Brain-specific repression of AMPKα1 alleviates pathophysiology in Alzheimer’s model mice

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AMP-activated protein kinase (AMPK) is a key regulator at the molecular level to maintain energy metabolism homeostasis. Mammalian AMPK is a heterotrimeric complex and its catalytic α subunit exists in two isoforms: AMPKα1 and AMPKα2. Recent studies suggest a role of AMPKα over-activation in AD-associated synaptic failure. However, whether AD-associated dementia can be improved by targeting AMPK remains unclear, and roles of AMPKα isoforms in AD pathophysiology are not understood. Here we showed distinct disruption of hippocampal AMPKα isoform expression patterns in post mortem human AD patients and AD model mice. We further investigated the effects of brain- and isoform-specific AMPKα repression on AD pathophysiology. We found that repression of AMPKα1 alleviated cognitive deficits and synaptic failure displayed in two separate lines of AD model mice. In contrast, AMPKα2 suppression did not alter AD pathophysiology. Using unbiased mass spectrometry-based proteomics analysis, we identified distinct patterns of protein expression associated with specific AMPKα isoform suppression in AD model mice. Further, AD-associated hyper-phosphorylation of eukaryotic elongation factor 2 (eEF2) was blunted with selective AMPKα1 inhibition. Our findings reveal isoform-specific roles of AMPKα in AD pathophysiology, thus providing insights into potential therapeutic strategy for AD and related dementia syndromes.
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Abstract

AMP-activated protein kinase (AMPK) is a key regulator at the molecular level to maintain energy metabolism homeostasis. Mammalian AMPK is a heterotrimeric complex and its catalytic α subunit exists in two isoforms: AMPKα1 and AMPKα2. Recent studies suggest a role of AMPKα over-activation in AD-associated synaptic failure. However, whether AD-associated dementia can be improved by targeting AMPK remains unclear, and roles of AMPKα isoforms in AD pathophysiology are not understood. Here we showed distinct disruption of hippocampal AMPKα isoform expression patterns in post mortem human AD patients and AD model mice. We further investigated the effects of brain- and isoform-specific AMPKα repression on AD pathophysiology. We found that repression of AMPKα1 alleviated cognitive deficits and synaptic failure displayed in two separate lines of AD model mice. In contrast, AMPKα2 suppression did not alter AD pathophysiology. Using unbiased mass spectrometry-based proteomics analysis, we identified distinct patterns of protein expression associated with specific AMPKα isoform suppression in AD model mice. Further, AD-associated hyper-phosphorylation of eukaryotic elongation factor 2 (eEF2) was blunted with selective AMPKα1 inhibition. Our findings reveal isoform-specific roles of AMPKα in AD pathophysiology, thus providing insights into potential therapeutic strategy for AD and related dementia syndromes.

Introduction

Alzheimer’s disease (AD) is the most common form of dementia and has become a global threat to public health. Currently there is no effective intervention to cure AD or slow disease progression, and the completed clinical trials have not succeeded in identifying disease-modifying strategies(1, 2). There is an urgent need to understand the molecular mechanisms underlying AD pathophysiology and accordingly identify novel therapeutic targets for this devastating neurodegenerative disease. Emerging evidence points to a role of the AMP-activated protein
kinase (AMPK) in AD pathophysiology (3-6). AMPK is a Ser/Thr kinase that functions as a central energy sensor at the molecular level. Low energy states lead to activation of AMPK to maintain cellular energy homeostasis(7). Mammalian AMPK is a heterotrimeric complex consisting of a catalytic α subunit, a scaffolding β subunit, and a regulatory γ subunit (8). AMPK is activated by two general mechanisms: binding of AMP to the Bateman domain of the γ subunit, causing a conformational change at the α subunit, or phosphorylation of the α subunit at the Thr172 site which involves either inhibition of protein phosphatases or activation of upstream kinases (9, 10). Dysregulation of energy metabolisms are implicated in the pathogenesis of multiple neurodegenerative diseases including AD(11, 12). Recent studies indicate upregulation of brain AMPK activity in AD, as evaluated by AMPKα phosphorylation (3, 13). Moreover, AMPK over-activation is linked to the loss of hippocampal dendritic spines caused by amyloid β (Aβ) oligomers(4). Consistently, impairments of hippocampal synaptic plasticity associated with Aβ treatment or AD model mice are alleviated by repression of AMPK signaling(3). Nevertheless, whether AD-associated cognitive deficits can be improved by targeting AMPK is not known.

There are two isoforms of the AMPK catalytic α subunit: AMPKα1 and AMPKα2. These two isoforms are encoded by distinct genes located on different chromosomes: PRKAA1 on chromosome 5, and PRKAA2 on chromosome 1, respectively(14). Both α1 and α2 are expressed in most tissues for the regulation of energy homeostasis, and previous work indicates isoform-specific substrate specificity (15-18). However, the specific downstream targets and roles for AMPKα isoforms in the central nervous system remain unclear.

AMPK interacts with multiple signaling cascades and has many downstream effectors involved in biosynthesis(19). Among the downstream effects of AMPK, regulation of mRNA translation (de novo protein synthesis) is of particular interest. Substantial studies have demonstrated that long-term synaptic plasticity and memory formation is dependent on de novo protein synthesis (20-22). In agreement, defects of mRNA translation are implicated in cognitive syndromes associated with neurodegenerative diseases including AD, prion disease, and
frontotemporal dementia (23-25). Briefly, AMPK activation results in the inhibition of mRNA translation through two potential mechanisms: 1) suppression of the mammalian target of rapamycin complex 1 (mTORC1) pathway, which controls synthesis of translational machinery and cap-dependent translation initiation; and 2) phosphorylation of eukaryotic elongation factor 2 kinase (eEF2K), leading to eEF2 phosphorylation and consequently disruption of the elongation step in mRNA translation(14). Moreover, recent studies indicate that interactions between AMPK and mTORC1 are dependent on eukaryotic initiation factor 2 α subunit (eIF2α) activation levels(26). Phosphorylation of eIF2α by its kinase PKR-like ER kinase (PERK) results in suppression of general protein synthesis, and has been linked to AD pathophysiology(23). Nevertheless, whether the role of AMPKα in the regulation of de novo protein synthesis is isoform-specific is unknown.

In the present study, we reported isoform-specific dysregulation of AMPKα in post mortem human AD brain tissue (including both sporadic AD or sAD, and familial AD or FAD). Using genetic approaches, we found that abnormal AMPKα1 upregulation plays a critical role in mediating AD-associated cognitive impairments and synaptic failure. Our findings reveal what we believe to be previously unrecognized isoform-specific role of AMPK dysregulation in AD pathogenesis, which may provide insights into novel therapeutic avenues for AD and related dementia syndromes.

