HHS Public Access

Author manuscript

J Neurochem. Author manuscript; available in PMC 2023 May 19.

Published in final edited form as:

J Neurochem. 2023 July ; 166(1): 47–57. doi:10.1111/jnc.15541.

Roles of eukaryotic elongation factor 2 kinase (eEF2K) in neuronal plasticity, cognition, and Alzheimer's disease

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Abstract

Understanding the molecular signaling mechanisms underlying cognition and neuronal plasticity would provide insights into the pathogenesis of neuronal disorders characterized with cognitive syndromes such as Alzheimer's disease (AD). Phosphorylation of the mRNA translational factor eukaryotic elongation factor 2 (eEF2) by its specific kinase eEF2K is critically involved in protein synthesis regulation. In this review, we discussed recent studies on the roles of eEF2K/eEF2 signaling in the context of regulation/dysregulation of cognitive function and synaptic plasticity. We specifically focus on the discussion of recent evidence indicating suppression of eEF2K signaling as a potential novel therapeutic avenue for AD and related dementias (ADRDs).

Keywords

Translation; eEF2K; eEF2 phosphorylation; protein synthesis; synaptic plasticity; cognition; memory; Alzheimer's disease

Introduction

Elucidation of the molecular basis for cognitive function and synaptic plasticity (persistent change in neuronal circuits) would help reveal mechanisms underlying the pathogenesis of cognitive syndromes such as Alzheimer's disease (AD). Findings from such studies could in turn help identify novel therapeutic strategies as well as diagnostic/prognostic biomarkers for neuronal disorders characterized with cognitive impairments. Extensive studies over the past few decades have established that protein synthesis (mRNA translation) is indispensable for long-lasting forms of synaptic plasticity and memory (Kelleher *et al.* 2004; Costa-Mattioli *et al.* 2009). Further, dysregulation of mRNA translation is linked to a

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Conflict of interests

The author declares no conflict of interest.

variety of human diseases including many neuronal diseases (Scheper *et al.* 2007; Tahmasebi *et al.* 2018).

As a fundamental cellular process critical for almost all biological functions, protein synthesis involves complex machinery and consumes a significant amount of energy and nutrients. Accordingly, the process of protein synthesis is subject to tight control to help maintain cellular homeostasis (Tahmasebi *et al.* 2019; Hershey *et al.* 2019). Overall protein synthesis takes place in three phases: initiation, elongation, and termination. Each phase requires the action of specific translational protein factors to regulate the process. Most (>95%) of the energy and amino acids utilized in the mRNA translation are consumed during the elongation phase (Browne & Proud 2002; Kenney *et al.* 2014). Consistently, accumulating evidence indicates that translation elongation control is critical in the modulation of protein synthesis, particularly during cellular responses to deficiency of nutrients and energy (Sutton & Schuman 2006; Biever *et al.* 2019). A key mechanism involved in elongation control is through phosphorylation of eukaryotic elongation factor 2 (eEF2). Phosphorylation of eEF2 on the Thr56 site by its specific kinase eEF2K hinders the engagement of eEF2 with ribosomes, and consequently inhibition of mRNA translation (Kenney *et al.* 2014; Liu & Proud 2016; Ryazanov 2002).

There are excellent review articles on basic biochemistry and biology of eEF2K/eEF2 signaling (Kenney *et al.* 2014; Drennan & Ryazanov 2004). In this review, we mainly discuss studies on the functional roles of eEF2K/eEF2 signaling in the context of regulation/dysregulation of synaptic plasticity and cognitive function. We specifically focus on the discussion of recent evidence indicating suppression of eEF2K signaling as a potential novel therapeutic strategy for AD and related dementias (ADRDs).

The eEF2K signaling: Upstream and Downstream

The vast majority of the eukaryotic protein kinases possess homologous catalytic domains (e.g. serine/threonine kinases and tyrosine kinases) and are usually referred to as the conventional protein kinases (CPKs). Unlike the CPKs, eEF2K belongs to an unusually small group of kinases (6 members in the human genome) termed “alpha-kinases” (α-kinases), whose catalytic domains are distinct from those in the CPKs (Drennan & Ryazanov 2004; Kenney *et al.* 2014; Ryazanov *et al.* 1999). Among all 6 members of the α-kinases family, eEF2K is the only one whose activity is associated with Ca^{2+} signaling. Indeed, eEF2K is originally known as Calmodulin-dependent protein kinase III (CaMKIII) (Nairn *et al.* 2001). One potential mechanism underlying eEF2K activation by Ca^{2+}/CaM involves autophosphorylation at the Thr348 and Ser500 sites (Tavares *et al.* 2012). In addition to autophosphorylation induced by Ca^{2+}/CaM, the activity of eEF2K is regulated through phosphorylation by multiple upstream signaling pathways under various situations. Particularly, eEF2K activity is inhibited by the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway and stimulated by the AMP-activated protein kinase (AMPK) signaling (Kenney *et al.* 2014). (Fig. 1).

