Neuropathogenic role of adenylate kinase-1 in Aβ-mediated tau phosphorylation via AMPK and GSK3β

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Abnormally hyperphosphorylated tau is often caused by tau kinases, such as GSK3β and Cdk5. Such occurrence leads to neurofibrillary tangle formation and neuronal degeneration in tauopathy, including Alzheimer’s disease (AD). However, little is known about the signaling cascade underlying the pathologic phosphorylation of tau by Aβ. In this study, we show that adenylate kinase 1 (AK1) is a novel regulator of abnormal tau phosphorylation. AK1 expression is markedly increased in the brains of AD patients and AD model mice and is significantly induced by Aβ in the primary neurons. Ectopic expression of AK1 alone augments the pathologic phosphorylation of tau at PHF1, CP13 and AT180 epitopes and enhances the formation of tau aggregates. Inversely, downregulation of AK1 alleviates Aβ-induced hyperphosphorylation of tau. AK1 plays a role in Aβ-induced impairment of AMPK activity and GSK3β activation in the primary neurons. Pharmacologic studies show that treatment with an AMPK inhibitor activates GSK3β, and a GSK3β inhibitor attenuates AK1-mediated tau phosphorylation. In a Drosophila model of human tauopathy, the retinal expression of human AK1 severely exacerbates rough eye phenotype and increases abnormal tau phosphorylation. Further, neural expression of AK1 reduces the lifespan of tau transgenic flies. Taken together, these observations indicate that the neuronal expression of AK1 is induced by Aβ to increase abnormal tau phosphorylation via AMPK-GSK3β and contributes to tau-mediated neurodegeneration, providing a new upstream modulator of GSK3β in the pathologic phosphorylation of tau.

INTRODUCTION

Alzheimer’s disease (AD) is the most common neurodegenerative disease and is characterized as a slow loss of cognitive function, leading to dementia and death (1). The neuropathologic hallmarks of AD include extracellular senile plaques composed of amyloid-beta (Aβ) and intracellular neurofibrillary tangles (NFTs) composed of the microtubule-associated protein tau (2,3). Aβ is derived from the amyloid precursor protein cleaved by β- and γ-secretases (4). Tau plays a role in promoting the assembly and maintenance of microtubules and its biologic function is regulated by phosphorylation. Under pathologic conditions, such as AD, tau becomes hyperphosphorylated, resulting in reduced affinity to microtubules and assembly into abnormal filaments (3). According to the amyloid cascade hypothesis, Aβ is the trigger for AD and thus facilitates the development of tau pathology (5). This concept linking Aβ and tau was supported by in vivo studies using AD mouse models. Enhanced neurofibrillary pathology was observed in double-transgenic mice, crossing mutant APP and tau (6), and administration of Aβ into mutant tau mice caused the formation of NFTs (7). Clearing Aβ by passive immunotherapy in 3xTg diminished pre-existing tau pathology (8). These findings strongly showed central roles of Aβ and tau in the pathogenesis of AD.
Abnormal hyperphosphorylation of tau appears to be mediated by the activation of several kinases, such as GSK3β (9), Cdk5 (10), MARK (11), etc. Among them, GSK3β generates pathologic phospho-epitopes on tau and co-localizes with aggregates of tau (12, 13). Until now, genetic evidence using transgenic mice model shows that GSK3β is important for tau-mediated pathology, including tau phosphorylation and aggregation (14). Further, pharmacologic studies utilizing GSK3β inhibitors suggest that GSK3β plays an essential role in tau pathology, thus providing therapeutic opportunity for the prevention of AD (15, 16). Interestingly, the observations that GSK3β activity is enhanced by Aβ in cultured neurons and mouse model indicate that GSK3β plays an important role in Aβ-mediated tau pathology (17, 18). However, how Aβ induces GSK3β activation is unanswered yet.

