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Suppression of the kinase for elongation factor 2 alleviates mGluR-LTD impairments in a mouse model of Alzheimer's disease

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Abstract

Impaired mRNA translation (protein synthesis) is linked to Alzheimer's disease (AD) pathophysiology. Recent studies revealed a role of increased phosphorylation of eukaryotic elongation factor 2 (eEF2) in AD-associated cognitive deficits. Phosphorylation of eEF2 (at the Thr56 site) by its only known kinase eEF2K leads to inhibition of general protein synthesis. AD is considered as a disease of "synaptic failure" characterized by impairments of synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD). Deficiency of metabotropic glutamate receptor 5-dependent LTD (mGluR-LTD) is indicated in cognitive syndromes associated with various neurological disorders including AD, but the molecular signaling mechanisms underlying the mGluR-LTD dysregulation in AD remain unclear. In this brief communication, we report genetic repression of eEF2K in aged APP/PS1 AD model mice prevented AD-associated hippocampal mGluR-LTD deficits. Using pharmacological approach, we further observed that impairments of mGluR-LTD in APP/PS1 mice were rescued by treating hippocampal slices with a small molecule eEF2K antagonist NH125. Taken together, our findings suggest a critical role of abnormal protein synthesis dysregulation at the elongation phase in AD-associated mGluR-LTD failure, thus providing insights into mechanistic understanding of synaptic impairments in AD and other related dementia syndromes.

Credit Author Statement

W.Y. conceptualized part of the experiments, collected and analyzed data, and wrote part of the manuscript. 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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Keywords

Alzheimer's disease; mGluR-LTD; eEF2K; protein synthesis; synaptic plasticity

1. Introduction

Mounting evidence points to a critical role of synaptic dysfunction in mediating the dementia syndrome associated with Alzheimer's disease (AD) (Ma and Klann, 2012; Rowan et al., 2007; Selkoe, 2002). Synaptic plasticity refers to the ability of the neuronal circuits to change (enhanced or weakened) in response to various stimuli and is widely considered as the primary mechanism for learning and memory (Cooke and Bliss, 2006; Malenka and Bear, 2004). Multiple lines of study indicate synapses as the target during the early stage of AD, and impairments of synaptic plasticity occur prior to neuronal death and cognitive deficits (Ma and Klann, 2012; Rowan et al., 2005; Selkoe, 2002; Teich et al., 2015). Thus, investigation of the molecular signaling mechanisms underlying AD-associated "synaptic failure" including impairments of synaptic plasticity may provide insights into development of novel strategies and biomarkers for early intervention and diagnosis/prognosis of this devastating disease. There are two established forms of synaptic plasticity: long-term potentiation (LTP) and long-term depression (LTD), both are known to be impaired in AD (Ma and Klann, 2012; Malenka and Bear, 2004). While extensive research has been carried out to elucidate mechanisms associated with LTP deficits in AD, much fewer studies have been reported on molecular mechanisms underlying AD-associated LTD impairments.

Similar to LTP, there are multiple forms of LTD. One well-characterized form is metabotropic glutamate receptor-dependent LTD (mGluR-LTD), which is often induced by application of selective group 1 mGluR agonists such as DHPG [(RS)-3,5-dihydroxyphenylglycine] (Huber et al., 2000; Lüscher and Huber, 2010; Yang et al., 2016). Modulation of mGluR is involved in cognitive function and abnormal mGluR-LTD has been linked to aging and cognitive syndromes associated with various neurological disorders including Fragile X syndrome and AD (Kumar et al., 2015; Kumar and Foster, 2007; Lüscher and Huber, 2010; Ribeiro et al., 2017). Interestingly, mGluR may function as a receptor/coreceptor for amyloid beta (Aβ), the main components of the hallmark brain pathological plaques associated with AD (Hu et al., 2014; Um et al., 2013). The molecular signaling mechanisms underpinning the mGluR-LTD dysregulation in AD remain unclear.

