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 Pi,2,5 Dan Liu,3,5 Yangling Mu,1,5 Qing Tian,4,5 Ling-Qiang Zhu,4,5 and Youming Lu1,5

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 ECIIPN of AD mice. Inhibition of DAPK1 by deleting a catalytic domain or a death domain of DAPK1 rescues the ECIIPN-CA1PV synaptic loss and improves spatial learning and memory in AD mice. This study demonstrates that activation of DAPK1 in ECIIPN 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

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 (Aβ) 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; Kamenetz 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 Aβ 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).
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(NP)), 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(P) are largely distributed in the EC layer II (ECP(NP)) and III (ECP(NP)) 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 EC(P) 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 ECP(P) synapses remain unknown. Our present studies demonstrate that death-associated protein kinase 1 (DAPK1) became activated selectively in the ECP(NP) of AD mice. We show that activation of DAPK1 is responsible for a selective degeneration of ECP(P)-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 ± 1°C) and humidity (50 ± 5%). Tg2576-AP mice (the AD mice), which express a mutant form of β-amyloid precursor protein (APP) (isofrom 695) with the Swedish mutation (KM670/671NL), were purchased from the 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/ECP(NP)DAPK1mice, we expressed ChR2-eGFP in the ECP(NP) of mutant mice that had a loxp-flanked STOP sequence followed by ChR2 (E123A)-eGFP. The Rosa-CAG-Flag-Chr2-eGFP-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 polyclon-A signal, and an attB-flanked PGK-FRT-Neo-poly-A cassette. This entire construct was inserted into the Gt(ROSA)26Sor locus via electroporation into C57BL/6J embryonic stem cells (ES cells). The targeted ES cells were selected and injected into C57BL/6 blastocysts, and chimeric animals were then bred to C57BL/6 (ChR2loxPloxP/mice) to generate the AD/ECP(NP)DAPK1mice. To generate the AD/ECP(NP)DAPK1mice, we created a mutant strain of DAPK1(CD) in an ECP(NP) transgenic background. DAPK1(CD) mice were crossed with the AD/ECP(NP)DAPK1mice, in which the DAPK1DD was selectively deleted in the ChR2loxPloxPmice. To generate the AD/ECP(NP)DAPK1mice, we created a mutant strain of mice with a selective deletion of the death domain (DD) of DAPK1 (DAPK1DDloxPloxP/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 recombinated allele containing the floxed DAPK1 allele and the Neo selection cassette. These mice were mated with Fpl recombinase-expressing C57BL/6J Flp mice to remove the Neo resistance cassette and to generate a line of Neo-excised DAPK1loxPloxPloxPloxPmice. These mice were bred with the AD/ECP(NP)ChR2loxPloxPmice, in which the offspring were AD/ECP(NP)DAPK1loxPloxPloxPloxPmice, in which the DAPK1 DD was selectively deleted in the ChR2loxPloxPloxPloxPmice.