Adenylate kinases (AK, EC 2.7.4.3) which catalyze the nucleotide phosphoryl exchange reaction (2ADP ↔ ATP + AMP) maintain the consistent concentration and fixed ratio of adenine nucleotides (19). These unique properties of AK function as a sensitive reporter of the cellular energy state and each of them has its distinct tissue and intracellular distribution (20). They are therefore major regulators of energetic, metabolic monitoring and cellular process in a living cell. Although maintaining the homeostasis of the cellular adenine nucleotide pool is critical to metabolic stress (20, 24). However, the role of AK1 to augment the pathologic species, including oligomeric Aβ42, are important factors responsible for tau pathogenesis (4). Therefore, we examined whether oligomeric forms of Aβ42 could regulate AK1 expression in neuronal cells. To assess the effect of Aβ42 on AK1 expression, mouse cortical neurons were cultured and treated with oligomeric Aβ42. From western blot analysis, we found that AK1 expression increased ~2-fold in the cortical neurons after exposure to Aβ42 (Fig. 2A and B). Interestingly, tau phosphorylation, which was detected by PHF-1 (Ser 396/404), CP13 (Ser 202) and 12E8 (Ser 262) antibodies, was also increased by Aβ42 (Fig. 2A). The induction kinetics of time-dependent tau phosphorylation by Aβ42 was similar with that of AK1 expression (Fig. 2C). The increased expression of AK1 by Aβ42 was observed in the NeuN-positive primary neurons (Fig. 2D) and in the neuronal cell lines, such as HT22 mouse hippocampal cells and SH-SY5Y human neuroblastoma cells (Supplementary Material, Fig. S2A). AK1 expression also increased in neuronal cells which were exposed to oxidative stress but not to other toxic insults, such as proteostasis stressors (Supplementary Material, Fig. S2B). Among AK isoforms, AK2 was not detected in the primary neurons and HT22 cells, whereas AK3 expression was observed in the neurons but not regulated by Aβ42 (Fig. 1E). When analyzed by reverse transcription-polymerase chain reaction, the level of AK1 mRNA, not AK1β mRNA, increased by Aβ42 in primary neurons (Supplementary Material, Fig. S2C). Thus, AK1 that is highly expressed in neuronal cells is upregulated by Aβ42.

AK1 regulates hyperphosphorylation and aggregation of tau via its enzyme activity

Soluble oligomers of Aβ42 serve as the prominent synapto-toxic form and induce tau hyperphosphorylation (29, 30). Our observation that Aβ42 increased AK1 expression and tau phosphorylation led us to examine the contribution of AK1 to Aβ42-induced tau phosphorylation. Thus, we directly targeted AK1 expression using siRNA in the primary cortical neurons

RESULTS

Neuronal expression of AK1 is upregulated in AD patients and is induced by Aβ42

Since previous reports showed that energy metabolism-related enzymes were frequently altered in AD (25, 26), we first examined AK1 expression in the brains of the AD model mice, such as Tg2576 (27) and APP-J20 mice (28), and AD patients. We collected paired samples of the hippocampus from Tg2576 mice and their littermates and performed western blot analysis. Compared with the control mice, the AK1 level was higher in the hippocampus of 9-, 12- and 15-month-old Tg2576 mice expressing Swedish mutant of amyloid precursor protein (hAPP) (Fig. 1A and B). AK1 also increased in the hippocampus of APP-J20, an AD model mouse expressing familial AD-mutant APP (Supplementary Material, Fig. S1A and B). With the immunohistochemical analysis, we found that the expression levels of AK1 markedly elevated in the NeuN-positive hippocampal neurons of AD patients (Fig. 1C). Quantitative comparison of AK1 expression level following western blotting showed that AK1 significantly increased in the hippocampus of AD patients (Fig. 1D and E). Interestingly, the phosphorylated form of tau at Ser 396/404 (PHF-1) was detected exclusively in AD patients, although total amounts of tau protein (TG5) were not changed (Fig. 1D). These results indicate that AK1 is upregulated in the brains of AD model mice and patients.

A number of studies have found that Aβ species, including oligomeric Aβ42, are important factors responsible for tau pathogenesis (4). Therefore, we examined whether oligomeric forms of Aβ42 could regulate AK1 expression in neuronal cells. To assess the effect of Aβ42 on AK1 expression,
and examined tau phosphorylation. Compared with control neurons, the effects of Aβ42 on tau phosphorylation at CP13, PHF-1 and AT180 epitopes were significantly ameliorated in AK1 knockdown cortical neurons (Fig. 3A). We obtained similar results in cortical neurons and SH-SY5Y cells in which AK1 expression was downregulated (Supplementary Material, Fig. S3A and B). These observations suggest that AK1 controls Aβ42-induced tau phosphorylation in neuronal cells.

To further characterize the role of AK1 as a novel regulator of tau phosphorylation, we examined the overexpression effects of AK1 on tau phosphorylation. Ectopic expression of AK1 in mouse cortical neurons increased immunoreactivity against phosphorylated tau at Ser396/404 (PHF-1), Ser202 (CP13) and Ser231/Thr235 (AT180) (Fig. 3B). Consistent with these changes, western blotting also showed that ectopic expression of AK1 induced similar patterns of tau phosphorylation in cortical neurons (Fig. 3C) and in SH-SY5Y cells (Supplementary Material, Fig. S4A). On the other hand, AK1 mutants, such as AK1 R132A, R138A and R149A, which are defective in AK activity (31), caused little change in tau phosphorylation (Fig. 3D). Therefore, AK1 regulates tau phosphorylation via its AK activity.