Substantial evidence demonstrates that long-lasting forms of synaptic plasticity, including mGluR-LTD, is dependent on de novo protein synthesis i.e. mRNA translation (Costa-Mattioli et al., 2009; Hou et al., 2006; Hou and Klann, 2004; Lüscher and Huber, 2010; Richter and Klann, 2009). Mounting evidence indicates a role of impaired mRNA translation ability in pathogenesis of neurodegenerative diseases such as prion disease, frontotemporal dementia (FTD) and AD (Beckelman et al., 2019; Ma and Klann, 2014; Ma et al., 2013; Moreno et al., 2012; Radford et al., 2015). Protein synthesis is a highly regulated process dependent on multiple specific translational factors at various stages i.e. initiation, elongation, and termination. While translation initiation usually is considered as the rate-limiting step, accumulating evidence suggests that regulation at the elongation phase is

critical in modulation of protein synthesis, particularly under circumstances with deficiency of nutrients and energy (Kenney et al., 2014; Sutton and Schuman, 2006; Taha et al., 2013; Zimmermann et al., 2018). Notably, a plenty of studies indicate that energy metabolism dysregulation is associated with AD pathogenesis (Lin and Beal, 2006; Ma et al., 2011; Massaad et al., 2009; Zimmermann, H. R. et al., 2020). A key factor involved in mRNA translation elongation is eukaryotic elongation factor 2 (eEF2). Phosphorylation of eEF2 on the Thr56 site by its only known kinase eEF2K prevents eEF2 from binding to the ribosome, resulting disruption of peptide growth and thus inhibition of general protein synthesis (Kenney et al., 2014; Taha et al., 2013). Previous studies demonstrated hyper-phosphorylation of eEF2 (at Thr56) in hippocampus of AD patients and animal models of AD (APP/PS1 and 3xTg-AD mice) (Jan et al., 2017; Ma, T. et al., 2014). Consistently, a recent study reported that genetic reduction of eEF2K alleviates deficiency of de novo protein synthesis and cognitive impairments in AD model mice (Beckelman et al., 2019). Additionally, chemical antagonists of eEF2K such as NH125 and AG-484954 have been developed, but their effects on AD-related pathophysiology have not been determined (Arora et al., 2003; Chen et al., 2011; Kenney et al., 2016).

In this study, we investigated whether impaired hippocampal mGluR-LTD in a mouse model of AD can be improved by reducing phosphorylation of eEF2 through inhibition of eEF2K. Both genetic and pharmacological approaches are applied to test the hypothesis and our results point to a previously unrecognized role of aberrant eEF2K/eEF2 signaling in mediating AD-associated mGluR-LTD deficits.

2. Materials & Methods

2.1. Mice

All mice were housed in a barrier facility dedicated to transgenic mice at Wake Forest University School of Medicine. The facility operates in accordance with standards and policies of the *US Department of Agriculture's Animal Welfare Information Center* (AWIC), and the *NIH Guide for Care and Use of Laboratory Animals.* The facility is kept on a 12 h light/dark cycle, with a regular feeding and cage-cleaning schedule. Both male and female mice were used at the age of 12-18 months. APP/PS1 transgenic mice (APPswe + PSEN1/ΔE9) were purchased from the Jackson Laboratory (Bar Harbor, ME) (Jankowsky et al., 2001). Homozygous $eEF2K^{-/-}$ mice were generated as described previously (Chu et al., 2014). Generation of APP/PS1/$eEF2K^{+/-}$ double mutant mice cohort was performed as described before (Beckelman et al., 2019). All genotypes were verified by polymerase chain reaction (PCR).

