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Repression of eEF2 kinase improves deficits in novel object recognition memory in aged mice.

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Abstract

The normal aging process is commonly associated with mild cognitive deficits including memory decline. Previous studies indicate a role of dysregulated mRNA translation capacity in cognitive defects associated with aging and aging-related diseases, including hyperphosphorylation of eukaryotic elongation factor 2 (eEF2). Phosphorylation of eEF2 by the kinase eEF2K inhibits its activity, hindering general protein synthesis. Here, we sought to determine whether cognitive deficits in aged mice can be improved by genetically deleting eEF2K (eEF2K KO) and consequently reduction of eEF2 phosphorylation. We found that suppression of eEF2K prevented aging-related deficits in novel object recognition memory. Interestingly, deletion of eEF2K did not alter overall protein synthesis in the hippocampus. Ultrastructural analysis revealed increase size and larger active zone lengths of postsynaptic densities (PSDs) in the hippocampus of aged eEF2K KO mice. Biochemical assays showed hippocampal eIF2α hyperphosphorylation in aged eEF2K KO mice, indicating inhibition of translation initiation. Our findings may provide insight into mechanistic understanding and thus development of novel therapeutic strategies for aging-related cognitive decline.

Keywords

Aging; protein synthesis; elongation factor 2; memory; signaling; synapse

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Credit Author Statement

S.G. conceptualized experiments, collected and analyzed data, and wrote the manuscript. H.J. collected and analyzed data, and contribute to the manuscript writing. X.Z. collected data and provided technical help. A.R. advised on eEF2K knockout mice. T.M. conceptualized experiments and wrote the manuscript.

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1. Introduction

Cognitive function declines with normal aging. With the aging population growing rapidly (Federal Interagency Form on Aging Related Statistics, 2016), the incidence of individuals with cognitive impairment is increasing markedly and represents a significant biomedical concern worldwide (Plassman et al., 2008). This growth demonstrates the need to uncover the molecular mechanisms associated with aging-related impairments of cognition, and accordingly identify therapeutic strategies that may alleviate cognitive decline to improve quality of life for the elderly and their families.

Pathological demented conditions such as Alzheimer's disease (AD) often involve massive neuronal loss or neurodegeneration. In comparison, alteration of neuronal structures occurring during normal aging are usually subtler and less aggressive, and previous studies in both humans and animals indicate that cognitive decline with normal aging is associated with sub-cellular synaptic alterations in hippocampus and prefrontal cortex (PFC) (Morrison and Baxter, 2012). Nevertheless, molecular signaling mechanisms underlying aging-associated cognitive deficits remains unclear. It is established that long-lasting forms of memory and synaptic plasticity depend on *de novo* protein synthesis (i.e. mRNA translation) (Alberini, 2008; Costa-Mattioli et al., 2009; Remaud et al., 2014; Rossato et al., 2007). The synthesis of new proteins is mediated partially by eukaryotic elongation factor 2 (eEF2). eEF2 is critical for the translocation step of elongation by facilitating the transfer of aminoacyl tRNA from the ribosomal A-site to the P-site via GTP hydrolysis. Phosphorylation of eEF2 at site Thr 56 by its only known kinase, eEF2K, results in inhibition of its activity and suppression of general protein synthesis (Proud, 2015; Ryazanov and Davydova, 1989). Activity of eEF2K itself can be regulated by multiple upstream signaling molecules including AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin complex 1 (mTORC1). In brief, eEF2K activity is inhibited by mTORC1, leading to de-phosphorylation of eEF2 and consequently promotion of general protein synthesis. On the other hand, AMPK increases activity of eEF2K, and results in eEF2 phosphorylation thus suppression of protein synthesis (Browne et al., 2004). Interestingly, a recent study reports hyperphosphorylation of eEF2 (indicative of impaired protein synthesis capacity) in hippocampi of aged mice that exhibit memory impairments and synaptic plasticity defects (Yang et al., 2019). Meanwhile, the roles of eEF2 hyperphosphorylation in aging-associated cognitive decline are unknown. Here, taking advantage of a unique aged mouse model with genetic deletion of eEF2K, we sought to determine whether repression of eEF2 phosphorylation could prevent cognitive deficits associated with normal aging.

2. Materials and Methods

2.1. Mice.

Homozygous *Eef2k*$^{-/-}$ mice were developed as described before (Beckelman et al., 2019; Chu et al., 2014). All mice were kept in the Wake Forest School of Medicine barrier facility under the supervision of the Animal Research Program. Mice followed a standard 12-hour light/12-hour dark cycle, with regular feeding, cage cleaning, and 24-hour food and water

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access. Both male and female mice were used for experimentation. All mice were subjected to behavioral and biochemical analyses at 21-23 months of age.

2.2. Open Field (OF).

All mice were handled for at least 5 days prior to behavioral testing and habituated to the testing room for 1 hour prior to experimentation. Animals were placed in an opaque plastic open field chamber (40 x 40 x 40 cm) and permitted to freely explore for 15 minutes. Time spent in the center and periphery of the chamber were measured and calculated as percentage of the total time. Velocity and distance moved were assessed using EthoVision XT Tracking Software (Noldus Information Technology). Data collection and analysis were performed blinded to genotype.