Single-cell Western blots, kinase assays, and coimmunoprecipitation. EC tissues were isolated from the AD/ECP(NP)DAPK1loxPloxPloxPloxPmice, the AD/ECP(NP)DAPK1loxPloxPloxPloxPmice, and control mice, in which the DAPK1 DD was selectively deleted in the ChR2loxPloxPloxPloxPmice. The isolated ECP(NP) were homogenized and diluted with a buffer containing 200 mU Tris-Cl, pH 7.6, 50 mM NaF, 1 mM Na2VO3, 1 mM edetic acid, 1 mM benzamidine, 1 mM PMFS, 1 mg/10 ml papain, and a mixture of aprotinin, leupeptin, and pepstatin A (10 μg/ml each) for 30 min. The cell extracts were then diluted with nonionic detergent (0.1% Igepal and 0.5% sodium deoxycholate) and an aliquot was automatically isolated using an S3e Cell Sorter (Bio-Rad). The isolated ECP(NP) were homogenized and diluted with a buffer containing 200 mU 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% bromphenol 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 onto nitrocellulose membranes using a Bio-Rad transblot apparatus. The membranes were washed three times with TBST buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) and incubated with anti-mouse primary antibodies overnight at 4°C. The membranes were washed three times with TBST buffer, incubated with the appropriate secondary antibodies (1:1000 dilution) 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), anti-phospho-MLC (1:500, Epitomics, catalog #3798-1). The band densities were quantitatively analyzed 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 Ser18/Thr20 by MLCK. DAPK1 was separated from the same conditions. Immunoprecipitation was used to determine the catalytic activity of DAPK1 with the same substrates. The lysates (~200 μg protein) were incubated with non-specific 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 CA1pv, brain slices from the AD mice, in which mCherry was expressed in the CA1pv cells was visualized using fluorescent IR-DIC using an Axioskop 2FS equipped with Hamamatsu C2400–07E optics. When stable whole-cell recordings were achieved with good access resistance (~20 MΩ), basic electrophysiological properties were recorded. All 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 ECIIPN, which had normal intrinsic properties in AD/ECIIpvChR2+ (resting membrane potentiation = 69.3 ± 2.1 mV; input resistance = 386 ± 21 MΩ, mean ± SEM, n = 36 cells/6 mice), AD/ECIIpvChR2+CD (resting membrane potentiation = 68.7 ± 2.0 mV; input resistance = 389 ± 19 MΩ, mean ± SEM, n = 33 cells/5 mice), and AD/ECIIpvChR2- mice (resting membrane potentiation = 69.9 ± 1.9 mV; input resistance = 401 ± 17 MΩ, mean ± 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 MΩ, mean ± 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 (antero-posterior 1.7 mm, mediolateral 1.0 mm, and dorsoventral 0.5–1.7 mm target to CA1). Mice were connected to the recording equipment via AC-coupled unity-gain operational amplifiers (Plexon). Signals were recorded simultaneously and isolated by using a 250 Hz 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 ECIIPN, which had normal intrinsic properties in AD/ECIIpvChR2+ (resting membrane potentiation = 69.3 ± 2.1 mV; input resistance = 386 ± 21 MΩ, mean ± SEM, n = 36 cells/6 mice), AD/ECIIpvChR2+CD (resting membrane potentiation = 68.7 ± 2.0 mV; input resistance = 389 ± 19 MΩ, mean ± SEM, n = 33 cells/5 mice), and AD/ECIIpvChR2- mice (resting membrane potentiation = 69.9 ± 1.9 mV; input resistance = 401 ± 17 MΩ, mean ± 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 MΩ, mean ± SEM, n = 32 cells/5 mice).

Mice were allowed to stay there for 30 s; if an animal did not find the platform 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. 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. Data analysis. All variance values in the text and figure legends are represented as the mean ± 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 ECIIPN of AD mice
To determine the molecular mechanisms underlying the selective degeneration of ECIIPN synapses in AD mice, we focused our studies on DAPK1, an enzyme cytoxic for synaptic losses in central neurons (Tu et al., 2010). To functionally and morphologically identify individual ECIIPN, we generated AD/ECIIpvChR2+ mice, in which channelrhodopsin-2-El213A (ChR2), a modified version of a light-gated cation channel, and an eGFP were selectively expressed in the vulnerable ECIIPN of the AD mouse. A mouse model of AD was chosen because these mice carry a transgene coding for the 69S amino acid isoform of the human Alzheimer β precursor protein with the Swedish mutation and exhibit plaque pathologies similar to those in AD patients.

Specifically, we constructed a conditional line of mutant mice that expressed a double-floxed inverted open reading frame of ChR2-eGFP (ChR2loxP/loxP mice; Fig. 1A). We also constructed a Type 1/2 recombinant adenovirus 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 ECIII of the AD/ChR2loxP/loxP mice, resulting in the ChR2-eGFP expression specifically in the ECIIPN (Fig. 1B). ECIIPN cells were named as island cells, in which Wfs1 is expressed (Kitamura et al., 2014). Consistent with this previous study, we showed that ECIIPN that directly innervate CA1pv were labeled with an antibody against Wfs1 protein (Fig. 1C). Thus, ECIIPNChR2+ neurons comprise a group of excitatory island cells.