2.2. Hippocampal slices preparation and electrophysiology.

Acute transverse hippocampal slices (400 μm thick) were prepared using a Leica VT1200S vibratome (Wetzlar, Germany) as described (Yang et al., 2016). Slices were maintained for 2 hours at room temperature prior to experimentation in artificial cerebrospinal fluid (ACSF) containing (in mM): 118 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 5 $NaHCO_3$ and 15 glucose, bubbled with 95% O_2 / 5% CO_2. For electrophysiology experiment, slices were transferred to recording chambers (preheated to 32 °C) where they were superfused

with oxygenated ACSF. Monophasic, constant-current stimuli (100 μsec) were delivered with a concentric bipolar microelectrode (FHC Inc., Bowdoin, ME) placed in the stratum radiatum of area CA3, and the field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum radiatum of area CA1. fEPSPs were acquired, and amplitudes and maximum initial slopes measured, using pClamp 10 (Axon Instruments, Foster City, CA). To induce mGluR-LTD, slices were perfused with DHPG (100 μM in ACSF) for 10 min. For LTD experiments, the "n" refers to number of slices, based on independent experiments from at least three mice.

2.3. Western blot

Mouse hippocampal tissue was flash-frozen on dry ice, followed by standard procedure for Western blot as described (Zimmermann, Helena R. et al., 2020). All primary and secondary antibodies were diluted in blocking buffer. Blots were probed with primary antibodies for phospho-eEF2 (1:1000; Cell Signaling; Cat# 2331), eEF2 (1:1000; Cell Signaling; Cat#2332), and β-actin (1:10,000, Cell Signaling; Cat#3700). Protein bands were visualized using chemiluminescence (Clarity™ ECL; Biorad) and the Biorad ChemiDoc™ MP Imaging System. Densitometric analysis was performed using ImageJ. For Western blot experiments, the "n" refers to number of mice.

2.4. Drug treatment

DHPG (Abcam, Cambridge, MA) was prepared as stock solution in distilled water and was diluted into a final concentration 100 μM immediately preceding the experiments. NH125 (Calbiochem) was prepared as stock solution in DMSO and diluted into ACSF to a final concentration of 1 μM. NH125 is applied to slices 30 minutes before DHPG treatment and left throughout the experiment. Only stock (both NH125 and DHPG) prepared within one week was used in the experiments.

2.4. Data analysis

Data are presented as mean ± SEM. Summary data are presented as group means with standard error bars. Prism 6 statistics software (GraphPad Software) was used to perform data analysis. For comparisons between multiple groups, ANOVA was used followed by individual *post hoc* tests when applicable. Error probabilities of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Genetic suppression of eEF2K alleviates impairments of hippocampal mGluR-LTD in APP/PS1 mouse model of AD

Previous studies show increased eEF2 phosphorylation in hippocampus of aged APP/PS1 mice, and abnormal increased phosphorylation of eEF2 is linked to multiple aspects of AD pathophysiology (Beckelman et al., 2019; Jan et al., 2017; Ma, Tao et al., 2014). To examine whether reduction of eEF2 phosphorylation can improve AD-associated mGluR-LTD impairment, we crossed APP/PS1 AD model mice with heterozygous eEF2K$^{+/-}$ transgenic mice to generate APP/PS1/eEF2K$^{+/-}$ double mutant mice, along with three relevant experimental groups (WT, APP/PS1, and eEF2K+$^{/-}$). Mice of both sex at the age of 12-18

months were used for experimentation. We recently demonstrated that eEF2 hyper-phosphorylation in hippocampus of aged APP/PS1 mice was restored to WT levels by genetically reducing eEF2K activity (Beckelman et al., 2019).