2.3. Morris Water Maze (MWM).

Morris water maze (MWM) test was performed as described (Yang et al., 2019). The training paradigm for the hidden platform version of the MWM consisted of 4 trials (60 sec maximum; interval 15 min) each day for 5 consecutive days. The probe trial was performed 2 hours after the completion of training on day 5. The visible platform task consisted of 4 trials each day for 2 consecutive days with the escape platform marked by a visible cue and moved randomly between four locations. The trajectories were recording with a video tracking system (EthoVision XT). Data collection and analysis were performed blinded.

2.4. Novel Object Recognition (NOR).

All mice were subjected to a 2-day familiarization protocol in which they were placed in an opaque plastic chamber (40 × 40 × 40 cm) with 2 identical and randomly selected objects and allowed to explore for 5 minutes. 24 hours after familiarization, animals were placed in the chamber again, except with one object replaced with a randomly chosen novel object. Placement of novel objects was counterbalanced. Time spent with each object was measured, both manually and using EthoVision 14 tracking software, and calculated as a percentage of total interaction time. Mice that exhibited less than 8 seconds of total interaction time were excluded from analysis. Novel object preference less than 50% indicates memory impairment (Antunes and Biala, 2012; Yang et al., 2019).

2.5. Western blots.

Tissues were dissected from appropriate brain structures and flash frozen on dry ice. Tissues were homogenized in lysis buffer and quantified using a BCA assay as previously described (Beckelman et al., 2019). Samples were loaded into 4-15% TGX™ Precast Gels (Bio-Rad), and transferred to nitrocellulose membranes. Membranes were blocked and probed with selected primary antibodies overnight at 4°C. Primary antibody was washed off the blot and HRP-conjugated secondary antibodies were added. Primary antibodies used: p-eEF2 (Thr 56) (1:1,000, Cell Signaling, catalog 2331S), eEF2 (1:1,000, Cell Signaling, catalog 2332S), Puromycin (1:10,000, Millipore, catalog MABE343), p-eIF2α (Ser 51) (1:1,000, Cell Signaling, catalog 3398S), eIF2α (1:1,000, Cell Signaling, catalog 9722S), p-mTOR (Ser 2448) (1:1,000, Cell Signaling, catalog 5536S), mTOR (1:1,000, Cell Signaling, catalog 2983S), p-4EBP1 (Thr 37,46) (1:1,000, Cell Signaling, catalog 2855S), 4EBP1 (1:1,000,

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Cell Signaling, catalog 9644S), p-p70S6K1 (Thr 389) (1:1,000, Cell Signaling, catalog 9234S), p70S6K1 (1:1,000, Cell Signaling, catalog 2708S), Arc/Arg3.1 (1:1,000, Cell Signaling, catalog 65650S), PKCζ (1:1,000, Santa Cruz Biotechnology, sc-17781), PSD95 (1:1,000, Cell Signaling, catalog 3450S), Synapsin (1:1,000, Cell Signaling, catalog 85852S), p-CaMKIIα (Thr 286) (1:1,000, Cell Signaling, catalog 12716S), CaMKII (pan) (1:1,000, Cell Signaling, catalog 3362S) and GAPDH (1:10,000, 2118S). All antibodies were diluted using either 5% wt/vol milk/TBST or 5% wt/vol BSA/TBST. Blots were visualized using chemiluminescence (Clarity™ ECL; Bio-Rad) and the Bio-Rad ChemiDoc™ MP Imaging System. Densitometry was conducted using ImageJ software (NIH). Data were normalized to relevant total proteins for phosphorylated protein analysis and GAPDH for total protein analysis.

2.6. Surface Sensing of Translation (SUnSET) assay.

Acute 400 μm transverse hippocampal slices were prepared using a Leica VT1200S vibratome as previously described (Beckelman et al., 2016). Slices were maintained in artificial cerebrospinal fluid (ACSF) for at least 2 hours at room temperature before experimentation. Slices were then incubated for 1 hour in bubble ACSF containing Puromycin (1 μg/mL) at 32°C. Area CA1 was micro-dissected and slices were flash frozen for Western blot analysis. Anti-puromycin antibody was used to detect Puromycin labeled proteins and *de novo* proteins synthesis was quantified from the total lane density (250 kDA to 15 kDa) using ImageJ.