Results

Brain AMPKα isoform homeostasis is disrupted in AD

To investigate regulation of AMPKα isoforms in AD, we first examined post mortem brain tissues from sporadic Alzheimer’s disease (sAD) patients (Table 1) and found that AMPKα1 levels were significantly increased, while AMPKα2 levels were markedly reduced in the hippocampus, as compared with age-matched controls (Figure. 1A). Consistently, immunohistochemical staining of post mortem brain sections revealed AD-associated increased AMPKα1 and decreased AMPKα2 staining throughout the hippocampus, including both soma and neurites (Figure 1B). In
contrast, no significant change in AMPKα isoforms was observed in the cerebellum (Figure 1C). We further assayed post mortem hippocampal tissue from patients with neuropathologically confirmed Lewy body dementia (LBD) and frontotemporal dementia (FTD), two non-AD neuronal diseases with dementia syndromes (Table 2-3). Notably, levels of AMPKα isoforms were not affected in either LBD or FTD, as compared with their respective age-matched controls (Figure 1D-E). Interestingly, in post mortem hippocampal tissue from familial Alzheimer’s disease (FAD) patients (Table 4), we observed significantly elevated AMPKα1 expression but no changes in AMPKα2 levels, as compared to tissue from age-matched controls (Figure 1F). Similarly, in hippocampal tissue from Tg19959 transgenic mice (Tg) that model FAD(27), there was a significant increase of AMPKα1 expression but no changes in AMPKα2 levels (Figure 1G). Additionally, no changes were found in Tg mice in either AMPKβ or AMPKγ protein levels or phosphorylation of either AMPKα isoform (Supplemental Figure 1A-C). Finally, immunofluorescence combined with confocal imaging revealed elevated AMPKα1 levels in both soma and dendrites in the hippocampus of Tg mice (Figure 1H). Taken together, these data demonstrate that AMPKα isoform homeostasis is disrupted in AD.

**Brain-specific suppression of AMPKα1 isoform alleviates learning and memory defects in Tg19959 AD model mice**

We further investigated whether there is an association between brain AMPK isoform dysregulation and AD pathophysiology. First, mice harboring loxP-flanked Prkaa1 or Prkaa2 were bred with mice expressing a brain specific Cre recombinase (Camk2a-cre)(23) to generate heterozygous AMPKα1 or AMPKα2 conditional knockout mice (referred to as α1/cre and α2/cre respectively) (Supplemental Figure 1D-E). Further, by crossing α1/cre and α2/cre mice with Tg19959 AD model mice, we generated Tg19959/AMPKα1+- (α1/Tg) and Tg19959/AMPKα2+- (α2/Tg) double mutant mice, along with other four experimental groups: WT, Tg19959 (Tg), α1/cre, and α2/cre mice. Western blot assay demonstrated that the increased levels of AMPKα1
in the hippocampi of Tg mice were restored to WT levels in α1/Tg mice, but remained elevated in α2/Tg mice (Figure 2A). Histological assays showed that the genetic reduction of AMPKα isoforms did not alter gross morphology of the hippocampus (Figure 2B, Supplemental Figure 1E).

To determine the consequence of selective AMPKα isoform reduction on AD-associated learning and memory impairments, we subjected the aforementioned mice to a series of behavioral tasks. First, we performed the open field (OF) test to assess general locomotor activity and baseline anxiety-like behavior, and did not find any differences among all genotypes (Figure 2C, Supplemental Figure 1F-G). We then tested the mice in the novel object recognition (NOR) task to evaluate their long-term hippocampus-dependent recognition memory(28). WT and α1/cre mice exhibited normal cognition as indicated by their preference for the novel object over the familiar object on the test day (i.e. significantly more interaction with the novel object) (Figure 2D). In contrast, Tg mice spent similar amounts of time with either the familiar or novel objects, indicating deficits in learning and memory (Figure 2D)(29). Notably, AD model mice with reduced AMPKα1 (α1/Tg) demonstrated normal cognition, as indicated by significant more time spent with the novel objects (Figure 2D).

Next we assessed spatial learning and memory by testing mice with the hidden platform Morris water maze (MWM) behavioral task(28), a well-validated test of hippocampus-dependent spatial memory. Consistent with results from NOR task, Tg mice displayed impaired learning and memory. During the acquisition (learning) phase, WT mice demonstrated marked day-to-day decrease in escape latency, whereas Tg mice exhibited longer escape latencies (Figure 2E). In the probe trial, Tg mice spent less time in the target quadrant as compared to WT mice (Figure 2F). Notably, impairments of spatial learning and memory associated with Tg mice were alleviated by suppressing AMPKα1, as indicated by decreased escape latency and improved target quadrant occupancy (not significantly different from WT) by α1/Tg mice (Figure 2E-F, Supplemental Figure 1H-J). In comparison, reduction of AMPKα2 did not alter cognitive deficits in Tg mice (Figure 2E-F). In order to examine potential memory-independent effects of AMPKα
isoform suppression such as swimming ability or visual impairments, mice were also run through the visible platform task (VP). No differences in latency to locate the visible platform were observed across all groups (Supplemental Figure 1K). Taken together, suppression of AMPKα1, but not AMPKα2 alleviates AD-associated learning and memory impairments.

**Suppression of AMPKα isoforms does not alter AD-associated brain Aβ pathology or tau phosphorylation**

We went on to investigate potential mechanisms associated with the beneficial effects on cognition with AMPKα1 suppression in Tg mice. First, we analyzed brain Aβ pathology in the cortical and hippocampal brain areas and found similar Aβ plaque deposition in Tg and α1/Tg mice (Figure 3A-B). Additionally, reduction of AMPKα2 did not alter brain Aβ plaque pathology either (Figure 3C-D). We then used ELISA to quantify the brain levels of Aβ1-40 and Aβ1-42. Compared to Tg mice, both α1/Tg and α2/Tg mice showed decreased Aβ1-40 levels, whereas levels of Aβ1-42 were not altered, and neither was the ratio of Aβ42:40 (Figure 3E-G). Furthermore, reduction of either AMPKα isoform did not alter levels of amyloid precursor protein (APP) and key APP processing enzymes including components of γ- and β-secretase (Supplemental Figure 2A-C). Levels of hippocampal Aβ monomers assessed by Western blot were not changed with AMPKα isoform suppression (Supplemental Figure 2D). Interestingly, phosphorylation of Tau at either S262 or S396 sites (known to be phosphorylated by AMPK(30)), was unaffected by repression of either AMPKα isoform (Figure 3H). Levels of total tau were unaffected as well (Supplemental Figure 2E). These data indicate that alleviation of cognitive impairments in Tg mice by reducing AMPKα1 is unlikely associated with effects on brain Aβ pathology or tau phosphorylation.

**Reduction of brain AMPKα1 corrects AD-associated deficits in synaptic density and dendritic spine morphology**
AD is considered as a disease of “synaptic failure” (31). Loss of synapses or synaptic density correlates with memory impairments in both human AD patients and animal models of AD (12, 32, 33). Dendritic spine morphology is critical for synaptic integrity and closely associated with neural plasticity and memory formation (34, 35). We used the rapid Golgi-Cox staining technique to assess spine density and morphology changes of dendritic spines within the area CA1 of hippocampus. Consistent with a previous study (28), overall dendritic spine density in Tg mice was significantly decreased as compared with that in WT controls (Figure 4B). Importantly, AD-associated reduction of dendritic spine density was restored by suppressing AMPKα1 but not AMPKα2 (Figure 4B). We further analyzed spine morphology changes based on established guidelines on classification of “mature” and “immature” spine types (Figure 4A) (36). We found that the density of “mature” spines (mushroom, stubby, and branched) in Tg mice was significantly decreased as compared to WT controls, and genetic reduction of AMPKα1 restored those defects in Tg mice (Figure 4C-F). Interestingly, density of overall “immature” spines (thin and filopodia) was higher in Tg mice compared to WT controls, and that increase was blunted by suppressing AMPKα1 (Supplemental Figure 3A-C).