We next isolated ECIIPNChR2+ cells from the brain tissues of the AD/ECIIpvChR2+ mice using flow cytometry and cell sorting techniques. To determine the catalytic activity of DAPK1 in the purified ECIIPNChR2+ 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 ECIIPNChR2+ cells from the AD/ECIIpvChR2+ mice and the controls (the control/ECIIpvChR2+ 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 ECIIPNChR2+ cells from the AD/ECIIpvChR2+ mice after 150 d of age (Fig. 1D), compared with the age-matched control/ECIIpvChR2+ mice (Fig. 1E). The pMLC was catalyzed by an activated DAPK1 as MLA kinase (MLCK) that also targets MLC at serine-20 was undetectable in the precipitates from the ECIIPNChR2+ cells of the AD/ECIIpvChR2+ mice (Fig. 1F). An increase of the pMLC was found in the ECIIPNChR2+ cells, but not in the frontal cortical neurons from the AD/ECIIpvChR2+.
mice (Fig. 1). Together, these data demonstrate that DAPK1 is activated selectively in ECIIPN cells of AD mice during aging.

Genetic inhibition of DAPK1 in ECIIPN of AD mice

We next determined whether activation of DAPK1 in the ECIIPN of AD mice contributes to impairments in synaptic transmission along the ECIIPN-CA1PV pathway. We developed two independent approaches to inhibiting DAPK1 function in the ECIIPN of AD mice. First, we generated a mutant strain of mice (DAPK1CDloxP/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/ECIIPNChR2/H11001 mice, which Cre recombinase was expressed in the ECIIPNChR2/H11001 cells (for details, see Fig. 1B), the CD of DAPK1 (DAPK1CD/H11002) was selectively deleted in the ECIIPNChR2/H11001 cells of the offspring (AD/ECIIPNCD/H11002 mice; Fig. 2C–E). To verify the successful generation of AD/ECIIPNCD/H11002 mice, we performed two independent lines of the studies. First, we stained the sections from the AD/ECIIPNCD/H11002 mice with antibody against D28K protein and showed that the DAPK1CD/H11002 mutant protein in the ECIIPNChR2/H11001 cells was recognized by anti-D28K (Fig. 2D). Second, we isolated the ECIIPNChR2/H11001 cells from the AD/ECIIPNCD/H11002 mice using flow cytometry and cell sorting techniques. The cell lysates were prepared from the isolated ECIIPNCD/H11002 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 ECIIPNChR2/H11001 cells of the AD/ECIIPNCD/H11002 mice (Fig. 2E).

Next, we generated the mutant mice (DAPK1DDloxP/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...
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$_{PN}$ DAPK1$^{DD-}$ 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$_{PN}$ DAPK1$^{DD-}$ mice with antibody against N-terminal fragment of DAPK1 (N-DAPK1; Fig. 2H). We next examined the enzymatic activity of DAPK1$^{DD-}$ mutant protein. We isolated the ECII$_{PN}$ ChR2$^+$ cells from the AD/ECII$_{PN}$ DAPK1$^{DD-}$ 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 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 stereotaxically injected with the rAAV1/2-D28K-Cre virus particles into the ECII region, resulting in the AD/ECII$_{PN}$ DAPK1$^{DD-}$ mice. D, Representative images showing the ECII$_{PN}$ of the AD/ECII$_{PN}$ DAPK1$^{DD-}$ mice that expressed the DAPK1$^{DD-}$ 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$_{PN}$ DAPK1$^{DD-}$ 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. M, An antibody against the N-terminal region of DAPK1 (N-DAPK1) recognizes the wild-type (~160 kDa) and mutant proteins (~140 kDa). Similar results were seen in each of the five experiments. J, 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 $\mu$g of protein from the ECII$_{PN}$ cell lysates without precipitation was loaded. Similar results were seen in each of the four experiments.
Figure 3. Inhibition of DAPK1 protects against the ECIIPN-CA1PV synaptic decay in AD mice. A, Illustration (top) represents the recording configurations. The mean amplitudes of the evoked NMDA receptor EPSCs in the CA1pv at a holding potential of 60 mV are plotted versus the light intensity increments (0.5 mV intervals) of stimulation of ECIIPN ChR2/H11001. 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 ECIIPN 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), ECIIPN form direct excitatory synapses with CA1PV and balance the excitatory/inhibitory synaptic transmission in CA1 circuits. During the disease progression of AD (E), ECIIPN-CA1PV synapses are degenerated. This degeneration disables the excitatory and inhibitory balance as a consequence of a loss of inhibitory inputs from CA1pv to CA1pv. Inhibition of DAPK1 in the ECIIPN (F) effectively intervenes in the degeneration of ECIIPN-CA1PV 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 CA1pv (G) and CA1pn (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 CA1pv (G) versus CA1pn (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 CA1pv (G) and CA1pn (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).
but this deletion blocked the binding of DAPK1 to DAPK1 substrates, such as the Tau and ERK proteins (Fig. 2).