2.7. Transmission electron microscopy (TEM).

Animal brains were removed and 1 mm thick transverse slices were cut using a Leica VT1200S vibratome. The CA1 was microdissected and fixed in 2.5% glutaraldehyde/1% paraformaldehyde in 0.1M Millonig's phosphate buffer (pH 7.3) overnight. The following day the samples were washed in buffer and post-fixed with 1% osmium tetroxide in phosphate buffer for an hour. Samples were then dehydrated through a graded series of ethanol solutions. For preparation of resin infiltration, the samples were incubated in propylene oxide for two 15-minute changes. The samples were subsequently infiltrated with 1:1, 1:2, and pure solutions of Spurr's resin and placed in a 70°C oven overnight to cure. A Reichert-Jung Ultracut E ultramicrotome was used to obtain 90 nm thick sections, stained with lead citrate and uranyl acetate, and viewed with a Tecnai Spirit transmission electron microscope operating at 80 kV (FEI Co.). Images were obtained with a 2Vu CCD camera (Advanced Microscopy Techniques) at ×11,000 magnification. Analysis was performed as previously described (Ostroff et al., 2002; Ostroff et al., 2018). Imaging and analysis were done by investigators blinded to animal groups.

2.8. 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. Development was performed according to kit instructions. Sections were dehydrated through a graded ethanol series and cleared in 100% xylene. Slides were coverslipped with VectaMount Permanent Mounting Medium (Vector Labs, catalog H-5000) and imaged at

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x100 on a Keyence BZ-X710 microscope. Area CA1 stratum radiatum apical dendrite segments (average 100-150 microns in length) were quantified. Images were blinded and spines were manually counted and sorted as previously described (Risher et al., 2014). This approach depends on individual geometric identities of mature and immature spines. Specifically, based on previously identified measurements, immature spines (i.e. filopodial, thin and long thin) are characterized by thin processes and the absence of a defined head. On the other hand, mature spines (stubby, mushroom and branched) are defined by a dendritic spine head.

3. Results

3.1. Genetic deletion of eEF2K results in reduction of phosphorylated eEF2 and improvement of recognition memory in aged mice.

Previous work has demonstrated that aged, behaviorally-impaired mice (18-20 months) exhibit hyperphosphorylated eEF2 in the hippocampus (Yang et al., 2019). We sought to determine if genetic suppression of eEF2K and presumably eEF2 phosphorylation could prevent age-related deficits in cognition. Using Western blot analysis, we first confirmed the reduction of phosphorylated eEF2 in hippocampal tissue from aged eEF2K KO mice compared with WT controls (Fig. 1A). To assess the behavioral effects of this genetic manipulation, mice were subjected to a series of tasks, including open field (OF), Morris Water Maze (MWM) and novel object recognition (NOR). In OF, a test to measure general locomotor activity and anxiety, there were no differences between the two groups (Supplemental Fig. 1A–C). We then assessed spatial learning and memory performance of the mice by applying hidden-platform MWM assay. Consistent with what we have reported recently (Yang et al., 2019), aged WT mice displayed impaired spatial learning and memory, as indicated by insignificant day-today escape latency, and less-than-normal target quadrant occupancy during probe trial test phase (Fig. 1B–C). Interestingly, aged eEF2K KO mice showed similar impaired spatial learning/memory assessed by hidden-platform MWM (Fig. 1B–C). We also observed similar time spent in non-target quadrants and no significant difference in day 2 platform latency in the visible platform task (Supplemental Fig. 1D–E). Next we employed NOR task to evaluate long-term hippocampus-dependent recognition memory. In agreement with recent findings (Yang et al., 2019), aged WT mice showed similar interaction with the familiar and novel object, indicating cognitive impairment (Fig. 1D–E). Importantly, aged eEF2K KO mice spent significantly more time with the novel object than with the familiar object, indicating preserved recognition memory (Fig. 1D–E). Interestingly, further analysis of the NOR data revealed potential gender-dependent effects of eEF2K KO (Supplemental Fig. 1F). Taken together, suppression of eEF2 phosphorylation alleviates aging-related recognition memory.

3.2. Suppression of eEF2K did not alter levels of de novo protein synthesis in hippocampi of aged mice.

We went on to investigate potential mechanisms underlying the beneficial effects of eEF2K suppression on recognition memory in aged mice. We asked whether overall new protein synthesis in hippocampus is boosted by eEF2K suppression. To measure levels of *de novo* protein synthesis, we employed a surface sensing of translation (SUnSET), a nonradioactive

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puromycin end-labeling assay (Schmidt et al., 2009). Compared to the WT group, no overall change in puromycin incorporation was detected in hippocampal slices from eEF2K KO mice, suggesting unaltered general *de novo* protein synthesis (Fig. 2A–B). We further use Transmission electron microscopy (TEM) to examine potential changes in hippocampal polyribosomes (clusters of ribosomes), which usually indicates active mRNA translation (Ostroff et al., 2002). Compared to the WT group, no change in hippocampal polyribosome count was observed in eEF2K KO mice, consistent with the data from the SUnSET experiment. Thus, overall protein synthesis in hippocampus of aged mice is unaltered upon genetic deletion of eEF2K.