Next, we explored whether AK1 could modulate the aggregation of tau protein in cultured cells. The effect of AK1 on tau solubility was examined using a filter-retardation assay to detect tau aggregates. Compared with SH-SY5Y control cells, the amount of insoluble tau significantly increased in SH-SY5Y/HA-AK1 stable cells but not in SH-SY5Y/HA-AK1 R132A stable cells, as measured by immunoblotting of the filters (Fig. 3E, Fil). The amounts of tau aggregates on the filters showed a good correlation with the levels of tau hyperphosphorylation on western blot in SH-SY5Y/HA-AK1 stable cells (Fig. 3E, Wes). Consistently, no significant increase in tau aggregates was observed in SH-SY5Y cells expressing AK1 R149A mutant (Supplementary Material, Fig. S4B and C). Furthermore, from fractionation assays using sarkosyl, we found that the amount of sarkosyl-insoluble GFP-htau increased in SH-SY5Y/HA-AK1 stable cells, whereas sarkosyl-soluble GFP-htau decreased (Fig. 3F). These changes were not observed in SH-SY5Y/HA-AK1 R132A stable cells. The data suggest that the increased expression of AK1 can enhance tau aggregation as well as tau phosphorylation.

AK1 impairs AMPK by regulating AMP/ATP ratio in neurons

To gain insight into how AK1 affects tau phosphorylation, we focused on AMPK since AK1 could regulate metabolic sensors, such as AMPK, by reading cellular adenine nucleotide balance (20). We were eager to know whether AMPK

**Figure 1.** Increased expression of AK1 in the brains of AD patients and Tg2576 mice. (A) AK1 expression is increased in the brains of Swedish mutant APP Tg2576 mice. Hippocampal lysates obtained from 9-, 12- and 15-month-old Tg2576 mice or age-matched littermates were subjected to western blotting with AK1, APP and β-actin antibodies. β-actin was used as a control for the consistency of protein loading. (B) Densitometric analysis of AK1. AK1 levels on the blots shown in (A) were measured by densitometric analysis and normalized by β-actin. Bars depict mean values ± SD. **P < 0.005; ***P < 0.0001. (C) Immunohistochemical analysis showing the increased levels of AK1 in the hippocampal tissue of AD patients. Hippocampal tissues of controls and AD patients were immunostained with AK1 and NeuN antibodies. Bar, 10 μm. (D) AK1 expression is elevated in the hippocampus of AD patients. Hippocampal lysates of controls and AD patients were analyzed with western blotting using the indicated antibodies. (E) Densitometric analysis of AK1 levels normalized by β-actin on the blots shown in (D). *P < 0.05.
was regulated by Aβ42. Interestingly, treatment with Aβ42 decreased the phosphorylation of AMPK at Thr172, whereas the total amount of AMPK was not altered in primary cortical neurons (Fig. 4A and B) and SH-SY5Y cells (Supplementary Material, Fig. S5A). Consistently, the phosphorylation of acetyl Co-A carboxylase, a well-defined downstream substrate of AMPK, was also reduced by Aβ42 (Fig. 4A and B). We found, in addition, that enzyme activity of AMPK was suppressed in the cortical neurons after exposure to Aβ42 (Fig. 4C). These results indicate that AMPK activity is impaired by Aβ42 in the neuronal cells in which tau phosphorylation increased. Moreover, the phosphorylation of AMPK at Thr172 tended to be reduced in AD patients, although statistical significance was marginal (Supplementary Material, Fig. S5B and C; P = 0.05).

We then addressed the influence of AK1 on the regulation of AMPK. Compared with control cells, AK1 overexpression markedly decreased AMPK phosphorylation at Thr172 in the primary neurons, whereas AK1 R132A mutant failed to do so (Fig. 4D and E). These results raise a possibility that AK1 exerts an inhibitory effect on AMPK. However, there was no direct protein–protein interaction between AK1 and AMPK subunits (Supplementary Material, Fig. S5D). As AK1 catalyzes the conversion of adenine nucleotides in the cytosol, we hypothesized that an imbalance in adenine nucleotide levels is caused by the abnormally increased expression of AK1, which may lead to the dysregulation of AMPK. To define the alterations in adenine nucleotide levels, we assessed nucleotide levels in the cortical neurons in which AK1 was overexpressed. The relative ratio of AMP/ATP was markedly lower in the cortical cells expressing AK1 (but not in AK1 R132A mutant) than control cells (Fig. 4F). The reduction in the ratios of AMP/ATP by AK1 was also observed in SH-SY5Y cells (Supplementary Material, Fig. S5E). Together,
our data indicate that AK1 may impair AMPK through the downregulation of AMP/ATP ratio.