**Protection against ECIIPN synaptic decay in AD mice**

To determine whether the genetic inhibition of DAPK1 might avert the ECIIPN synaptic decay in AD mice, we performed whole-cell patch-clamp recordings with the CA1PV (Fig. 3A) from the AD/ECII\textsubscript{PN} CD\textsuperscript{+} and AD/ECII\textsubscript{PN} DD\textsuperscript{−} mice at 180 ± 5 d of age as well as age-matched controls (AD/ECII\textsubscript{PN} \textsuperscript{ChR2+}, the control/ECII\textsubscript{PN} DD\textsuperscript{−}, and the control/ECII\textsubscript{PN} \textsuperscript{ChR2+} mice). EPSCs were evoked by delivering blue laser light directly to the ECIIPN in brain slices. To exclude disturbances from other projections (i.e., the ECIIPN-DG-CA3-CA1PV 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 presence of 50 μM D-101 to saturate AMPA receptors.
ence of 20 μM CNQX and 20 μM 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 ECIIPN and CA1PV 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 AD mice did not alter synaptic transmission in the control mice (the peak values of the mean amplitudes were 99 ± 13 pA in the control/ECII_PN^ChR2_+ mouse vs 92 ± 15 pA in the control/ECII_PN^CD− mouse), but it was effective in reversing the ECII_PN−CA1PV synaptic decline in the AD mice (peak values of the mean amplitudes were 65 ± 8 pA in the AD/ECII_PN^ChR2_+ vs 93 ± 9 pA in the AD/ECII_PN^CD− mouse).

Next, we examined the structure of ECII_PN−CA1PV synapses in the AD/ECII_PN^CD− mouse (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 μm × 200 μm) in the AD mice at 180 ± 5 d of age was 29.1 ± 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 μm segment) from the antibody-labeled CA1PV cells (50 segments per animal) in the stratum lacunosum-moleculare region of the AD mice decreased by 35.1 ± 3.9% compared with those in the age-matched controls (5.3 ± 0.63 vs 3.3 ± 0.36, mean ± SEM, n = 50 dendritic branches/5 mice/group, p < 0.001; Fig. 3C). The inhibition of DAPK1 completely rescued the synaptic loss of the CA1PV in the AD mice (5.1 ± 0.59 in the AD/ECII_PN^CD− mice, 4.9 ± 0.62 in the AD/ECII_PN^DD− mice vs 5.2 ± 0.58 in the controls, mean ± SEM, n = 50 dendritic branches/5 mice/group, p > 0.05; Fig. 3C).

Impairments of synaptic transmission along the ECII_PN−CA1PV excitatory pathway, which primarily target the dendrites of CA1 excitatory pyramidal neurons (CA1PN) 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−CA1PV 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 ± 5 d of age by using extracellular single-unit recording techniques (Yang et al., 2016). Action potentials that originated from the CA1PN versus the CA1PV of freely moving mice were classified on the basis of the properties of the action potentials, as recently described (Yang et al., 2016). In the AD mice, the probability of action potential firing was dramatically reduced in the CA1PV (11.8 ± 1.6 vs 16.9 ± 1.9, mean ± SEM, n = 36 units/9 mice/group, p < 0.001; Fig. 3G) and increased in the CA1PN (5.6 ± 0.68 vs 3.3 ± 0.51, mean ± SEM, n = 36 units/9 mice/group, p < 0.001; Fig. 3H), compared with the age-matched controls.