Moreover, we investigate potential alterations of postsynaptic densities (PSDs) by using transmission electron microscopy (TEM). PSDs are located at the heads of spines and are vital for synaptic function (34, 37). Ultrastructural analysis revealed decreased PSD density in area CA1 of hippocampus in Tg mice, which was restored in α1/Tg mice, but unaffected in α2/Tg mice (Figure 4G). Analysis of hippocampal PSD-95 levels by Western blot assay showed a similar deficit in Tg mice, which was also restored by genetic suppression of AMPKα1 but not AMPKα2 (Figure 4H). Taken together, these data suggest that genetic repression of AMPKα1 alleviated defects in hippocampal spine density/morphology and PSD formation associated with Tg19959 AD model mice. These findings are consistent with the results from the behavioral experiments described above (Figure 2).
Repression of brain AMPKα1 restores abnormal eEF2 phosphorylation and *de novo* protein synthesis deficits in Tg19959 mice

*De novo* protein synthesis is essential for memory formation and synaptic plasticity, and is notably impaired in AD (23, 28, 38). As described in the introduction, AMPK may regulate protein synthesis via two main downstream signaling pathways: mTORC1 and eEF2K/eEF2(14). Biochemical assay showed that brain mTORC1 signaling was not affected by reducing either AMPKα1 or AMPKα2, as indicated by unaltered phosphorylation levels of mTOR and TSC2 in α1/Tg or α2/Tg mice, as compared to those in WT or Tg mice (Supplemental Figure 4A-B). Additionally, we examined hippocampal protein levels of the structural components of protein phosphatase 2A (PP2A), a potential phosphatase and key regulator for AMPK activity(39, 40), and did not observe any alterations (Supplemental Figure 4C-E) Levels of eEF2 phosphorylation were elevated in hippocampi of Tg mice, in agreement with recent studies(28, 41). Markedly, AD-associated eEF2 hyper-phosphorylation was restored with selective AMPKα1 but not AMPKα2 suppression (Figure 5A).

To directly measure *de novo* protein synthesis, we used the surface sensing of translation (SUnSET, a nonradioactive puromycin end-labeling assay) on living acute hippocampal slices(42, 43). In agreement with previous studies(44), *de novo* protein synthesis in hippocampal slices (assessed by puromycin labeling) was reduced in Tg mice as compared with WT controls (Figure 5B). Notably, AD-associated protein synthesis defects were alleviated by reduction of AMPKα1 (Figure 5B). AMPKα2 repression did not improve *de novo* protein synthesis deficiency in Tg mice (Figure 5B). We further examined potential isoform specific effects of AMPKα repression on protein synthesis with mass spectrometry-based proteomic experiments. Altered proteins were summarized using a heat map (Figure 5C). Briefly, proteins identified as significantly altered from the WT in other genotypes were pooled, and for each protein a deviation from the mean was calculated and mapped using PermutMatrix software(45). Of all the proteins identified (2041 in total), 27 were commonly dysregulated (17 up-regulated, 10 down-regulated). From the pooled
proteins, 10 proteins were identified to be uniquely restored with AMPKα1 repression including proteins involved in apoptosis, Ca\textsuperscript{2+} signaling, cytoskeletal dynamics, and oxidative stress (Figure 5C-E, Table 5). Thus, AD-associated abnormal eEF2 phosphorylation and de novo protein synthesis deficits were lessened by genetic reduction of AMPKα1 isoform.

**Reduction of brain AMPKα1 mitigates impairments of cognition and synaptic plasticity associated with APP/PS1 AD model mice**

We further determined whether the above findings related to selective AMPKα isoform suppression can be replicated in another rodent model of AD. We crossed APP/PS1 AD model mice(46) with either α1/cre or α2/cre mice to generate APP/PS1/AMPKα1+- (α1/APP) and APP/PS1/AMPKα2+- (α2/APP) double mutant mice. AMPKβ and AMPKγ levels were unaffected among different genotype (Supplemental Figure 5A-B). As was seen in the Tg mice, APP/PS1 mice also exhibited elevated levels of eEF2 phosphorylation, which was corrected by AMPKα1 (but not AMPKα2) repression (Figure 6A). To verify the effects of selective AMPKα isoform suppression on AD-associated learning and memory deficits, mice were subjected to the same behavioral battery as the Tg mice cohort. Briefly, baseline anxiety-like behavior, assessed by OF task, was indistinguishable among all groups (Figure 6B, Supplemental Figure 5C-D). Compared to WT control mice, APP/PS1 mice displayed learning and memory impairments based on results from both NOR and hidden platform MWM behavioral tasks (Figure 6C-E). Importantly, AD-associated cognitive deficits were alleviated by selective suppression of AMPKα1, as demonstrated by normal learning and memory performance in α1/APP mice in both NOR and MWM behavioral tests (Figure 6C-E). In contrast, α2/APP mice showed similar impaired cognition in both tests, as compared to APP/PS1 mice (Figure 6C-E). We also evaluated regulation of long-term synaptic plasticity in these mice by examining hippocampal long-term potentiation (LTP), one of the most intensively studied cellular models for learning and memory(47, 48). APP/PS1 mice showed LTP impairments that were improved by suppression of AMPKα1, but not AMPKα2.
Interestingly, suppression of AMPKα2 (but not AMPKα1) resulted in LTP impairment (Figure 6F-H). Moreover, brain Aβ pathology in APP/PS1 mice was unaltered with repression of either AMPKα isoform (Figure 6I-L, Supplemental Figure.6A-C). Additionally, there was no change in tau phosphorylation or total tau levels across groups (Supplemental Figure 6D-E). These results are consistent with the findings in Tg19959 AD model mice described above, indicating that brain-specific genetic suppression of AMPKα1 is able to prevent AD-associated cognitive defects and synaptic plasticity impairments.

Discussion

Among the top ten causes of death, AD is the only disease without any effective therapeutic approach(1). The incidence of AD has been increasing dramatically with global aging, and it is imperative to identify novel therapeutic targets based on solid mechanistic studies. In the current study, we report that brain-specific suppression of AMPKα1 selectively alleviated cognitive deficits and synaptic failure displayed in two separate lines of AD model mice, revealing isoform-specific roles of AMPKα in AD pathophysiology. Energy metabolism dysregulation is linked to neurodegenerative diseases including AD(11, 12, 49). AMPK is the central energy sensor at the molecular level, and is considered to regulate all aspects of cellular function(19). As a master kinase functioning to maintain energy homeostasis, AMPK plays a key role in many biological processes. Considering all the downstream effectors of AMPK, dysregulation of AMPK signaling may be connected to many, if not all cellular abnormalities and functional impairments in AD(5).

It is noteworthy that disruption of the AMPKα isoform expression levels in hippocampus was found in AD, but not in LBD or FTD post mortem brain tissue (Figure 1). LBD cases are characterized by hippocampal α–synuclein without substantial levels of p-tau (50, 51). All FTD patients reported here were diagnosed with progressive supranuclear palsy, and typically have increased levels of tau phosphorylation in hippocampus(52). Furthermore, neither LBD nor FTD cases exhibit significant hippocampal Aβ deposition(51, 52). Our data indicate that dysregulation
of AMPKα isoform homeostasis might be specific to AD-related pathological processes, such as Aβ or phosphorylation of AD-type tau, but not related to α-synuclein, frontotemporal lobar degeneration-tau (FTLD-tau), or nonspecific conditions associated with neurodegenerative diseases in general. Impaired brain energy metabolisms have been implicated in many neurodegenerative diseases (11). Our current study suggests AD-specific dysregulation of brain AMPKα isoform expression, which may provide insights into development of novel biomarkers and therapeutic approaches for AD. Meanwhile, our findings do not exclude a role of (aberrant) AMPK signaling in pathogenesis of other neurodegenerative diseases. Furthermore, it is possible that upregulation of AMPKα1 expression in sAD is compensatory in response to downregulation of AMPKα2 expression to maintain homeostasis, or vice versa. Interestingly, while increased levels of hippocampal AMPKα1 were observed in both sAD and FAD cases, reduction of hippocampal AMPKα2 levels was seen only in sAD, but not in FAD. Further, the pattern of hippocampal AMPKα isoform dysregulation in Tg19959 AD model mice, which are models of FAD, matches those of FAD patients. These differences could be attributed to factors such as aging (FAD cases are younger than sAD in our study) or certain comorbidites in sAD cases, and future studies are warranted to elucidate the nature and source of this deviation.

