) disrupt the excitatory and inhibitory balance in the CA1 neural circuits of AD mice (Lesne et al., 2006; Mucke, 2007; Palop and Mucke, 2009). Therefore, protecting the ECII_{PN}-CA1_{PV} pathway from synaptic loss in AD mice should restore the excitatory and inhibitory balance in the CA1 circuits (Fig. 3D–F). To test this hypothesis, we monitored the activity of CA1 neuronal cells in freely moving mice at 180 $\pm$ 5 d of age by using extracellular single-unit recording techniques (Yang et al., 2016). Action potentials that originated from the CA1_{PN} versus the CA1_{PV} of freely moving mice were classified on the basis of the properties of the action potential, as recently described (Yang et al., 2016). In the AD mice, the probability of action potential firing was dramatically reduced in the CA1_{PV} (11.8 $\pm$ 1.6 vs 16.9 $\pm$ 1.9, mean $\pm$ SEM, $n = 36$ units/9 mice/group, $p < 0.001$; Fig. 3G) and increased in the CA1_{PN} (5.6 $\pm$ 0.68 vs 3.3 $\pm$ 0.51, mean $\pm$ SEM, $n = 36$ units/9 mice/group, $p < 0.001$; Fig. 3H), compared with the age-matched

control mice, thus showing that the excitatory and inhibitory balance was disrupted in the CA1 circuits of the AD mice. In the AD/ECII_{PN}^{CD−} mice, in which DAPK1 was inactivated specifically in the ECII_{PN}, the excitatory and inhibitory balance in the CA1 circuits was restored; the probabilities of action potential firings in both the CA1_{PV} (16.2 ± 1.8 vs 17.5 ± 1.8 , mean $\pm$ SEM, $n = 36$ units/9 mice/group, $p > 0.05$) and the CA1_{PN} (3.7 ± 0.52 vs 3.7 ± 0.58 , mean $\pm$ SEM, $n = 36$ units/9 mice/group, $p > 0.05$; Fig. 3*H,G*) were identical to those in the age-matched controls. In the AD/ECII_{PN}^{DD−} mice, action potential firing in both the CA1_{PV} and CA1_{PN} was comparable with that in the age-matched controls. Together, these data demonstrate that the inhibition of DAPK1 by selective deletion of either the DAPK1 CD or DAPK1 DD within the ECII_{PN} prevents the decay of the ECII_{PN}-CA1_{PV} synaptic transmission and hence restores the excitatory and inhibitory balance in the CA1 circuits of the AD mice.

Improvements in spatial learning and memory in AD mice

We next determined whether preventing the synaptic impairments via the inhibition of DAPK1 in the ECII_{PN} improves spatial learning and memory in the AD mice. We analyzed the task performance of mice at 180 ± 5 d of age in a hidden version of the Morris Water Maze test. The AD/ECII_{PN}^{CD−} mice showed better performance in all the measured indices (Fig. 4*A–G*); the latency and swim length to reach a hidden platform during the training session (Fig. 4*A–G*), and the percentage of time spent in search of a hidden platform in each quadrant during the probe trial (Fig. 4*D–G*) were comparable between the AD/ECII_{PN}^{CD−} mice (latency = 16.9 ± 1.7 s; length = 192 ± 21 cm; percentage time = 37 ± 3.5) and the control AD/ECII_{PN}^{CD+} mice (latency = 15.7 ± 1.3 s; length = 183 ± 19 cm; percentage time = 35.5 ± 3.6). These data indicate that the inhibition of DAPK1 effectively averts the decay of spatial learning and memory in the AD mice. Consistently with this conclusion, the AD/ECII_{PN}^{DD−} mice with a deletion of the DAPK1 DD in the ECII_{PN} also exhibited significant improvements in acquiring spatial information during the Morris Water Maze tests (latency = 15.0 ± 1.6 s; length = 191 ± 18 cm; percentage time = 38 ± 3.2 in the AD/ECII_{PN}^{DD−} mice vs latency = 17.1 ± 1.6 s; length = 189 ± 20 cm; percentage time = 34.9 ± 3.3 in the AD/ECII_{PN}^{ChR2+} mice; Fig. 4*H,I*). To determine the specific effects of DAPK1 inhibition on spatial learning and memory, we also performed learning-unrelated behavioral tests, including open field, rotarod, elevated plus maze, and forced swimming. Our data revealed that all the groups of mice, including the AD mice at 180 ± 5 d of age, performed normally in all of the learning-unrelated tests, compared with the age-matched controls (Fig. 5*A–D*).

Discussion

ECII_{PN} are some of the earliest affected brain cells in AD, and the selective expression of human mutant APP in the ECII_{PN} of mice impairs spatial learning and memory (Harris et al., 2010). Consistently, our data revealed that DAPK1 was selectively activated in the ECII_{PN} of AD mice and that the genetic inhibition of DAPK1 effectively protected against impairments in the ECII_{PN}-CA1_{PV} synaptic transmission and improved spatial learning and memory.

The key findings in the present study include the following: (1) DAPK1 is selectively activated in ECII_{PN} in the early stages of AD mice; and (2) the specific inhibition of DAPK1 in the ECII_{PN} of AD mice results in therapeutic effects against declines in spatial learning and memory. DAPK1 is a Ca²⁺/calmodulin-dependent protein kinase and was originally

identified by functional cloning on the basis of its involvement in interferon- γ -induced apoptosis (Shohat et al., 2001). Previously, we have reported that DAPK1 is activated in central neurons and contributes to neuronal death (Tu et al., 2010). More recently, we have reported that activated DAPK1 directly binds and phosphorylates the Tau protein on Ser262 and induces synaptic degeneration (Pei et al., 2015). Hyperphosphorylation of Tau (tau inclusions, pTau) results in the self-assembly of tangles of paired helical filaments and straight filaments in the brain, which are involved in the early pathogenesis of AD (Alonso et al., 2001; Clavaguera et al., 2009; Hoover et al., 2010; de Calignon et al., 2012; Spire-Jones and Hyman, 2014). In the present study, we found that genetic deletion of the DAPK1 DD in the AD/ECII_{PN}^{DD−} mice disrupted binding of DAPK1 to the Tau protein in ECII_{PN}. Thus, the DAPK1-Tau interaction may be a crucial signaling event underlying the early degeneration of excitatory synaptic transmission at ECII_{PN}-CA1_{PV} synapses and may be a promising target for therapeutic interventions to treat disease progression.

The present study analyzed task performance in a hidden version of the Morris Water Maze test and revealed that AD mice exhibit spatial learning and memory defects beginning at 6 months of age (Hsiao et al., 1996; Langston et al., 2010). Previous studies have reported that AD mice show deficits in task performance at 9 months of age (Hsiao et al., 1996) or after 14 months of age (Holcomb et al., 1999). However, several other studies have described the presence of behavioral deficits in AD mice as early as 3–6 months of age (King and Arendash, 2002; Lindner et al., 2006; Fritsch et al., 2010). The discrepancies among these studies may be due to differences in the experimental paradigms and in the genetic backgrounds of the mice used in the different laboratories. For example, some studies used a training schedule with 9 trials per day for 6 d, whereas others applied 10 trials per day for 4 d. The genetic background of the AD mice also affects the outcome of behavioral tests. In the present study, the AD mice and the nontransgenic controls were housed under the same conditions and were derived from the same litters; therefore, these parameters should not affect our conclusion that the activation of DAPK1 in ECII_{PN} contributes to impairments in spatial learning and memory in AD mice.

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