3.3. Reduction of eEF2 phosphorylation improves hippocampal synaptic morphology in aged mice.

Integral postsynaptic densities (PSDs) are critical for normal synaptic and cognitive function (Okabe, 2007). Additionally, the area of PSD correlates with the spine head volume and total number of presynaptic vesicles, including vesicles fused at the presynaptic active zone (Harris and Weinberg, 2012). We examined whether eEF2K deletion may alter properties of hippocampal PSDs in aged mice. Ultrastructural analysis on TEM images revealed that while genetic reduction of eEF2 phosphorylation in aged mice did not alter overall PSD count (Fig. 3A–B), it resulted in significantly larger active zone lengths (Fig. 3C) and increased size of PSD (Fig. 3D). We also investigated the effects of eEF2K suppression on dendritic spine morphology in hippocampus by performing the rapid Golgi staining protocol (Risher et al., 2014). We did not find differences in the total spine count between WT and eEF2K KO groups (Fig. 3F). Furthermore, there was no significant difference in prevalence of overall “mature” (e.g. stubby, mushroom and branched) and “immature” (e.g. thin and filopodial) dendritic spines between the two groups (Fig. 3E–H). It is worth mentioning that there was a trendy ($p=0.0518$) increase in stubby spine count in eEF2K KO mice compared to WT controls (Fig. 3I). These findings indicate that genetic reduction of eEF2 phosphorylation improves hippocampal synaptic morphology in aged mice.

3.4. Genetic reduction of eEF2 phosphorylation correlates with eIF2α hyperphosphorylation.

We next examined the activity of mTORCI signaling, which plays a critical role in regulating protein synthesis and is well connected with eEF2K/eEF2 phosphorylation (Proud, 2013). Biochemical experiments showed that hippocampal mTORC1 signaling in aged mice was not affected by eEF2K suppression, as indicated by unaltered levels of phosphorylation of mTOR (S2448, Fig. 4A) and its two established downstream substrates p70S6K (Thr389, Fig. 4B) and 4EBP1 (Fig. 4C). Moreover, we investigated the effects of genetic eEF2K deletion in aged mice on eIF2α phosphorylation. Phosphorylation of eIF2α leads to inhibition of translation initiation and plays an important role in regulation of cognition and synaptic plasticity (Costa-Mattioli et al., 2007; Ma et al., 2013; Zimmermann et al., 2018). Surprisingly, we found significant hyperphosphorylation of eIF2α in hippocampi of aged eEF2K KO mice compared to WT group (Fig. 4D). Lastly, genetic deletion of eEF2K did not alter levels of multiple established plasticity related proteins compared to the WT group (Supplemental Fig. 2). Collectively, these findings suggest

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genetic suppression of eEF2K leads to eIF2α hyperphosphorylation, which may contribute to the observation of unaltered general protein synthesis.

4. Discussion

With remarkable increase of average lifespan worldwide, there is an urgent need to identify molecular mechanisms underlying aging-related cognitive impairments and develop corresponding therapeutic approaches to curb such decline. In the current study, we report that genetic suppression of eEF2K, the kinase for mRNA translation factor eEF2, alleviated recognition memory impairments in aged mice. These findings are consistent with recent studies indicating aging- and AD-related eEF2 hyperphosphorylation (Beckelman et al., 2019; Yang et al., 2019). Phosphorylation of eEF2 is usually linked to reduction of *de novo* protein synthesis and eEF2 de-phosphorylation is considered to boost protein synthesis. Therefore, we expected an increase of protein synthesis with genetic deletion of eEF2K and consequently suppression of eEF2 phosphorylation in aged mice. Interestingly, we did not observe significant alterations in overall *de novo* protein synthesis in hippocampi of aged eEF2K KO mice based on results from two independent assays to assess protein synthesis (Fig. 2). The unexpected findings on new protein synthesis might be attributed to several mechanisms including mainly “local” (i.e. dendritic or synaptic) protein synthesis or increased synthesis of specific pools of proteins that are not precisely reflected by assays (i.e. SUnSET) done with lysate from whole hippocampus. Future studies with more comprehensive techniques such as those in cellular imaging combined with proteomics shall help resolve this puzzle. Moreover, we measured protein synthesis in hippocampus under resting state. It is possible that eEF2 phosphorylation (by eEF2K) contributes to de novo protein synthesis more significantly during intense neuronal activities (e.g. induction of certain forms of synaptic plasticity or cognition). Additionally, we found increased phosphorylation of eIF2α in hippocampi of aged eEF2K KO mice (Fig. 4D).

Hyperphosphorylation of eIF2α hinders formation of mRNA translation initiation complex and thus results in suppression of general protein synthesis (Dever, 1999; Hinnebusch, 2000). Therefore, increased phosphorylation of eIF2α could be a compensatory response to reduction of eEF2 phosphorylation, leading to unchanged overall protein synthesis. It was reported that brain-specific deletion of eIF2α kinase PERK leads to reduction of eEF2 phosphorylation (Zimmermann et al., 2018). Taken together with findings in the current study that eEF2K deletion results in eIF2α phosphorylation, it strongly suggests a retrospective regulation between eEF2K and eIF2α. Future studies are warranted to investigate the mechanisms responsible for the crosstalk between these signaling cascades.