We induced mGluR-LTD at the CA3-CA1 synapses (Schaffer collateral pathway) in acute hippocampal slices by treating slices with group 1 mGluR agonist DHPG (100 μM for 10 minutes). Consistent with previous findings (Yang et al., 2016), DHPG treatment induced sustained (>1 hour) LTD in slices from wild type (WT) mice, but only transient, detrimental LTD in slices derived from APP/PS1 AD model mice (Fig. 1A–E). Importantly, AD-associated mGluR-LTD impairment was alleviated by reducing eEF2K activity, as indicated by improved LTD (e.g. measurement of fEPSP slopes at 60 minutes after DHPG treatment) in slices from APP/PS1/eEF2K$^{+/-}$ double mutant mice (one-way ANOVA followed by post hoc test, $p<0.01$ for WT vs. APP/PS1 and APP/PS1 vs. APP/PS1/eEF2K+/−, 60 min post-DHPG) (Fig. 1A–E). Interestingly, we observed a trend of reduced LTD in slices from eEF2K$^{+/-}$ mice compared to WT group (one-way ANOVA followed by post hoc test, $p=0.1928$, WT vs. eEF2K +/−, 60 min post-DHPG) (Fig. 1A–E). To assess potential alterations in basal synaptic transmission, we determined synaptic input-output relationship by plotting slope of fEPSP with fiber volley amplitude, and did not find difference among the four experimental groups (Fig. 1F). We also measured paired pulse facilitation (PPF), a form of calcium-dependent pre-synaptic activity elicited by two temporally linked stimuli at various intervals (Katz and Miledi, 1968). We observed no difference in PPF performance in hippocampal slices from mice of the four groups (Fig. 1G). Taken together, inhibition of eEF2 phosphorylation through genetic reduction of its kinase eEF2K alleviates AD-associated hippocampal mGluR-LTD deficits, without effects on basal synaptic transmission.

3.2. Hippocampal mGluR-LTD failure in APP/PS1 mice is rescued by eEF2K antagonist NH125

In addition to genetic knockdown of eEF2K, we applied a pharmacological approach to investigate the effects of suppressing eEF2K/eEF2 signaling on the mGluR-LTD deficits in APP/PS1 mice. NH125 is a selective small molecule inhibitor of eEF2K, and it was demonstrated that Aβ-induced LTP failure is rescued by NH125 (1 μM) treatment (Arora et al., 2003; Ma, Tao et al., 2014). First, we did not observe difference in mGluR-LTD performance in hippocampal slices from WT mice treated with vehicle or NH125 (Fig. 2A–E). In slices from APP/PS1 mice, NH125 treatment is able to rescue AD-associated mGluR-LTD deficits, as compared to vehicle-treated slices (one-way ANOVA followed by post hoc test, $p<0.05$ for WT vs. APP/PS1 and $p<0.01$ for APP/PS1 vs. APP/PS1 with NH125, 60 min post-DHPG) (Fig. 2A–E). Additionally, analysis of synaptic input/output relationship and PPF response did not reveal differences among the four experimental groups (Fig. 2F, G). Such findings are consistent with the results above using genetic approach, supporting a link between mGluR-LTD impairment and elevated eEF2 phosphorylation in AD.

4. Discussion

Despite decades of intensive research, detailed molecular mechanisms underlying AD pathogenesis remain elusive, which potentially hinders development of effective therapies and early diagnostic biomarkers for this devastating neurodegenerative disease (2020; Abeysinghe et al., 2020; Holtzman et al., 2011; Teich et al., 2015). Accumulating evidence supports the conception that AD is “a disease of synaptic failure” characterized by impairments of synaptic plasticity (Ma and Klann, 2012; Selkoe, 2002; Teich et al., 2015). Elucidation of the signaling mechanisms underlying AD-associated synaptic failure may provide insights into novel therapeutic strategies for cognitive impairments in AD. In the current study, we report that hippocampal mGluR-LTD defects in a mouse model of AD were alleviated through repressing elongation factor 2 kinase with genetic or pharmacological approaches.