Multiple studies indicate a role of AMPK in AD-associated Aβ and tau pathology (5, 6, 53, 54). AMPK regulation may affect AD brain pathology, although controversy arises regarding whether Aβ accumulation and tau hyper-phosphorylation can be alleviated or aggravated by activation of AMPK (5, 6, 53, 54). On the other hand, increased AMPK activity (assessed by AMPKα phosphorylation) was observed in brain tissue from AD patients, transgenic mouse models of AD, and induced by exogenous Aβ application (3, 13). Thus, there probably exists a reciprocal relationship between AMPK dysregulation and AD brain pathology. It is worth mentioning that these studies were conducted across multiple experimental models and species, which could contribute to the seemingly inconsistent findings (55). While AMPK expression is conserved across many species, little is known about the exact roles of AMPK isoforms and their
subcellular distribution in neuronal system. To the best of our knowledge, none of the aforementioned studies actually clarifies potential distinct roles of AMPK isoforms in AD-associated impairments of both cognition and synaptic plasticity in their experimental paradigms. In the current study, reducing AMPKα1 or AMPKα2 does not alter brain levels of either Aβ$_{42}$ or p-tau in AD model mice. Future studies to elucidate the complexity of AMPK isoforms in the central nervous system shall provide insights into understanding on some of the controversy surrounding the effects of AMPK and AMPK-related drug (e.g. metformin) on AD (56-58). The current study indicates that brain-specific repression of AMPKα2 does not improve cognitive deficits and synaptic plasticity impairments in AD model mice. It was previously reported that LTP failure induced by application of exogenous synthetic Aβ in acute hippocampal slices was prevented in slices derived from global AMPKα2 knockout mice(3). These results suggest different roles of AMPKα2 regulation in AD-related synaptic plasticity impairments under acute or chronic circumstances. Additionally, potential compensatory signaling mechanisms (e.g. upregulation of other AMPK isoforms) associated with global AMPKα2 knockout may contribute to the different performance for LTP.

The findings that AD-associated hippocampal eEF2 hyper-phosphorylation was blunted by selectively reducing AMPKα1 is particularly interesting. As an mRNA translational factor, eEF2 mediates the translocation step during the elongation phase of protein synthesis, catalyzing movement of tRNA from the ribosomal A-site to the P-site(59, 60). Phosphorylation of eEF2 at Thr56 leads to disruption of new peptide growth and thus inhibits overall protein synthesis(59). AMPK is known to be activated in low energy status, leading to the inhibition of protein synthesis/mRNA translation(14). Repression of mRNA translation by eEF2 phosphorylation (under the condition of AMPK activation) may initially serve as a protective strategy to conserve energy resources (by turning off major energy-consuming processes such as protein synthesis) for cells to cope with low energy states. However, a prolonged increase of eEF2 phosphorylation and inactivation (e.g. in the case of AD) would be detrimental for cognitive function since de novo
protein synthesis is indispensable for long-lasting forms of synaptic plasticity and memory consolidation. Indeed, cognitive impairments associated with multiple neurodegenerative diseases are linked to defects in capacity of de novo protein synthesis (23-25). Consistently, a recent study shows that repression of eEF2K and thus eEF2 phosphorylation is sufficient to improve multiple aspects of pathophysiology in AD model mice (28).

The results of the proteomic experiments may drive future research into AMPK isoforms in AD in several ways. Each of the proteins found to be uniquely restored with selective AMPKα1 repression indicates potential mechanisms through which AMPKα1 repression is beneficial in AD. A variety of pathways are also implicated, including those in apoptosis, cell signaling, mRNA translation, and oxidative stress (Table 5). Among the identified proteins restored in selective AMPKα1 repression is S100 calcium-binding protein B (S100B). S100B overexpression is seen in inflammation and astrocyte activation surrounding Aβ plaques and is thought to help drive disease progression (61, 62). Also identified in the proteomics screen were proteins involved in membrane and spine dynamics, including Calcium-activated potassium channel subunit α-1 (KCa1.1). Levels of KCa1.1 was significantly reduced in Tg mice and was restored by suppression of AMPKα1 but not α2. KCa1.1 is activated by cytosolic Ca^{2+} increases or membrane depolarization and opens to export K^{+}, and is implicated in intellectual and seizure disorders (63). Notably, aberrant intracellular calcium signaling has been linked to AD pathogenesis(64, 65).

In summary, our findings indicate that targeting brain dysregulation of AMPK isoforms, particularly AMPKα1, might be a feasible therapeutic strategy for AD-associated cognitive impairments. Future endeavors are critical to identify small molecule modulators which are selective for AMPKα isoforms to be tested in AD models. Moreover, given the central role of AMPK in energy metabolism regulation and the complexity of the AMPK signaling network, future work is necessary to determine the detailed molecular mechanisms underlying the interactions between AMPK isoforms and AD pathogenesis at different stages (early vs. late) or forms (familial vs. sporadic).
Materials and Methods

Post mortem tissue samples

Post mortem human tissue was obtained from the University of Washington School Of Medicine
Brain Bank and the NIH NeuroBioBank affiliated with University of Maryland. Samples were
collected in accordance with approved Institutional Review Board protocols. Diagnoses were
based on cognitive testing, post mortem Braak (AD stages V-VI) stage, and Consortium to
Establish a Registry for Alzheimer’s disease (CERAD) scores. Studies were performed using
hippocampal tissue from male and female patients clinically diagnosed with AD and age-matched
controls. Mean age of death was 89.6 years. Postmortem interval (PMI) ranged between 2 and
10 h with a mean of 5.3 h. For hippocampal tissue from FTD patients and age-matched controls,
mean age of death was 80.4 years with a PMI between 3 and 9 h and average of 4.8 h. For
hippocampal tissue from LBD patients and age-matched controls, mean age of death was 85.2
years with a PMI between 3.5 and 9 h and an average of 5.6 h. Patient information was presented
in Tables 1-4.

Mice

All mice were housed at the Wake Forest School of Medicine barrier facility under the supervision
of the Animal Research Program. Mice were kept in compliance with the NIH Guide for Care and
Use of Laboratory Animals. Mice adhered to a 12 h light/dark cycle with regular feeding, cage
cleaning, and 24 h food and water access. Both male and female mice were used for
experimentation. Breeders for Tg19959 AD model mice were generously provided by Dr. George
Carlson of McLaughlin Research Institute (Great Falls, MT)(27). APP/PS1 mice were purchased
from Jackson Labs (Br Harbor, ME) and expressed human APP (KM670/671NL) and presenilin-
1(66). The following mice were purchased from the Jackson Laboratory (Bar Harbor, ME): B6.Cg-
Tg(Camk2a-cre)T29-1St/J (Camk2a-cre mice), stock No. 005359; Prkaa1tm1.1Sjm/J (loxP-flanked Prkaa1 mice), stock No. 014141; Prkaa2tm1.1Sjm/J (loxP-flanked Prkaa2 mice), stock No. 014142. Mice harboring loxP-flanked Prkaa1 or Prkaa2 were bred with mice expressing a brain specific Cre recombinase (Camk2a-cre) (23) to generate AMPKα1 or α2 conditional knockout mice (referred as α1/cre and α2/cre). These mice were further crossed with AD model mice to generate the double mutant mice. Genotypes were verified with polymerase chain reaction (PCR). Tg19959 mice of ages 6-9 months and APP/PS1 mice of ages 12-16 months were used for all experiments (28, 66).