It is notable that suppression of eEF2 phosphorylation improves novel object recognition memory (assessed by NOR task) but not spatial learning and memory (evaluated by hidden platform MWM) in aged mice (Fig. 1). While both NOR and MWM are considered to be hippocampal-dependent behavioral assays, the two tasks also involved different brain structures (i.e. PFC) (Akirav and Maroun, 2006; Buzsáki and Moser, 2013). Future studies are necessary to help elucidate whether there exists neuronal structure- or brain region-specific roles of eEF2 phosphorylation in cognition. For instance, it would be interesting to determine whether functions associated with frontal cortex (as compared to hippocampus)

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are more sensitive to eEF2K suppression. Furthermore, the behavioral paradigms in MWM (i.e. swimming for multiple days) are more stressful than those in NOR, which relies on the rodent's innate ability to discern novelty in an open-box environment (Harrison et al., 2009). Thus, it is possible that the potential beneficial effects on cognition associated with eEF2K reduction are overwhelmed by "stress", resulting in "ceiling" effects in stressful tasks such as MWM. It is noteworthy that a recent study demonstrates genetic suppression of eEF2K in AD model mice alleviates AD-associated cognitive impairments in both NOR and MWM tasks (Beckelman et al., 2019). A close examination of such differences may help provide insights in to the understanding of distinct molecular mechanisms (e.g. protein synthesis) in mediating cognitive decline associated with "normal aging" vs. "pathological aging". While mounting evidence demonstrates that neuronal alterations that underlie aging-related cognitive decline are less significant compared to those in aging-related neuronal diseases such as AD, these "subtle" alterations (e.g. PSD changes) might make neurons vulnerable to degeneration in certain disease processes. A better understanding of the mechanisms underlying cognitive changes in normal aging can set a solid background for evaluating situations in pathological diseases. Unlike many other kinases, eEF2K is the only known kinase for eEF2 (at the Thr56 site), and eEF2 is the only known substrate for eEF2K. This unique 1:1 relationship makes it feasible and attractive to explore the possibility of targeting eEF2K/eEF2 phosphorylation as a potential therapeutic strategy for aging-related cognitive impairments in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Suppression of eEF2K alleviates novel recognition memory defects in aged mice.
- Aging-related spatial memory impairments are not affected by eEF2K suppression.
- Inhibition of eEF2K improves hippocampal synaptic morphology in aged mice.
- Reduction of eEF2 phosphorylation correlates with eIF2α hyper-phosphorylation.

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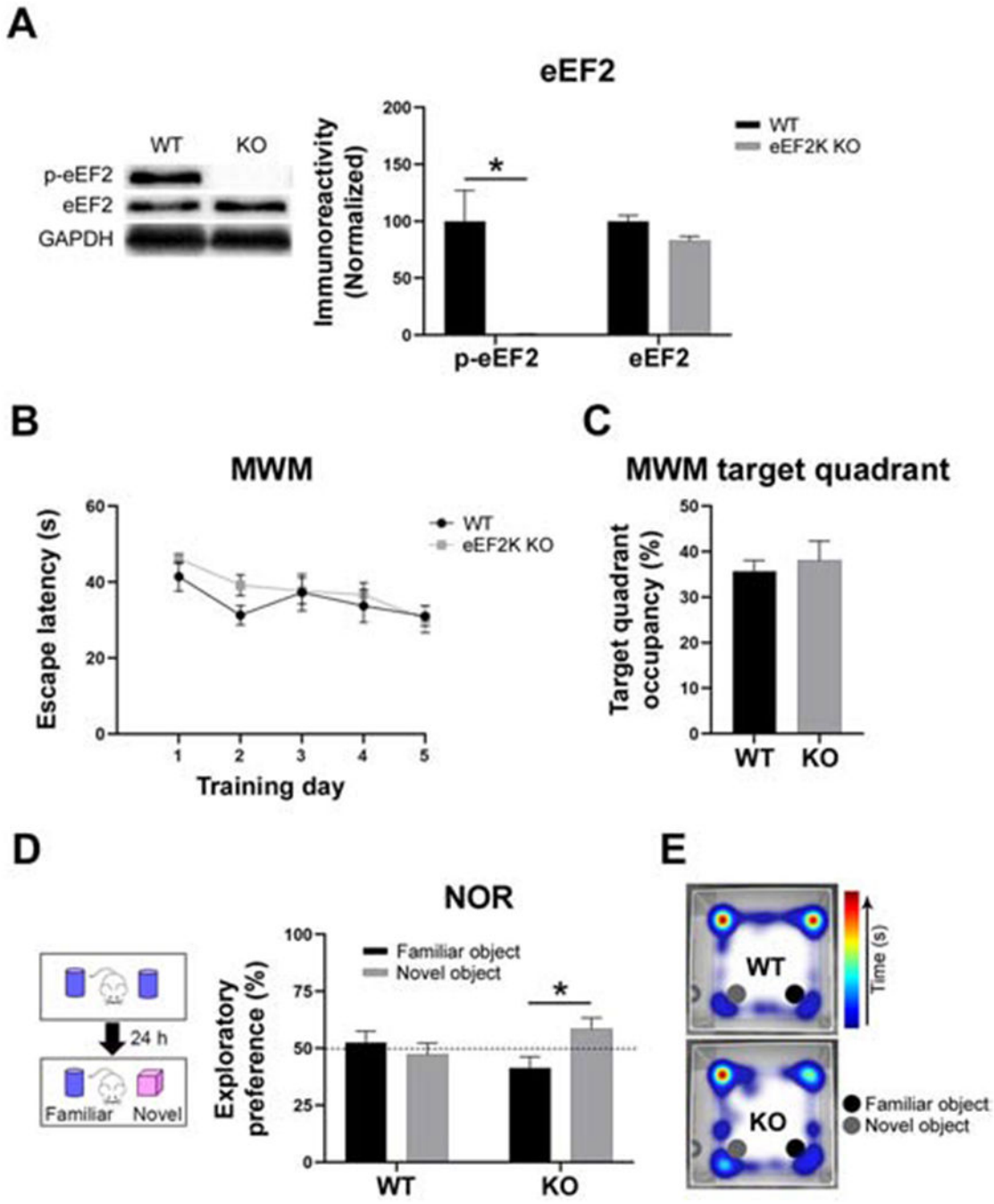