Previous studies indicate that memory deficits in early stage of AD is related to loss of functional synapses in hippocampus along with dysregulation of LTP and LTD. Opposite to LTP, LTD decreases the synaptic efficiency and may equally contribute (as LTP) to generation of cognitive network connections (Malenka and Bear, 2004; Ribeiro et al., 2017). Studies in rodents indicate that normal aging is associated with mGluR-dependent LTD (Ménard and Quirion, 2012). Most if not all studies so far point to inhibition of LTP in AD. In comparison, much fewer studies on LTD were carried out in the context of AD, and generated seemingly inconsistent results. Various factors may contribute to such inconsistent LTD phenotypes in AD. For example, acute application of exogenous Aβ can induce enhancement of NMDAR-dependent LTD but no significant effects on mGluR-LTD (Li et al., 2009; Ma, T. et al., 2014; Shankar et al., 2008; Yang et al., 2016). Further, most of LTD studies in AD model were conducted in animals at relatively young age (<1 year old) (Megill et al., 2015). Here we reported occlusion of hippocampal mGluR-LTD in an aged (1 year old) AD model mice, which is consistent with previous findings (Yang et al., 2016). It was reported that DHPG treatment (50 μM, 5 min treatment) failed to induce long-lasting hippocampal mGluR-LTD in young eEF2K homozygous KO mice (eEF2K−/−) (Park et al., 2008). In agreement, we found that aged, heterozygous eEF2K mice (eEF2K+/−) exhibit trendy hippocampal mGluR-LTD impairments (Fig. 1), though not as significant as reported in the aforementioned study. Interestingly, treatment of WT slices with eEF2K antagonist NH125 does not affect mGluR-LTD (Fig. 2), suggesting different underlying mechanisms associated with acute or chronic inhibition of eEF2K/eEF2 signaling.

What would be the mechanisms contributing to the correction of hippocampal mGluR-LTD deficits in APP/PS1 AD model mice by inhibition of eEF2K? The parsimonious explanation would be through the improvement of protein synthesis, in light of our previous findings that AD-associated deficits in protein synthesis and eEF2 phosphorylation in hippocampus were alleviated by genetic reduction of eEF2K (Beckelman et al., 2019). If so, it logically raises two significant questions. First, are there specific proteins (presumably controlled by the eEF2K/eEF2 signaling) whose synthesis is critical for the mGluR-LTD phenotype findings here? As a beginning point to address the question, it would be necessary to perform comprehensive proteomic analysis of newly synthesized proteins at the hippocampus under the aforementioned conditions. We recently reported results from mass spectrometry (MS)-

based proteomic experiments in hippocampus of an AD mouse model with genetic reduction of eEF2K (Beckelman et al., 2019). Functional classification revealed that among the top proteins that were significantly dysregulated in AD model mice and restored with eEF2K suppression, quite a few proteins were critically involved in synaptic function and calcium buffering (Beckelman et al., 2019). However, what are the exact roles of these proteins in AD-related LTP/LTD deficits (e.g. causal relationship?) would require further in-depth investigation. Further, it is worth of mentioning that most if not all proteomic studies in the field are aimed to examine overall protein profiling alteration and do not differentiate "newly synthesized proteins", which are considered to be the key for maintenance of long-term synaptic plasticity and memory (Alberini, 2008; Klann and Dever, 2004; Sutton and Schuman, 2006). Second, is such improvement on mGluR-LTD in APP/PS1 mice related to "general" protein synthesis capacity change or something specific to regulation of elongation phase by eEF2K/eEF2 phosphorylation? In another word, can boost of protein synthesis through regulation on other phases of protein synthesis (e.g. initiation) result in mGluR-LTD improvement as well? We previously reported that hippocampal mGluR-LTD impairments in APP/PS1 AD model mice were alleviated by repressing activity of PERK, a kinase for mRNA translation initiation factor eIF2 (Yang et al., 2016). Repression of PERK reduces phosphorylation of eIF2 at its α subunit (eIF2α), leading to increased general protein synthesis (Wek et al., 2006). Moreover, decades of intensive studies in search of the "plasticity-related proteins (PRPs)" have not yielded consensus (Okuda et al., 2020). Taken together, we speculate that a boost of "general" protein synthesis (through regulation on mRNA translation capacity) instead of synthesis of particular proteins (e.g. PRPs) might be critical for improving synaptic plasticity impairments and consequently cognitive deficits in AD. Finally, given the specificity of eEF2K on eEF2 phosphorylation (as compared to a wide array of substrates for PERK, for example), targeting eEF2K could be an appealing and efficient strategy to improve synaptic and cognitive defects in AD and related dementia syndromes.