**Western blots assay**

Tissues were removed from appropriate structures and flash frozen on dry ice. Tissues were then homogenized in an appropriate lysis buffer and quantified as previously described (43). Samples were loaded on 4-15% TGX™ Precast Gels (Bio-Rad), and transferred to nitrocellulose membranes. Membranes were blocked and then probed overnight at 4°C using primary antibodies of interest. Blots were washed and HRP-labeled secondary antibodies were added. Primary antibodies used: AMPKα1 (1:1000; Cell Signaling, Cat#2795S), AMPKα2 (1:1000; Cell Signaling, Cat#2757S), AMPKα1 (1:1000; Abcam, Cat#ab3759), AMPKα2 (1:1000; Ab cam, Cat#3760), p-Tau (Ser396) (1:1000; Thermofisher, Cat# 44-752G), p-Tau Ser262 (1:1000; Thermofisher, Cat#44-750G), Amyloid-Beta (6E10) (1:1000; Sigma Cat#Sig39320), APP (1:1000; Cell Signaling , Cat#CS2452), BACE1(1:1000; Cell Signaling, Cat#CS5606), Presenilin 2 (PS2) (1:1000; Cell Signaling, Cat#CS9979), PSD-95 (1:1000; Cell Signaling , Cat#Cs3450), Puromycin (1:5000; Millipore, Cat#AB3258), p-eEF2 (Thr 56) (1:1000; Cell Signaling, Cat#CS2331), eEF2 (1:1000; Cell Signaling, Cat#CS2332), PP2AA (1:1000; Cell Signaling, Cat#CS2041), PP2AB (1:1000; Cell Signaling, Cat#cs4953), PP2AC (1:1000; Cell Signaling, Cat#2038), p-mTOR (Ser2448) (1:1000; Cell Signaling, Cat# CS2971), mTOR (1:1000; Cell Signaling, Cat#CS2983), p-TSC2 (Thr 1462) (1:1000; Cell Signaling, Cat#CS2983), β-Actin (1:10000, Sigma, Cat#A2228),
and GAPDH (1:10,000, Cell Signaling, Cat#CS5174). AMPKβ1 (1:1000; Abcam, Cat#71C10) AMPKγ (1:1000; Invitrogen, Cat#PA5-36314), Tau (1:1000; Sigma Cat#T5530), Catalase (1:1000; Cell Signaling, Cat#14097S), and KCaα1 (1:1000; Abcam Cat#ab3586). All antibodies were diluted in either 5% wt/vol Milk/TBST or 5% wt/vol BSA/TBST. The blots were visualized using chemiluminescence (Clarity™ ECL; Bio-Rad) and the Bio-Rad ChemiDoc™ MP Imaging System. Densitometric analysis was performed using ImageJ software. Data were normalized to β–actin or GAPDH (for total protein analysis) or relevant total proteins (for phospho-protein analysis) unless otherwise specified.

**Immunohistochemistry for post mortem human tissue**

*Post mortem* tissue sections from patients were prepared at the University of Washington. Brains were fixed in 10% neutral buffered formalin. Hippocampal samples were embedded in paraffin and sectioned at 5 μm thickness. Sections were mounted on positively charged slides and baked for 30 min at 60°C. For staining, sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Slides were boiled in citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 25 min. Slides were incubated in a humid chamber in primary antibody for AMPKα1 (1:500; Cell Signaling) or AMPKα2 (1:250, Cell Signaling) overnight at 4°C. Sections were then incubated in biotinylated α-rabbit secondary antibody (1: 200; Vector Labs) for 30 min at room temperature followed by Vectastain® Elite ABC reagent (Vector Labs) for another 30 min. Primary and secondary antibodies and ABC reagent were diluted in 1% BSA/PBS. Diaminobenzidine (DAB) was diluted in Tris buffer (pH 7.7) and 3% hydrogen peroxidase in a working DAB solution. Sections were developed in DAB for 10 min in a 42 °C water bath. Slides were counterstained using Mayer’s hematoxylin and blued with 0.2% lithium carbonate. Negative controls were incubated in 1% BSA with rabbit IgG as the primary antibody. Sections were dehydrated in an alcohol series and cleared.
with xylene, coverslipped, and dried overnight. Slides were imaged at 20X and 60X on a Keyence BZ-X710 microscope (Keyence, Japan).

Immunohistochemistry for mouse tissue

Following euthanasia, mouse brains were hemisected and fixed overnight in ice-cold PFA and transferred to 70% EtOH. Paraffin embedding was performed by Wake Forest Pathology. Paraffin embedded mouse sections were de-paraffinized in xylene and rehydrated through graded ethanol series. Antigen retrieval utilized citrate buffer (pH 6.0) in a standard 10 min microwave procedure. Blocking was done for 2 h with 10% NGS in 1%BSA/TBS. Slides were incubated in a humidified chamber in primary antibody for Amyloid-β (6E10) (1:200) overnight at 4°C. Following second 15 min 3% hydrogen peroxide blocking, sections were then incubated in biotinylated α mouse secondary antibody (1:200; Vector Labs, Burlingame, CA) for 1 hr at room temperature followed by Vectastain Elite ABC reagent (Vector Labs, Burlingame, CA) for another 30 minutes. Primary and secondary antibodies as well as ABC reagent were diluted in 1% BSA/TBS. Sections were developed in DAB (Vector Labs, Burlingame, CA) for 30s-3 min with monitoring. Slides were counterstained using Mayer’s hematoxylin for 60 s and blued with 0.2% lithium carbonate for 20s. In between each step of immunohistochemistry, sections were rinsed using distilled water or TBSTX (pH 7.4). Negative controls were incubated in 1% BSA with no primary antibody. Sections were dehydrated in an alcohol series and cleared with xylene, coverslipped, and dried overnight. Imaging was performed using BZ-X710 all-in-one fluorescent microscope (Keyence, Japan).

Immunofluorescence and confocal microscopy

Hippocampal slices were fixed overnight in ice-cold 4% paraformaldehyde in PBS. Fixed free floating sections were subsectioned to 40 μm using a Leica VT1200S vibratome and permeabilized with 0.3% TritonX-100. Sections were blocked with 10% normal goat serum, 0.1% sodium azide in 1% BSA in PBS for 5 h and incubated overnight with primary antibody for
AMPKα1 (12.5 μg; R&D Systems; Cat#AF3197). Alexa Fluor 633 secondary antibodies (1:250 Thermo Fisher Cat#A21082) was used. The sections were imaged using an Olympus FV1200 Confocal microscope at 20X. All parameters (pinhole, contrast, gain, and offset) were held constant for all sections across the same experiment.