Fig. 1.

Genetic reduction of eEF2K results in elimination of p-eEF2 and improved recognition memory in aged mice. (A) Aged eEF2K KO mice exhibit decreased levels of hippocampal eEF2 phosphorylation compared to age-matched wild-type controls. ($n = 8$. $^*P < 0.05$, unpaired t test.) (B) Escape latency in hidden-platform MWM. (WT, $n = 10$; eEF2K KO, $n = 14$). (C) Target quadrant occupancy during MWM probe trial. (D) Novel object recognition task schematic and exploratory preference for familiar and novel object. (WT, $n = 7$; eEF2K

KO, $n = 8$. $^{*}P < 0.05$, unpaired t test.) (E) Heat map indicating interaction frequency with novel or familiar object.

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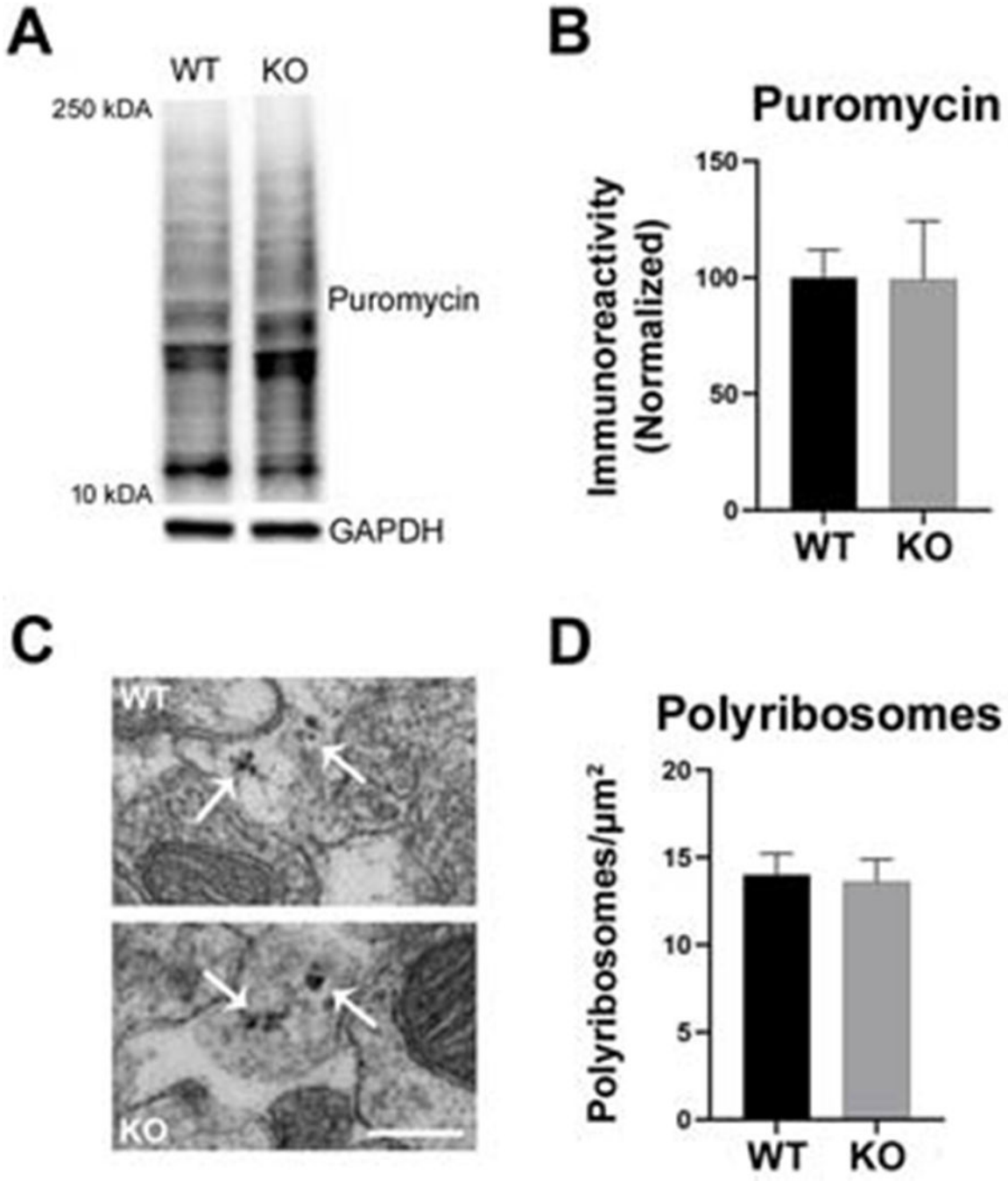