**AK1 controls Aβ42-induced GSK3β activation via AMPK**

In addition, we found that transient expression of AK1 (but not AK1 R132A mutant) markedly reduced the inhibitory phosphorylation of GSK3β at Ser9 (Fig. 4D and Supplementary Material, S6A), indicating that AK1 may regulate GSK3β activity. Therefore, we tested whether or not AK1 regulated AMPK and GSK3β in the neuronal cells exposed to Aβ42. We generated SH-SY5Y/AK1 knockdown (shAK1) stable cells and examined the regulation of these kinases by AK1. As shown in Figure 5A and B, treatment of control...
cells with Aβ42 reduced the levels of AMPK phosphorylation at Thr172 and the inhibitory phosphorylation of GSK3β at Ser9. On the contrary, these alterations triggered by Aβ42 were not observed in SH-SY5Y/AK1 knockdown cells, showing no changes in the levels of the phosphorylated AMPK and GSK3β. These results suggest that AK1 plays a crucial role in the regulation of AMPK and GSK3β in the neuronal cells exposed to Aβ42.

With the notion that AMPK may inhibit GSK3β activity (32), we tested whether AMPK regulates GSK3β and tau phosphorylation. The treatment of cortical neurons with compound C, a selective AMPK inhibitor (33), reduced the inhibitory phosphorylation of GSK3β at Ser9 and markedly increased tau phosphorylation at Ser396/404 (Fig. 5C). Conversely, treatment with AICAR (5-aminomidazole-4-carboxamide ribonucleoside), an AMPK activator (34), increased the inhibitory phosphorylation of GSK3β and reduced tau phosphorylation at Ser396/404 in SH-SY5Y cells and cortical neurons (Supplementary Material, Fig. S6B and C). These observations indicate that AMPK negatively regulates GSK3β activity and tau phosphorylation. Further, we found that tau phosphorylation at Ser 396/404 triggered by AK1 overexpression was significantly inhibited in cortical neurons by SB-415286, a GSK3β inhibitor (Fig. 5D), suggesting that AK1 increases tau phosphorylation at Ser 396/404 in a GSK3β-dependent manner. On the other hand, treatment with roscovitine, a Cdk5 inhibitor, had no significant effect on the tau phosphorylation (Fig. 5D) and there was no proteolytic cleavage of p35, a Cdk5 regulatory protein, by transient expression of AK1 in primary neurons (Supplementary Material, Fig. S6A). We also observed that GSK3β activation triggered by AK1 overexpression was attenuated by AICAR.
flies with tau rough eye flies (gl-tau2.1) (35). Compared with sing human cytoplasmic AK1 (UAS-AK1) and crossed the Drosophila model organism, we generated transgenic To assess the neuropathogenic role of AK1 in a tauopathy tau-mediated neural degeneration in flies Increased expression of AK1 enhances human control flies (gmr-GAL4) (Fig. 6Aa), there was no obvious phosphorylation. (Supplementary Material, Fig. S7), indicating that AK1 controls GSK3β activation via AMPK.

**Increased expression of AK1 enhances human tau-mediated neural degeneration in flies**

To assess the neuropathogenic role of AK1 in a tauopathy model organism, we generated transgenic Drosophila expressing human cytoplasmic AK1 (UAS-AK1) and crossed the flies with tau rough eye flies (gl-tau2.1) (35). Compared with control flies (gmr-GAL4) (Fig. 6Aa), there was no obvious phenotypic difference in the appearance on the eyes of flies overexpressing AK1 (Fig. 6Ad). The flies with the targeted retinal expression of tau (gl-tau2.1) showed moderate toxicity, characterized by the loss of photoreceptor neurons, leading to observation in adults as a rough surface (Fig. 6Ab). Co-expression of an AK1 transgene with tau in flies (gl-tau2.1/UAS-AK1) markedly enhanced tau-induced retinal degeneration (Fig. 6Ac), indicating that AK1 enhances tau toxicity in the fly retina. In addition, from western blot analysis of fly brains, we found that tau phosphorylation at PHF-1, CP13 and AT180 epitopes highly increased in the flies co-expressing tau and AK1 compared with tau transgenic flies (Fig. 6B). Quantification analysis revealed an increase of ~3-fold in the phosphorylation at CP13, PHF-1 and AT180 epitopes, and 1.5-fold at 12E8 epitope in the flies co-expressing tau and AK1 (Fig. 6C). These results indicate that AK1 exacerbates rough eye phenotype and tau hyperphosphorylation in a tauopathy model organism and that there is a close correlation between the exacerbated rough eye phenotype and tau phosphorylation.