**Open field (OF) and Novel object recognition (NOR)**

Mice were handled 7 days prior to behavioral testing and habituated to the behavior room for at least an hour prior to experimentation. Animals were placed in an opaque arena (40 cm³) for 15 mins. The distance travelled, average velocity and the duration spent by the animal in the center were analyzed by Noldus software (Noldus Information Technology, Leesburg, VA). Following OF, mice underwent two d familiarization with identical ceramic objects in the testing arena. Mice were given 5 mins to explore the objects. 24 h following familiarization, animals were tested in the arena with one object randomly replaced with a novel object. Time spent with each object was manually calculated and reported as a percentage of total interaction time. An interaction of < 50% with the novel object indicates memory impairment(29). Mice with < 10s total interaction time or > 90% object preference were excluded. Data collection and analysis were performed blinded.

**Morris Water Maze (MWM)**

Morris Water Maze was performed as previously described(28). The training paradigm consisted of 4 trials (60 s maximum, 15 min intertrial interval) with average escape latency (s) assessed per trial over 5 consecutive days. 2 hours following the final trial, mice underwent a probe trial. Probe trajectories, quadrant occupancy, velocity, and distance were monitored by Ethovision XT (Noldus Information Technology, Leesburg, VA). The visible platform (VP) task consists of 4 trials per day (60s max, 15 min interval) for 2 days, with the escape platform marked by a visible cue and moved randomly throughout the quadrants. Data collection and analysis were performed blinded.
**Hippocampal slice preparation and electrophysiology**

Acute 400 µm transverse hippocampal slices were prepared using a Leica VT1200S vibratome (Wetzlar, Germany) as described (67). Briefly, slices were maintained at room temperature prior to experimentation (2 hr) in artificial cerebrospinal fluid (ACSF) containing (in mM): 118 NaCl, 3.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1.25 NaH₂PO₄, 5 NaHCO₃, and 15 glucose, bubbled with 95% O₂ / 5% CO₂. For electrophysiology, slices were maintained at 32 °C, and monophasic current stimuli of 100 µs were delivered with a bipolar silver electrode in the stratum radiatum of area CA3. Field excitatory post-synaptic potentials (fEPSPs) were recorded using a glass microelectrode from the stratum radiatum of area CA1. Long term potentiation (LTP) was induced using high-frequency stimulation (HFS) comprised of two 1s 100 Hz trains, with a 60s interval, delivered at 70-80% of evoked spike intensity.

**Aβ ELISA**

Frozen mouse forebrain samples were sonicated as previously described (28). Samples were centrifuged at 16,000 x g for 20 min at 4 °C. The supernatant was collected for ELISA. Aβ 1-42 (Thermo Fisher Scientific, Cat#KMB3441) and Aβ 1-40 (Thermo Fisher Scientific, Cat# KMB3481) ELISAs were performed according to the manufacturer’s instructions. 96-well plates were read at 450 nm using an iMark™ microplate reader (Bio-Rad).

**Golgi-Cox stain**

Brains were processed with the Rapid Golgi Kit (FD NeuroTechnologies, MD, USA, Cat#PK401) in accordance with manufacturer’s instructions. Transverse hippocampal sections (100 µm) were cut using a Leica VT1200S vibratome and mounted to gelatin coated slides. Sections were developed and fixed in accordance with kit instructions. Imaging was performed using BZ-X710 all-in-one fluorescent microscope (Keyence, Japan) with a 100x/NA 1.45 oil lens. 5 different view fields containing approximately 200 µm of dendrites were imaged in the stratum radiatum of area
CA1 per hippocampal slice. Dendritic spine density was calculated by the number of total spines per dendritic region of interest. Spines were morphologically classified based on published guidelines (34, 68). Spine analysis was done blinded.

**Transmission electron micrographs**

Samples for electron microscopy were prepared as previously described (44). Briefly, freshly dissected 1 mm transverse hippocampal sections had area CA1 dissected and fixed in 1% PFA + 2.5% glutaraldehyde in 0.1M Millonig’s phosphate buffer (pH 7.3) overnight. Samples were washed and post-fixed in 1% osmium tetroxide in PBS 1 hr. Samples were dehydrated through graded ethanol series and incubated in propylene oxide for two 15 min changes. Samples were subsequently infiltrated with Spurr’s resin and cured overnight at 70ºC. 90 nm sections were made with a Reichert-Jung Ultracut E ultramicrotome, stained with lead citrate and uranyl acetate and viewed with a FEI Tecnai Spirit TEM operating at 80 kV (FEI Company, Hillsboro, OR). Images were obtained with a 2Vu CCD camera (Advanced Microscopy Techniques, Woburn, MA) at 11,000X. Three mice were used per genotype, and 20 images of the stratum radiatum of area CA1 were taken. ImageJ was used to analyze the PSD counts. Imaging and analysis were done blinded.

**Surface sensing of translation (SUnSET) assay**

Acute 400 µm transverse hippocampal slices were prepared using a Leica VT1200S vibratome as described (67). Slices were maintained at room temperature prior to experimentation (2 hr) in artificial cerebrospinal fluid (ACSF). Slices were incubated at 32ºC for 1 hr in bubbling ACSF containing puromycin (1µg/mL), slices were flash frozen and area CA1 was micro-dissected for Western Blot analysis (see above). Puromycin labelled proteins were detected using an anti-puromycin antibody and *de novo* protein synthesis was determined with the total lane density from 250 kDa to 15 kDa using ImageJ.
Mass spectrometry and Proteomics Analysis

Whole hippocampi were flash frozen on dry ice and dissected in ice-cold PBS and lysed immediately in 500 µL of PBS with protease/phosphatase inhibitor using a Bead Mill Homogenizer (Bead Ruptor, Omni International, Kennesaw, GA). 500 µL of 2X radioimmunoprecipitation (RIPA) buffer was added and the mixture was incubated on ice for 30 minutes before centrifugation at 18,000 x g for 10 mins. Protein amount was measured in supernatant and 50 µg of protein was subjected to tryptic digestion.

Reducing alkylation was performed in the presence of 10 mM dithiothreitol and 30 mM iodoacetamide. Four times the sample volume of cold acetone was added to the tube which was incubated at -20 °C overnight. Tubes were centrifuged at 14,000 x g for 10 mins to obtain protein pellet, which was re-suspended in 50 mM ammonium bicarbonate. 1 µg of sequencing-grade modified trypsin was added (1:50 enzyme to substrate) and incubated at 37 °C overnight. Tryptic digest was purified using a C18 desalting spin-column, and then prepared in 5% (v/v) ACN containing 1% (v/v) formic acid for LC-MS/MS analysis.

The LC-MS/MS system consisted of a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Rockford, IL) and a Dionex Ultimate-3000 nano-UPLC system (Thermo Scientific, Rockford, IL) employing a Nanospray Flex Ion Source (Thermo Scientific, Rockford, IL). An Acclaim PepMap 100 (C18, 5 µm, 100 Å, 100 µm x 2 cm) trap column and an Acclaim PepMap RSLC (C18, 2 µm, 100 Å, 75 µm x 50 cm) analytical column were used for the stationary phase. Peptides were separated employing a linear gradient consisting of mobile phases A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) where the gradient was from 5% B at 0 min to 40% B at 170 min. MS spectra were acquired by data dependent scans consisting of MS/MS scans of the twenty most intense ions from the full MS scan with a dynamic exclusion option, which was 10 seconds.
Spectra were searched using the Sequest HT algorithm within the Proteome Discoverer v2.1 (Thermo Scientific) in combination with the mouse UniProt protein FASTA database (annotated 16,747 entries, December 2015). Search parameters were as follows; FT-trap instrument, parent mass error tolerance of 10 ppm, fragment mass error tolerance of 0.02 Da (monoisotopic), variable modifications of 16 Da (oxidation) on methionine and fixed modification of 57 Da (carbamidomethylation) on cysteine.