The study has some limitations. Mainly due to the low yield of the aged "double mutant" (i.e. APP/PS1/eEF2K+/−) mice, we performed experiments on animals of both sex at the age range of 12-18 month. It would be informative in the future (when sufficient samples are available) to further analyze the effects of biological variables such as gender and age on the LTD phenotype. Additionally, we chose the dose of NH125 (1 μM) based on previous studies using the inhibitor under similar experimental paradigms (Ma, Tao et al., 2014). Future more comprehensive studies (biochemical and electrophysiological) with different doses of eEF2K inhibitor (s) would provide further mechanistic understanding of the roles of eEF2K/eEF2 phosphorylation in AD-associated synaptic plasticity impairments.

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Highlights

- Hippocampal mGluR-LTD is impaired in aged APP/PS1 AD model mice
- Genetic reduction of eEF2 kinase alleviates mGluR-LTD failure in APP/PS1 mice.
- AD-associated mGluR-LTD deficiency is rescued by an eEF2K inhibitor.

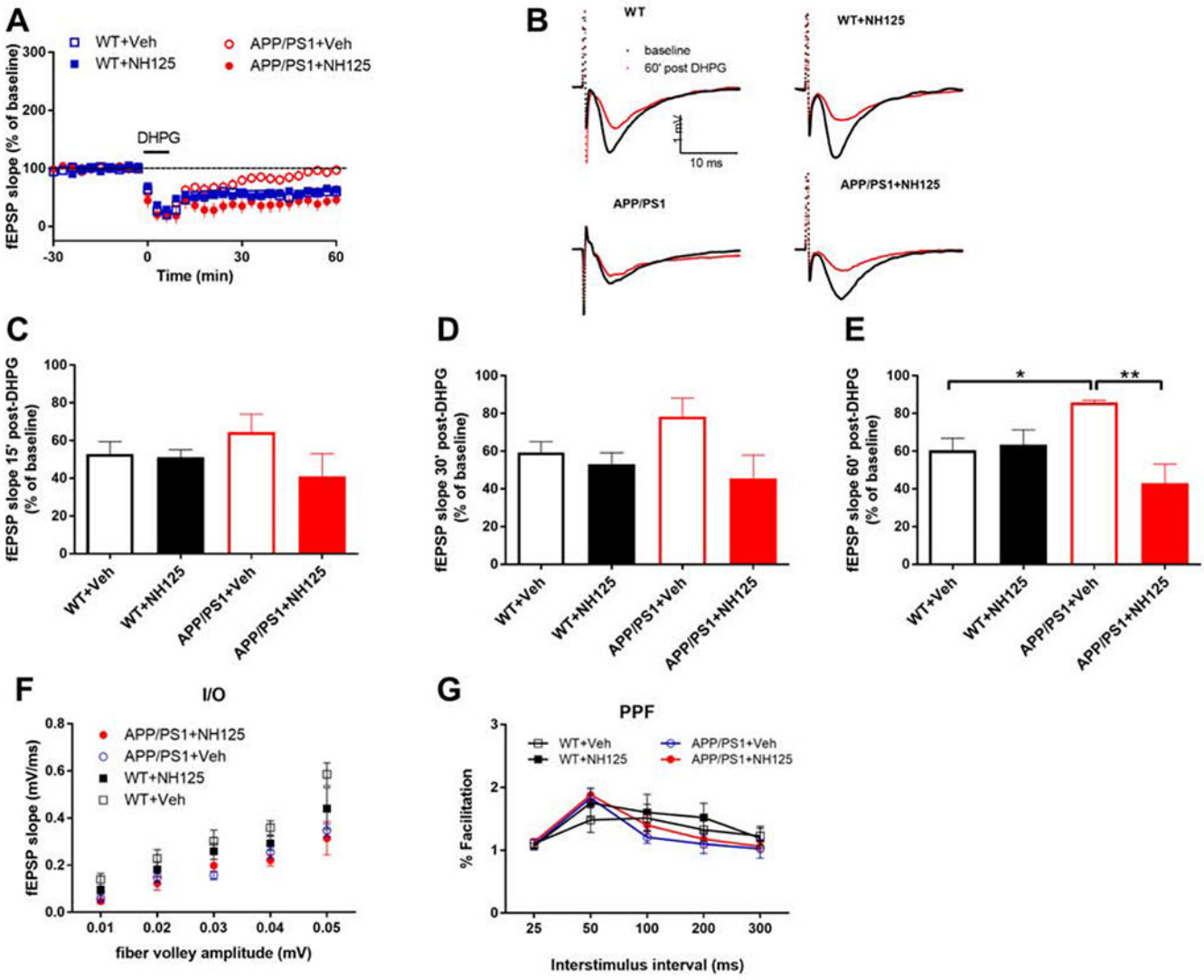

Fig 1.