Further, we raised adult transgenic flies which expressed tau, AK1, or both tau and AK1 in a pan-neural pattern using elav-GAL4 driver and followed their survivals on standard food. The flies overexpressing AK1 show similar lifespan with control flies (average 45 days) (Fig. 6D). Consistent with the previous report (36), the flies over-expressing tau (TaulWT) showed reduced lifespan (average 34 days) compared with control flies, whereas the flies co-expressing tau and AK1 (TaulWT/AK1) had 25% shorter lifespan (average 26 days) than tau flies. These results indicate that AK1 also promotes the neurotoxicity of tau in a fly model. Together, these results suggest that upregulated AK1 plays a crucial role in the abnormal hyperphosphorylation of tau and tau-mediated neurodegeneration in a model organism.

**DISCUSSION**

Intracellular accumulation of hyperphosphorylated tau and neurofibrillary degeneration are hallmark lesions in tauopathies. In AD, especially, the relationship between amyloid accumulation and neurofibrillary degeneration is a major unresolved question in that Aβ42 affects tau pathology and tau is required for Aβ42-induced neurotoxicity in cultured cells and transgenic mouse models (6–8). In this study, we placed AK1 as a crucial player in Aβ42-mediated tau phosphorylation and tau-associated pathology. AK1 is originally known to be a major cytosolic AK catalyzing the nucleotide phosphoryl exchange especially in skeletal muscle. AK1 knockout mice showed diminished AMP production during muscle contraction, although AMP contents were not changed or increased in AK1-deficient mice in resting muscle condition (20,24,37). Thus, the role of AK1 in AMP dynamics may differ in the distinct states of energy metabolism. Our observation that the increased AK1 decreases the AMP/ATP ratio in neuronal cells further shows tissue-specific role of AK1 in altering AMP dynamics. Like the change in the muscle tissue of AK1 knockout mice, it will be interesting to determine whether AMP contents are also altered in the brain of AD patients.
AMPK also functions in the maintenance of energy metabolism (38). A large body of literature has shown that AMPK acts as a neuroprotective factor under several pathologic conditions, and AMPK deficiency causes neurodegeneration in Drosophila (38,39). In this regard, the AMPK-activating hormone, leptin, reduces tau phosphorylation in the neuronal cell lines (40). On the other hand, a recent report proposed the detrimental aspects of AMPK, showing that AMPK is a tau kinase and is activated by aggregated Aβ42 (41). Despite those contradictory results in the roles of AMPK, our observations that AMPK is impaired by oligomeric Aβ42 to activate GSK3β and to induce tau hyperphosphorylation provide a mechanistic basis for the function of AMPK in tau phosphorylation under pathologic conditions (29,30).

How then does AK1 regulate AMPK? Though AK1 usually regulates the equilibrium state of nucleotides, we believe that AK1 activity seems not to be overtly linked to tau phosphorylation in the range below the natural and endogenous AK1 levels. AK1 knockdown in neuronal cells did not affect the nucleotide ratios (data not shown), as well as tau phosphorylation. Lack of such relation in AK1 deficiency may also be associated with a range of compensatory changes in energy metabolism as previously reported (20,24). On the other hand, we observed that abnormally overexpressed AK1 could change AMP/ATP ratio and dysregulate AMPK to enhance tau phosphorylation in the differentiated cortical neurons. Likewise, enhancement of AK1 is connected with AMPK signaling in metabolic stress response and cardiac stem cell differentiation (20,42). Unlike AK1 in cardiac cells, however, upregulated AK1 in neuronal cells may distinctly dysregulate AMPK in the neuron-specific compartments such as dendrites and axons of the differentiated neurons. In addition, it was reported that AMPK is regulated by conformational change caused by the binding of AMP to AMPK.
which promoted phosphorylation and inhibited dephosphorylation (38). Recent studies also showed that ADP could also regulate AMPK by binding to the nucleotide-binding pocket in the γ-subunit of AMPK (43,44). Thus, we believe that abnormally overexpressed AK1 may distort the signaling of AMPK by changing the nucleotide ratios under pathologic condition.