Fig. 2.

Reduction of eEF2 phosphorylation did not alter overall *de novo* protein synthesis in hippocampi of aged mice. (A) Representative image of puromycin Western blot from SUnSET assay. Image represents 10-250 kDa range. (B) Quantification of protein synthesis using SUnSET assay. (WT, $n = 9$; eEF2K KO, $n = 10$). (C) Representative TEM images for polyribosomes in area CA1 of hippocampus. Arrows indicate polyribosomes. ($n = 3$. Original magnification, x11,000. Scale bar: 250 nm.) (D) Quantification of polyribosomes counts. (WT, $n = 3$; eEF2K KO, $n = 3$.)

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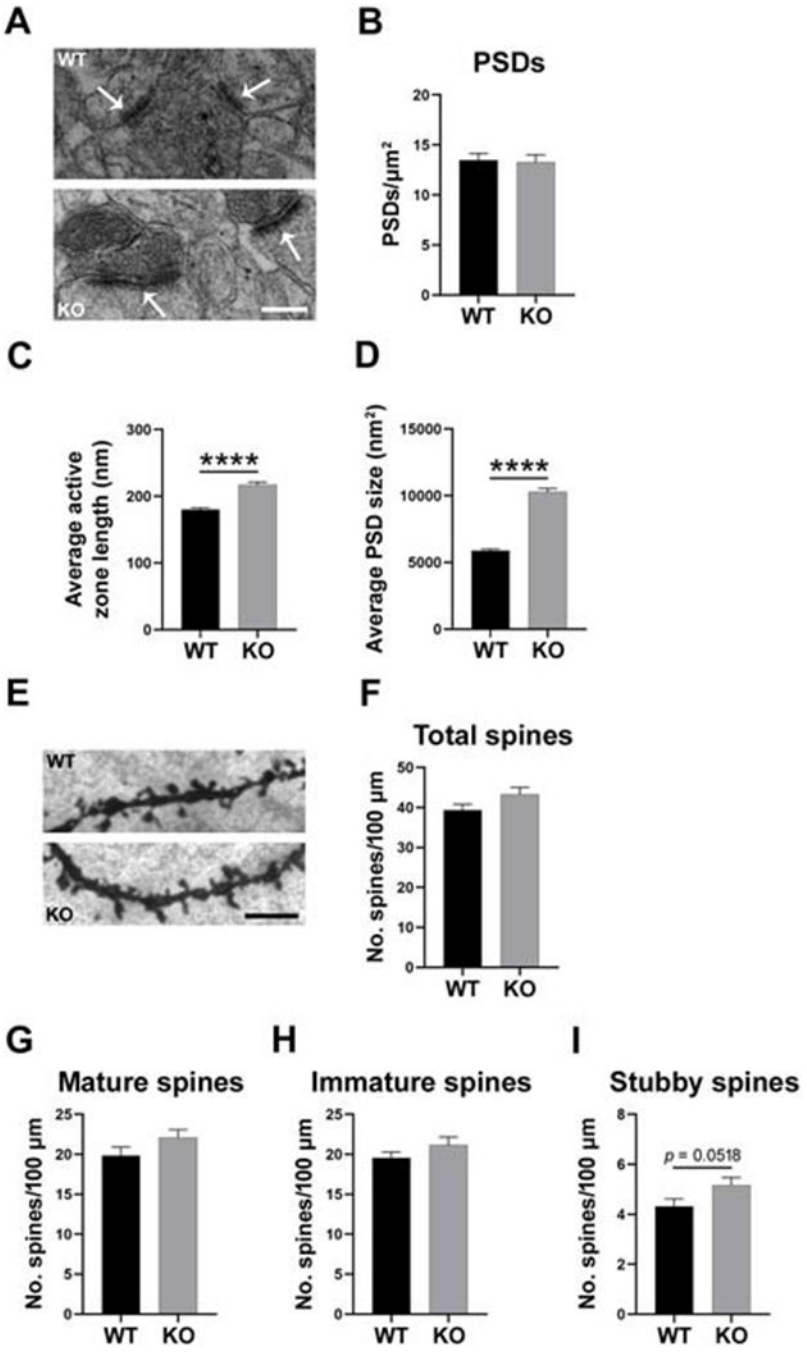

Fig. 3.