Significantly altered proteins were calculated as previously described\(^2\), proteins with a fold change >1.5 or <0.667 and a \( p < 0.08 \) were considered significant. Proteins significantly different from the WT condition were calculated and the signal intensity was used to generate a heat map using PermutMatrix(69) (Montpellier Bioinformatics Platform: http://www.atgc-montpellier.fr/permutmatrix). Venn diagrams were generated using BioVenn (http://www.biovenn.nl)(70).

**Statistical analyses**

Data are presented as box and whisker plots which represent the interquartile range, with the line across the box indicating the median. Whiskers show the highest and lowest values detected. For comparisons between groups, a two-tailed unpaired student’s \( t \)-test was used. For multiple groups, one-way ANOVA with Tukey’s post-hoc analysis (when applicable) were used. Error probabilities of \( p < 0.05 \) were considered statistically significant. Outliers were determined via Grubbs test. Statistics were performed using Prism 7 statistics software (GraphPad Software, San Diego, CA).

**Study Approval**
Mice were kept in compliance with the NIH Guide for Care and Use of Laboratory Animals. All animal experiments were performed in accordance with and with the approval of the Institutional Animal Care and Use Committee at Wake Forest University (protocol number: A17-116).

Acknowledgments

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Author Contributions

H.R.Z. conceptualized experiments, collected and analyzed data, and wrote the manuscript. W.Y., B.C.B, N.P.K., X.Z., and X.W. collected and analyzed data. J.L. and C.M.F. collected and help analyze the proteomics data. C.D.K. advised on pathology and provided human tissue samples. T.M. conceptualized experiments and wrote the manuscript.
References


### Table 1. AD Patient Demographics

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Control (CT); Alzheimer’s disease (AD); Consortium to Establish a Registry for Alzheimer’s disease score (CERAD)
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Control (CT); Lewy Body Dementia (LBD); Postmortem Interval (PMI); Consortium to Establish a Registry for Alzheimer’s disease score (CERAD)
Table 3. FTD Patient Demographics

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Control (CT); PSP (Progressive Supranuclear Palsy (PSP); Consortium to Establish a Registry for Alzheimer’s disease score (CERAD)
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Top altered proteins in hippocampi of Tg19959 mice (as compared to WT) which were uniquely restored by AMPKα1 reduction, n = 3 mice per genotype.
Figure Legends and Figures