Among tau kinases, GSK3β is one of the most prominent kinases responsible for tau phosphorylation in vitro and in vivo. Conditional transgenic mice overexpressing GSK3β displayed tau hyperphosphorylation and disrupted microtubules, whereas administration of LiCl, a GSK3β inhibitor, reduced tauopathy and neuronal degeneration in JNPL3, mutant human tau transgenic mice (9,15). Furthermore, previous reports have highlighted GSK3β as a possible linker between Aβ42 and tau toxicity (17,18). However, the upstream regulator of GSK3β remains unclear until now. We provide several lines of evidence that AK1 is an upstream of GSK3β in Aβ42-mediated tau phosphorylation: (i) AK1 increased tau phosphorylation at CP13, AT180 and PHF-1 epitopes, which overlapped with those of GSK3β; (ii) AK1-mediated tau phosphorylation was diminished by SB-415286, a GSK3β inhibitor; (iii) likewise, inhibitory phosphorylation of GSK3β at Ser9 was altered by AK1; (iv) depletion of AK1 prevented Aβ42-induced GSK3β activation.

In conclusion, we show a novel function of neuronal AK1 that regulates GSK3β via AMPK to modulate tau hyperphosphorylation by Aβ42 and that enhances neurodegeneration in a Drosophila model of tau (Fig. 7). Based on the present study, it is tempting to speculate that the inhibition of AK1 activity offers a new therapeutics to rescue tau pathology.

**MATERIALS AND METHODS**

**Cell culture and DNA transfection**

HEK293T (human embryonic kidney) cells, HT22 (mouse hippocampal) cells and SH-SY5Y (human neuroblastoma) cells were cultured in DMEM (Hyclone) supplemented with 10% (v/v) fetal bovine serum (Hyclone). Primary cortical neurons were prepared from mouse brains at embryonic day 16. The neurons were seeded on poly-L-lysine (0.01% in 100 mM borate buffer, pH 8.5)-coated glass cover slips and maintained in neurobasal medium containing 2% B-27 supplement (Invitrogen) and 0.5 mM l-glutamine. Half of the medium was exchanged every 3 days. Primary neurons were treated with the following compounds: Aβ42, compound C or AICAR (Sigma-Aldrich). Oligomeric Aβ42 was first solubilized in DMSO at 2.2 mM, diluted in phosphate-buffered saline (PBS) at 250 μM, incubated at 4°C for 48 h and stored at −80°C until use. HEK293T and SH-SY5Y cells were transfected using the Polyfect reagent (Qiagen), whereas primary neurons were transfected using the LipofectAMINETM 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

**Generation of stable cell lines**

SH-SY5Y cells were transfected with pcDNA3-HA, pHA-AK1, pHA-R132A, pSuper-neo or pAK1 shRNAs for 36 h and then cultivated in selection medium containing 1 mg/ml G418 (Invitrogen) for 2 weeks. A single cell was further cultivated to form stable cell clones, and the expression level of each was analyzed by western blotting.

**Construction of plasmids and shRNAs**

Construction of tau was previously described (45). Human tau (0N4R) and AK1 cDNAs were subcloned into pcDNA3, pcDNA3-HA and pEGFP (Invitrogen) to generate HA or GFP-fusion protein (pHA-htau, pGFP-htau, pAK1, pHA-AK1 and pGFP-AK1, respectively), and mouse AK1 (a generous gift from Dr B. Wieringa, NCMLS University of Medical Center, the Netherlands) was cloned into pcDNA3 vector. Point mutants of AK1 (R132A, R138A and R149A) were generated by site-directed mutagenesis. All mutants were confirmed by DNA sequencing analysis.

Mouse AK1 siRNAs (sense 5′-GUU A-3′; antisense 5′-313GGC AAG AAG CTG TCG GAA A 331-3′; #1, 5′-CGG GGA UCA AGG CCA UGU A-3′; antisense 5′-UAA CAU CUC GGA GCA UGU A-3′; #2, 5′-GAC GUG GGA UCA AGG ACC A-3′; antisense 5′-UGG UCC UUG AUC ACA CCA C-3′; #3, 5′-UUG GGA UCA AGG ACC A-3′; antisense 5′-GUU A-3′) were purchased from Bioneer Corp. (Daegu, South Korea). The pAK1 shRNAs were constructed; forward and reverse 64-nucleotides (#1, 5′-313GGC AAG AAG CTG TCG GAA A 331-3′; #2, 5′-157GAA GGA GAG AAG CGT AAG AAA A175-3′) were synthesized, annealed and cloned into the BglII and HindIII sites of pSUPER.neo (pshRNA) (OligoEngine).