Genetic suppression of eEF2K alleviates impairments of hippocampal mGluR-LTD in aged APP/PS1 mouse model of AD. (A) Treatment of hippocampal slices with DHPG (100 μM for 10 minutes) induced sustained mGluR-LTD in WT mice (open squares), but only transient mGluR-LTD in APP/PS1 mice (filled squares). In slices from APP/PS1/eEF2K+/− mice (filled circles), mGluR-LTD was improved compared to the APP/PS1 group. In slices from eEF2K+/− mice (open circles), mGluR-LTD was inhibited. (B) Representative fEPSP traces before and after DHPG treatment (60 minutes post DHPG) to induce mGluR-LTD for the experiments shown in A. (C-E) Cumulative data showing mean fEPSP slopes 15, 30, and 60 minutes after DHPG application for the mGluR-LTD experiments in A. n=7 for WT, n=7 for APP/PS1, n=13 for eEF2K+/−, n=9 for APP/PS1/eEF2K+/−. $^{**}p<0.01$. One-way ANOVA followed by Tukey's post hoc test. (F) Basal synaptic transmission alteration assessed by input-output (I/O) relationships plotted as fiber volley amplitude vs. fEPSP slope. (G) Performance of paired-pulse facilitation (PPF). n=5-8. (H-I) DHPG-induced LTD was unaltered in younger (8-9 months) cohort of mice. n=7 for WT, n=7 for APP/PS1, n=10

for eEF2K+/−, n=8 for APP/PS1/eEF2K+/−. p>0.05. One-way ANOVA. (K) Levels of eEF2 phosphorylation in younger (8-9 months) cohort of mice. n=3 for WT, n=4 for APP/PS1, n=3 for eEF2K+/−, n=3 for APP/PS1/eEF2K+/−. p>0.05. One-way ANOVA.