Suppression of eEF2K activity improves hippocampal synaptic morphology of aged mice. (A) Representative TEM images for postsynaptic densities (PSDs) in area CA1 of hippocampus. Arrows indicate PSDs. ($n = 3$. Original magnification, x11,000. Scale bar: 250 nm.) (B) Quantification of PSDs per µm^2. (C) Quantification of average active zone length in nm. (****$P < 0.0001$, unpaired t test.) (D) Quantification of average PSD size in nm^2. (****$P < 0.0001$, unpaired t test.) (E) Representative images from Golgi-Cox stain of dendritic spines in hippocampal area CA1. ($n = 3$. Original magnification, x100. Scale bar:

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10 μm.) (F) Quantification of total spines in area CA1 per 100 μm. (WT, $n = 153$ dendrites; eEF2K KO, $n = 132$ dendrites. Unpaired t test.) (G) Mature spine density per 100 μm in which branched, mushroom and stubby spines were categorized as mature. (H) Immature spine density per 100 μm in which thin, long thin and filopodial spines were categorized as immature. (I) Stubby spine density per 100 μm.

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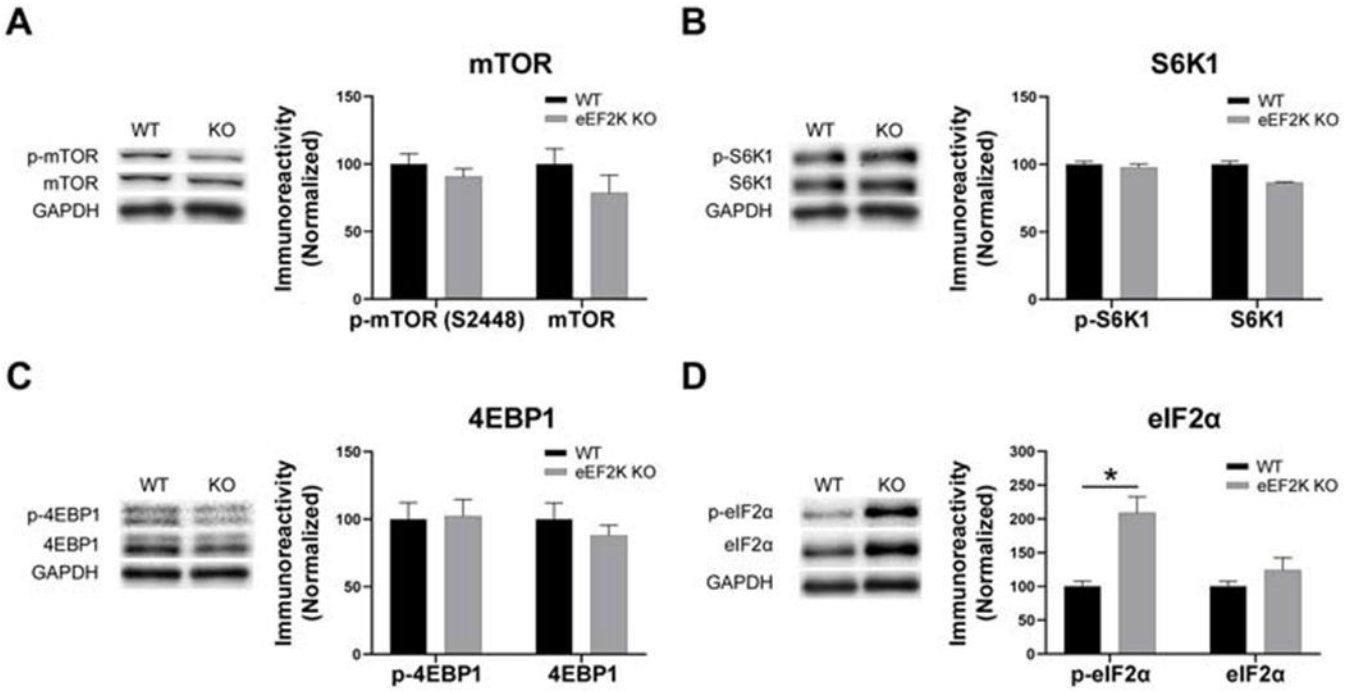

Fig. 4.

Reduction of eEF2 phosphorylation correlates with eIF2α hyperphosphorylation. (A) mTOR phosphorylation (S2448) is unaltered between groups. ($n = 9$) (B) Phosphorylation of p70S6K is not different between groups ($n = 9$) (C) 4EBP1 phosphorylation is unchanged between groups ($n = 9$) (D) Aged eEF2K KO mice exhibit hyperphosphorylated eIF2α in hippocampus when compared to age-matched WT controls ($n = 9$, $^*P < 0.05$, unpaired *t* test.)