**Reverse transcription-PCR**

Cells were lysed and total RNA was purified using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. PCR was performed using following synthetic oligonucleotide sets: AK1 (5′-GGG GCA AGA AGC TGT CCG-3′, 5′-GAC CTT CCG CAC AAT GCC-3′), AK1β (5′-CTG CTT GTG TGC TAG TGA ACC-3′, 5′-CCT CGG GTA GCC GTG CAT C-3′), AK2 (5′-ATG GCT CCC AGC GTG C-3′).
CCA-3', 5'-GAT AAA CAT AAC CAA GTC-3') and AK3 (5'-CGA GCT GAA GCA CCT CTC C-3', 5'-CAG GTC ATC AAT GCC CAC-3').

**Generation of recombinant adenovirus**

A recombinant adenovirus was generated as described previously (46). Briefly, GFP-human tau was subcloned into the pShuttle-CMV and transformed with the adenoviral backbone vector pAdEasy-1 into BJ5183 cells for homologous recombination. Viral production was performed using HEK293 cells and monitored under fluorescence microscopy. Cells were harvested, resuspended in PBS, lysed by freeze-thawing and purified by CsCl gradient centrifugation.

**Western blotting and antibodies**

Cells were lysed in lysis buffer (50 mM Tris−Cl, pH 7.4, 30 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF and 1 μg/ml each of aprotinin, leupeptin and pepstatin A). The lysates were clarified by centrifugation, separated by SDS−PAGE and blotted onto PVDF membrane. The blots were blocked for 1 h and incubated with following antibodies: CP13, PHF-1, TG5 (a generous gift from Dr P. Davies, Albert Einstein College of Medicine, New York), 12E8 (a generous gift from Dr P. Seubert, Elan Pharmaceuticals), AT180 (Innomogenetics), AK1 (a gift from Dr B. Wieringa, NCMLS University of Medicine, New York), p-GSK3β (Cell Signaling), GSK3β (BD Biosciences), p-AMPK, AMPK, p-ACC, ACC, P38/P25, AMPKβ, AMPKγ (Cell Signaling), α-tubulin, β-actin (Sigma) and GFP (Santa Cruz Biotechnology) antibodies. Membranes were rinsed and incubated for 1 h with peroxidase-conjugated anti-mouse or rabbit antibody and visualized using the ECL detection system.

**Filter trap assay for tau aggregates**

SH-SY5Y stable cells expressing pcDNA3-HA, pHA-AK1 or pHA-R13A were infected with GFP-htau adenovirus. Cells were harvested and washed twice with PBS. Cells were lysed in 50 mM Tris−Cl (pH 7.4), 120 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% Nonidet P-40 and protease inhibitors mixture. After brief sonication, equal amount of cell lysates were passed through a nitrocellulose filter (0.2 μm). The resultant membrane was washed three times with 1% SDS and blocked in the 5% non-fat milk for 1 h followed by immunoblotting using the TG5 antibody. The samples were also analyzed with western blotting using tau antibodies for the changes in tau phosphorylation.

**Isolation of sarkosyl-insoluble tau**

Preparation of sarkosyl insoluble tau was previously described (47). Briefly, SH-SY5Y/pcDNA3, HA-AK1 or HA-R132A stable cells were infected with GFP-htau adenovirus. Cells were homogenized in sucrose buffer containing 50 mM Tris−Cl (pH 7.4), 0.8 M NaCl, 1 mM EGTA (pH 8.0), 10% sucrose and protease and phosphatase inhibitors. The homogenates were centrifuged at 15 000 g for 30 min at 4°C. The supernatants were incubated with 1% sarkosyl for 1 h at 37°C with gentle stirring. After ultracentrifugation at 100 000 g for 1 h (Beckman Coulter; TL-100), the sarkosyl-soluble or -insoluble tractions were subjected to western blotting using TG5 antibody.

**Immunocytochemistry**

Mouse primary cortical neurons were fixed in 4% paraformaldehyde (PFA) (Sigma) for 15 min, rinsed three times with PBS and permeabilized with 0.1% Triton X-100. After blocking in 5% BSA in PBS, neurons were incubated for 3 h at 4°C with the following antibodies: AK1 (1:500), NeuN (Chemicon, CA, USA; 1:500), CP13 (1:500), PHF-1 (1:500) or AT180 (1:250). After rinsing with PBS three times, cells were incubated with FITC-conjugated or TRITC-conjugated secondary antibodies (Jackson Laboratory, Inc.) at 4°C for 1 h. After rinsing three times with PBS, cover slips were placed with a mounting medium (Sigma). Samples were observed on a confocal laser scanning microscope (LSM510, Carl Zeiss, Inc.).