**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 ± 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 ± SEM, n = 8 mice per group). C, Mice are normal in elevated plus maze tests. Bar graphs represent that the performance for foot stay and fault on rotarod tests is comparable between genotypes (mean ± SEM, n = 8 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 ± SEM, n = 9 mice per group).
control mice, thus showing that the excitatory and inhibitory balance was disrupted in the CA1 circuits of the AD mice. In the AD/ECCI\textsubscript{pN}\textsuperscript{CD-} mice, in which DAPK1 was inactivated specifically in the ECCI\textsubscript{pN}, the excitatory and inhibitory balance in the CA1 circuits was restored; the probabilities of action potential firings in both the CA1\textsubscript{pV} (16.2 ± 1.8 vs 17.5 ± 1.8, mean ± SEM, n = 36 units/9 mice/group, p > 0.05) and the CA1\textsubscript{pV} (3.7 ± 0.52 vs 3.7 ± 0.58, mean ± SEM, n = 36 units/9 mice/group, p > 0.05; Fig. 3 H, G) were identical to those in the age-matched controls. These data are consistent with this conclusion, the AD/ECCI\textsubscript{pN}\textsuperscript{CD-} mice, action potential firing in both the CA1\textsubscript{pV} and CA1\textsubscript{pV} was comparable with that in the age-matched controls. Together, these data demonstrate that the inhibition of DAPK1 by selective deletion of the DAPK1 DD in the ECIIPN also exhibited significant improvements in acquiring spatial information during the probe trial (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/ECCI\textsubscript{pN}\textsuperscript{CD-} mice (latency = 16.9 ± 1.7 s; length = 192 ± 21 cm; percentage time = 37 ± 3.5) and the control AD/ECCI\textsubscript{pN}\textsuperscript{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/ECCI\textsubscript{pN}\textsuperscript{DD-} mice with a deletion of the DAPK1 DD in the ECCI\textsubscript{pN} also exhibited significant improvements in acquiring spatial information during the Morris Water Maze test (latency = 15.0 ± 1.6 s; length = 191 ± 18 cm; percentage time = 38 ± 3.2 in the AD/ECCI\textsubscript{pN}\textsuperscript{DD-} mice vs latency = 17.1 ± 1.6 s; length = 189 ± 20 cm; percentage time = 34.9 ± 3.3 in the AD/ECCI\textsubscript{pN}\textsuperscript{DD+} 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

ECCI\textsubscript{pN} are some of the earliest affected brain cells in AD, and the selective expression of human mutant APP in the ECCI\textsubscript{pN} of mice impairs spatial learning and memory (Harris et al., 2010). Consistently, our data revealed that DAPK1 was selectively activated in the ECCI\textsubscript{pN} of AD mice and that the genetic inhibition of DAPK1 effectively protected against impairments in the ECCI\textsubscript{pN}-CA1\textsubscript{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 ECCI\textsubscript{pN} in the early stages of AD mouse; and (2) the specific inhibition of DAPK1 in the ECCI\textsubscript{pN} of AD mice results in therapeutic effects against declines in spatial learning and memory. DAPK1 is a Ca\textsuperscript{2+}/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; Spires-Jones and Hyman, 2014). In the present study, we found that genetic deletion of the DAPK1 DD in the AD/ECCI\textsubscript{pN}\textsuperscript{DD-} mice disrupted binding of DAPK1 to the Tau protein in ECCI\textsubscript{pN}. Thus, the DAPK1-Tau interaction may be a crucial signaling event underlying the early degeneration of excitatory synaptic transmission at ECCI\textsubscript{pN}-CA1\textsubscript{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). These 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 ECCI\textsubscript{pN} contributes to impairments in spatial learning and memory in AD mice.

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