Figure 1. Expression of AMPKα isoforms is dysregulated in AD hippocampus. (A) Hippocampus lysate from sAD patients showed increased AMPKα1 and decreased AMPKα2 levels as compared to those of age-matched controls (n = 10 with up to 4 technical replicates, *p=0.0119, **p=0.0014, unpaired t-test). (B) Representative images of AMPKα isoform dysregulation in area CA1 of hippocampus in AD and age matched control patients (scale bar = 50 μm). Immunohistochemical experiments were replicated independently 3 times. (C) AMPKα isoform expression was unaffected in cerebellum samples from AD patients (n = 5 with up to 3 technical replicates, p=0.8457 for AMPKα1, and p=0.9870 for AMPKα2, unpaired t-test). (D) Hippocampal lysate from LBD patients had unaffected AMPKα isoform levels (n = 4 for CT and n=3 for LBD with 1 technical replicate, p=0.9146 for AMPKα1, and p=0.5635 for AMPKα2, unpaired t-test). (E) Levels of AMPK isoforms were unaltered in hippocampal tissue from FTD patients (n = 8 for CT with 1 technical replicate, n = 5 for FTD with up to 3 technical replicates, p=0.9283 for AMPKα1 and p=0.335 for AMPKα2, unpaired t-test). (F) AMPKα1 levels were significantly increased in cortical lysates from FAD patients while AMPKα2 levels are unaffected (n = 5 with 4 technical replicates, **p=0.0060 for AMPKα1 and p=0.9412 for AMPKα2, unpaired t-test). (G) AMPKα1 levels are significantly increased in hippocampal lysates from Tg19959 AD model mice compared to WT controls. AMPKα2 levels were unaffected (n = 7 with up to 2 technical replicates, **p=0.0023 for AMPKα1 and p=0.9094 for AMPKα2, unpaired t-test). Box and whisker plots represent the interquartile range, with the line across the box indicating the median. Whiskers show the highest and lowest values detected. (H) Immunofluorescent labeling of DAPI (Blue), and AMPKα1 (red) distribution in area CA1 mouse hippocampal slices (n = 3, scale = 200 μm).
Figure 2. Brain-specific suppression of AMPKα1 alleviates learning and memory defects in Tg19959 AD model mice. (A) Brain-specific genetic reduction of AMPKα1 and AMPKα2 in Tg19959 AD model mice. Non-congruous WT, Tg19959 (Tg), AMPKα1(+/−)/Tg19959 (α1/Tg), AMPKα2(+/−)/Tg19959 (α2/Tg) (n = 10, 10, 6, 7), up to three technical replicates. For AMPK α1: WT vs Tg *p=0.0212, WT vs α2/Tg **p=0.0029, Tg vs α1/Tg *p=0.0130, α1/Tg vs α2/Tg **p=0.0016. One-way ANOVA with Tukey’s post-hoc test, F = 8.218. For AMPKα2: WT vs α2/Tg **p=0.005, Tg vs α2/Tg, *p=0.043, α1/Tg vs α2/Tg, **p=0.007, one-way ANOVA with Tukey’s post-hoc test, F = 7.585. (B) Representative H&E stain of hippocampal structure (n = 3, scale bar = 50 μm). (C) Percentage of time spent in the periphery for the open field (OF) test (n = 25, 21, 17, 14, 19, 13). (D) Percentage time spent with familiar (white) and novel (purple) objects in the Novel Object Recognition (NOR) task during the testing phase. Preference < 50% indicates cognitive impairment. (n=19,13, 10, 9, 10, 8). Statistical preference for novel or familiar object; WT *p<0.0001, Tg p=0.5523, α1/cre *p=0.0004, α1/Tg *p=0.0008, α2/cre *p=0.0465, α2/Tg *p=0.1497, unpaired t-test. (E) Escape latency (s) over 5 days of training in the hidden platform Morris Water Maze (MWM) (4 trials/day, 5 days, n=19, 17, 13, 17, 19, 13). WT vs α1/Tg *p=0.0256, WT vs α2/cre *p=0.0121, α1/cre vs α2/cre *p=0.0371, Tg vs α1/Tg, **p=0.0094, α1/Tg vs α2/Tg ***p=0.0009, ****p<0.0001 one-way ANOVA with Tukey’s post-hoc test, F = 18.16). (F) Percentage of time spent in the target quadrant during probe trial phase of MWM task. WT vs α2/Tg *p=0.0186, WT vs Tg **p=0.0027, one-way ANOVA with Tukey’s post-hoc test, F = 4.525. Box and whisker plots represent the interquartile range, with the line across the box indicating the median. Whiskers show the highest and lowest values detected.
Figure 3. Suppression of AMPKα does not alter AD-associated brain Aβ pathology or tau phosphorylation. (A) Representative images of cortical Aβ plaque deposition in WT, Tg, α1/Tg, and α2/Tg mice. Scale bars = 100 μm for 20x images, 50 μm for 60x images. (B) Percentage of cortex covered by Aβ plaques in Tg, α1/Tg, and α2/Tg mice, n = 9 slices / 3 mice. (C) Representative images of hippocampal Aβ plaque deposition in WT, Tg, α1/Tg, and α2/Tg mice. Scale bars = 100 μm for 20x images, 50 μm for 60x images. (D) Percentage of hippocampal area covered by amyloid plaques in Tg, α1/Tg, and α2/Tg mice, n = 9 slices / 3 mice. (E) ELISA of prefrontal cortex lysate showed decreases of Aβ1-40 in both α1/Tg and α2/Tg mice, as compared to Tg mice (Tg vs α1/Tg *p=0.0356, Tg vs α2/Tg *p =0.0226, one-way ANOVA with Tukey’s post-hoc test, F = 5.034). (F) ELISA showed no differences in levels of Aβ1-42 in Tg, α1/Tg and α2/Tg mice (one-way ANOVA with Tukey’s post-hoc test F = 1.771). (G) The ratio of Aβ42:40 was unaltered in Tg, α1/Tg and α2/Tg mice. (One-way ANOVA with Tukey’s post-hoc test F = 1.635) (Tg n = 12, α1/Tg and α2/Tg n = 9). (H) Western blot analysis of p-Tau (S396 and S262) levels in hippocampus showed no differences between WT, Tg, α1/Tg, and α2/Tg mice (WT n = 8, Tg n = 6, α1/Tg n = 6, α2/Tg n =7, one-way ANOVA). Box and whisker plots represent the interquartile range, with the line across the box indicating the median. Whiskers show the highest and lowest values detected.
Figure 4. Reduction of brain AMPKα1 corrects AD-associated deficits in synaptic density and dendritic spine morphology. (A) Classification of mature (mushroom, stubby, and branched) and immature (filopodia, long and short thin) spine types on dendrite (scale bar = 20 μm). (B) Representative images from Golgi-Cox stain of area CA1 dendritic spines (100X, scale bar = 5 μm) and quantification of total (mature & immature) spine density. Tg and α2/Tg spines have significantly decreased spine density as compared to WT and α1/Tg mice (**p<0.0001, one-way ANOVA with Tukey’s post-hoc test F = 107.2). (C-E) Quantification of sub-classification of mature spines. Tg and α2/Tg mice have significantly fewer stubby (F = 18.84), branched (F = 20.87), and mushroom (F = 64.33) spines than WT and α1/Tg mice. ***p<0.0001, one-way ANOVA with Tukey’s post-hoc test. (F) Quantification of mature spine density. (WT n = 4, Tg, α1/Tg, and α2/Tg n = 3, 200 μm spine length analyzed from 5 ROIs per slice, 3-7 slices per mouse). ***p<0.0001, one-way ANOVA with Tukey’s post-hoc test. (G) Representative transmission electron micrographs (TEM) from hippocampal CA1 dendrites and quantification of post-synaptic densities (PSDs), (n = 3 animals per group, scale bar= 500 μm), blue arrows indicate PSDs. Number of PSDs are significantly decreased in Tg and α2/Tg mice as compared to WT and α1/Tg mice (**p<0.0001, one-way ANOVA with Tukey’s post-hoc test, F = 49.77). (H) Western Blot analysis of hippocampal lysate showed significantly reduced levels of PSD95 in Tg and α2/Tg mice as compared to WT and α1/Tg mice, (WT n = 10, Tg n = 9, α1/Tg n = 6, α2/Tg n = 7, *p=0.0305, **p=0.0047, Tg vs α1/Tg ***p=0.0006, Tg vs α2/Tg ***p<0.0001 one-way ANOVA with Tukey’s post-hoc test, F = 9.572, non-congruous). Box and whisker plots represent the interquartile range, with the line across the box indicating the median. Whiskers show the highest and lowest values detected.
Figure 5. Repression of brain AMPKα1 restores abnormal eEF2 phosphorylation and de novo protein synthesis deficits in Tg19959 mice. (A) Genetic reduction of AMPKα1 but not AMPKα2 corrected eEF2 hyper-phosphorylation in hippocampi of Tg19959 AD model mice (n = 5 with three technical replicates, non-congruous). WT vs Tg, *p=0.0297, ***p<0.001, one-way ANOVA with Tukey's post-hoc test, F = 13.88. (B) Representative images and quantification for surface sensing of translation (SUnSET) puromycin incorporation assay. Assay was performed on area CA1 of hippocampus. (WT, Tg, and α2/Tg n = 5, α1/Tg n = 3. *p=0.0231, **p=0.0027, ***p <0.001, one-way ANOVA with Tukey’s post-hoc test, F = 12.85). Box and whisker plots represent the interquartile range, with the line across the box indicating the median. Whiskers show the highest and lowest values detected. (C) Patterns of protein expression in hippocampi of WT, Tg, α1/Tg, and α2/Tg mice, reported as normalized deviation from the mean. (D-E) Venn diagrams showing proteins that were significantly altered (up-regulated or down-regulated) in the Tg condition that were restored to WT levels in either α1/Tg, α2/Tg, or both α1/Tg and α2/Tg hippocampi.
Figure 6. Reduction of brain AMPKα1 mitigates impairments of cognition and synaptic plasticity associated with APP/PS1 AD model mice. (A) Genetic reduction of AMPKα1 but not AMPKα2 corrected eEF2 hyper-phosphorylation in hippocampi of APP/PS1 mice. n=4, WT vs α2/APP, *p=0.0464, WT vs APP **p=0.0029, α1/APP vs α2/APP **p=0.006, α1/APP vs APP ***p=0.0003, one-way ANOVA with Tukey’s post-hoc test, F = 8.455. (B) Unaltered periphery time in OF task (n = 28, 20, 13, 20). (C) Preference for familiar (white) or novel (purple) objects in the NOR task. (n = 12, 12, 10, 10). WT, *p<0.0001, APP p=0.7602, α1/APP *p<0.0001, α2/Tg *p=0.0123, unpaired t-test). (D) Escape latency (s) in the hidden-platform Morris water maze (MWM), escape latency of day 5 was significantly higher in APP and α2/APP mice (n = 18, 18, 9, 15). α1/APP vs α2/APP *p=0.0259, WT vs α2/APP **p=0.0013, APP vs α1/APP **p=0.0064, WT vs APP ***p<0.0001. F = 10.53. (E) Target quadrant occupancy during probe trial of MWM task. WT vs APP *p=0.0298, WT vs α2/APP **p =0.003, one-way ANOVA with Tukey’s post-hoc test, F = 5.259. (F) Hippocampal LTP (HFS denoted by arrow, n=18, 12, 12, 7, 8, 9). (G) Representative fEPSP traces pre- and post-HFS. (H) Quantification of the fEPSP slope 90 mins post-HFS (WT vs APP ***p=0.0009, WT vs α2/APP **p=0.0011, α1/cre vs APP **p=0.0017, α1/cre vs α2/APP **p=0.0017, one-way ANOVA with Tukey’s post-hoc test F = 7.519). (I-J) Representative images and quantification of cortical Aβ plaque deposition. n=9 slices/3 mice (K-L) Representative images and quantification of hippocampal Aβ plaque deposition. n=9 slices /3 mice. Scale bars: 100 µm for 20x images, 50 µm for 60x images. Box and whisker plots represent the interquartile range, with the line across the box indicating the median. Whiskers show the highest and lowest values detected.