**Preparation of human brain sample and model mice**

Hippocampal tissues of AD (Braak V–VI) patients (aged 71–93 years; postmortem intervals 2–16 h) and age-matched control were kindly provided by Harvard Brain Tissue Resource Center (McLean Hospital, MA, USA). Hippocampal tissues of AD patients or cortex and hippocampal tissues from TG 2576 and J20 mice were homogenized in ice-cold Tris-buffered saline containing of 20 mM Tris–Cl (pH 7.4), 150 mM NaCl and protease inhibitors. The homogenates were clarified by centrifugation at 4°C, aliquoted and stored at −80°C. The supernatants were resolved by SDS−PAGE.

**Immunohistochemistry**

Immunohistochemical studies of brain sections from AD patients were described previously (48). The brains were fixed in PFA for 48 h before serial coronal sections and cut on a freezing microtome. Samples were placed on the slides, dried and refixed for 30 min in 4% PFA. The sections were incubated first in 0.5% Triton X-100 for 30 min and then in 1% BSA for 1 h before the application of a primary antibody. Blocked sections were incubated with AK1 (1:200) and NeuN (Chemicon; 1:200) antibodies, washed and incubated with FITC-conjugated and TRITC-conjugated secondary antibodies (Jackson Laboratory, Inc.). Samples were washed, mounted and observed under the Zeiss LSM510 microscope.

**Adenine nucleotide assay**

Cellular levels of adenine nucleotides were measured by a coupled enzymatic assay as described previously (49). In brief, primary cortical neurons or SH-SY5Y cells were washed once with ice-cold PBS and harvested into a buffer containing 20 mM Hepes with 3 mM MgCl2 (pH 7.75). Trichloroacetic acid (TCA) was added into the lysates to a final concentration of 5%, vortex-mixed, incubated on ice for 5 min and then centrifuged at 5000 g for 5 min. Supernatant
was neutralized by diluting with 1 m Tris–Cl (pH 7.5). The sample (10 μl) was added into 90 μl of 20 mM Hepes with 3 mM MgCl₂ (pH 7.75) in a 96-well plate. For ADP measurement, 1.5 mM phosphoenolpyruvate (PEP) and 2.3 U/ml pyruvate kinase (PK) were added. For AMP measurement, the sample was mixed with PEP, PK and 36 U/ml of AK. The plate was incubated for 40 min and then 100 μl of the CellTiter-Glo® luminescent assay reagent (Promega) was added and incubated for an additional 10 min. Luminescence was measured using a microplate spectrophotometer. Relative ADP and AMP levels were calculated by difference.

Immunoprecipitation assay
HEK293T cells were lysed in a lysis buffer containing protease inhibitors and then centrifuged at 12 000 r.p.m. for 30 min at 4°C. The supernatants were incubated with HA antibody at 4°C for 2 h and pulled down by Protein G Sepharose beads (GE Healthcare).

AMPK activity assay
AMPK activity was measured using the SAMS peptide (HMRSAMSGLHLVKRR) phosphorylation according to the procedure described in the work of Witters and Kemp (50). In short, lysates of primary cortical neurons were immunoprecipitated with AMPKα antibody and protein G-agarose beads. Beads were washed and the immunoprecipitated enzyme was assayed in the presence of 5 mM MgCl₂, 0.2 mM ATP and [32P]ATP for 20 min at 30°C. Aliquots of the reaction mixture supernatant were spotted on Whatman filter paper (P81). The filters were washed with ice-cold 1% phosphoric acid for 20 min for four times followed by a 5 min washing with acetone and then 5 min with petroleum ether. Dried papers were counted as scintillation counter (PerkinElmer).

Transgenic Drosophila
The gl-tau 2.1 line expressing wild-type human tau 4R in pExpress-gl modification of the GMR expression vector was obtained from Dr Daniel Geschwind (University of California, Los Angeles, CA, USA) (35). UAS-tau fly line for the generation of elav-tau was obtained from Dr Mel Feany (Harvard Medical School, Boston, MA, USA) (36). The human cytoplasmic AK1 cDNA was cloned into the GAL4-responsive pUAST expression vector. Transgenic strains for AK1 were created by embryonic injection (KAIST, Daejeon), obtained and analyzed. All crossbreeding experiments were carried out at 25°C.

Western blotting of Drosophila
To analyze the phosphorylation status of tau, fly heads were homogenized in 10 mM Tris–Cl (pH 7.4), 0.8 mM NaCl, 1 mM EGTA (pH 8.0), 10% sucrose and protease inhibitor cocktail (Roche). Homogenates were spun for 20 min at 4°C and the supernatants were collected and protein concentrations were determined by the Bradford assay (Bio-Rad). Equal protein concentrations were analyzed by western blotting.

Longevity assay
More than 200 flies of each genotype were collected, divided into tubes of 10 flies and incubated at 29°C on the standard food. The culture medium was changed every 2 days and the numbers of dead files were recorded.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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