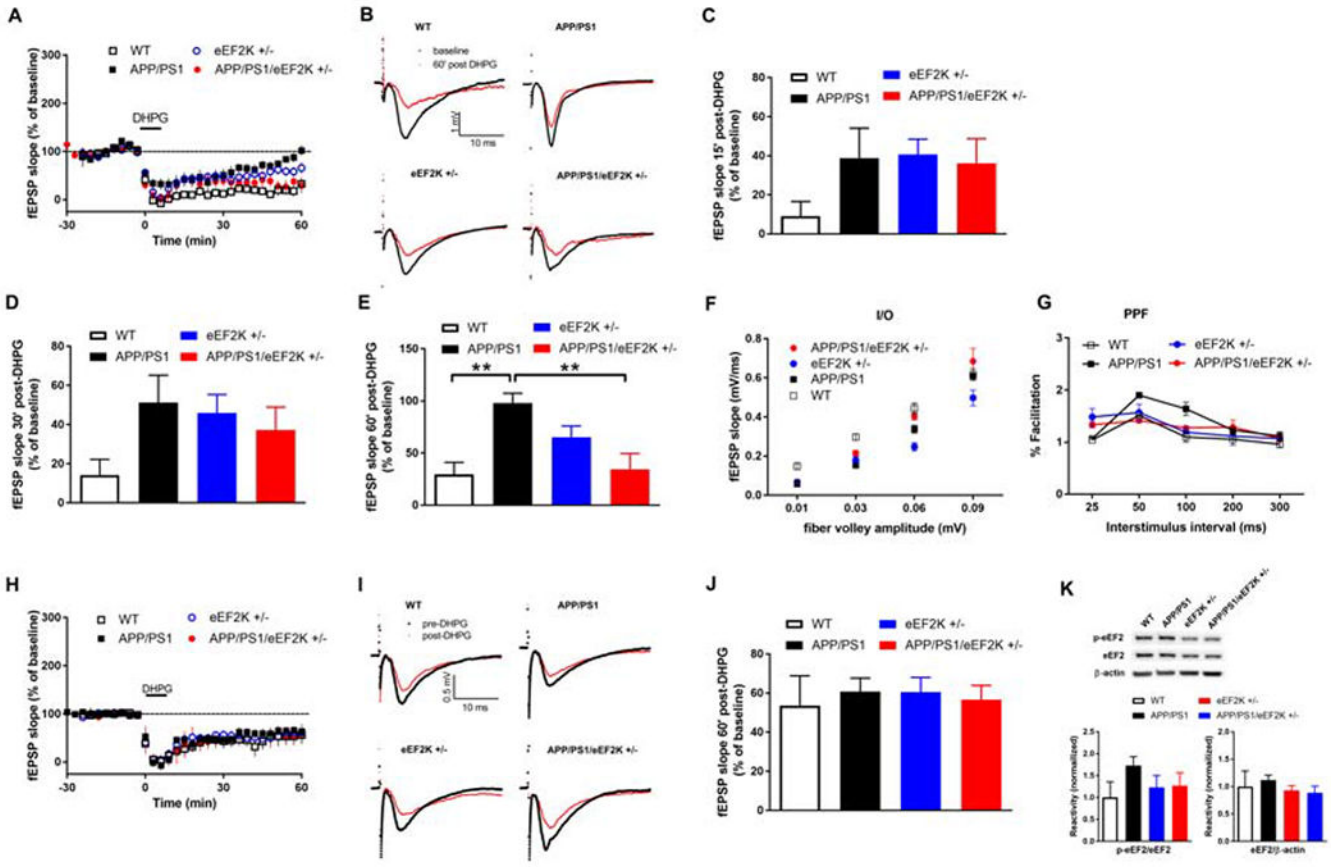

Fig 2.

Hippocampal mGluR-LTD failure in APP/PS1 mice is rescued by eEF2K antagonist NH125. (A) In APP/PS1 mice, treatment of slices with eEF2K antagonist NH125 (1 μM, filled circles) alleviated mGluR-LTD impairments, as compared to vehicle-treated slices (open circles). In WT mice, DHPG induced similar LTD in slices treated with vehicle (open squares) and NH125 (filled squares). (B) Representative fEPSP traces before and after DHPG treatment (60 minutes post DHPG) for the LTD experiments shown in A. (C-E) Cumulative data showing mean fEPSP slopes 15, 30, and 60 minutes after DHPG application for the mGluR-LTD experiments in A. n=17 for WT, n=6 for APP/PS1, n=12 for WT+NH125, n=9 for APP/PS1+NH125. *p<0.05, **p<0.01. One-way ANOVA followed by Tukey's post hoc test. (F) Basal synaptic transmission alteration assessed by input-output (I/O) relationships plotted as fiber volley amplitude vs. fEPSP slope. (G) Performance of paired-pulse facilitation (PPF). n=4-6.