The mTORC1 signaling is involved in a wide range of cellular processes such as autophagy and protein synthesis (mainly through translation initiation) (Hall 2016; Yang & Guan

2007). Mounting evidence indicates an important role of the mTORC1 signaling in synaptic plasticity and memory formation (Hoeffer & Klann 2010; Graber *et al.* 2013). Studies from the Proud laboratory revealed that eEF2K is phosphorylated at the Ser366 site by S6 kinase1 (S6K1 or p70S6K), an established substrate of mTORC1 (Wang *et al.* 2001). A later study from the same group discovered that eEF2K is phosphorylated at the Ser78 site in a mTORC1-dependent manner (Browne & Proud 2004). Phosphorylation of eEF2K at either Ser366 or Ser78 site by mTORC1 results in inhibition of eEF2K activity, leading to dephosphorylation of eEF2 and subsequently increase of general protein synthesis (Kenney *et al.* 2014). A most recent study suggested that a scaffolding protein Homer1b/c regulates eEF2K activity, and the process is associated with the mTORC1 pathway through phosphorylation of eEF2K at the Ser396 site (Gladulich *et al.* 2021). Previous studies in rodents indicate that the involvement of mTORC1 in the regulation of long-term hippocampal synaptic plasticity requires inputs from multiple kinases including AKT (protein kinase B) and ERK (Ma *et al.* 2011b; Tsokas *et al.* 2007). In addition, studies from non-neuronal systems reveal that ERK can inactivate eEF2K through direct or indirect phosphorylation (Liu & Proud 2016). (Fig. 1).

AMPK is a central molecular sensor critical for the maintenance of cellular energy homeostasis (Hardie 2011). In a low energy state (e.g. decreased levels of ATP and increased levels of AMP), AMPK is activated to phosphorylate a range of substrates to reduce energy consumption and increase energy supply (Hardie *et al.* 2012). It is perhaps unsurprising that AMPK regulation is linked to eEF2K signaling given that the protein synthesis process consumes a substantial amount of energy as mentioned above (Hershey *et al.* 2019). Activation of AMPK stimulates eEF2K through at least two mechanisms: 1) Inhibition of the mTORC1, which is a negative regulator of eEF2K as discussed above (Horman *et al.* 2002); and 2) Direct phosphorylation of eEF2K (mainly at the Ser398 site), which is independent of the mTORC1 signaling (Browne *et al.* 2004). Stimulation of eEF2K by AMPK leads to phosphorylation of eEF2 and inhibition of general protein synthesis. (Fig. 1).

Roles of eEF2K in synaptic plasticity and cognition: Knockdown and Overexpression

A plethora of studies has demonstrated that maintenance of synaptic plasticity and memory is dependent on *de novo* protein synthesis (Klann & Dever 2004; Richter & Klann 2009; Sossin & Costa-Mattioli 2019; Rosenberg *et al.* 2014). As also mentioned above, energy consumption during elongation is dominant for the protein synthesis process. Moreover, regulation of protein synthesis via elongation is particularly important in cellular environments (e.g. neuronal dendrites) where both initiation and elongation processes need to be upregulated to fulfill the substantial requirements of new protein synthesis associated with synaptic plasticity (Sutton & Schuman 2006; Tsokas *et al.* 2005). Consistently, it was demonstrated that treatment of neurons with brain-derived neurotrophic factor (BDNF), a key player in neuronal plasticity, reduced eEF2 phosphorylation, leading to the promotion of translation elongation and general protein synthesis (Takei *et al.* 2009). A more recent study from the Proud group using proteomics approach reported that eEF2K/eEF2 signaling

controls the synthesis of microtubule-related proteins, which play an important role in regulating dendritic spine morphology that is modified during synaptic plasticity, learning, and memory (Kenney *et al.* 2016). On the other hand, a previous study performed in cultured neurons revealed that inhibition of eEF2K resulted in decreased dendritic BDNF protein expression (Verpelli *et al.* 2010). Another interesting finding, in light of the role of eEF2K signaling and general protein synthesis in synaptic plasticity discussed above, is that activation of the NMDA receptor led to increased protein expression of alphaCamK II via phosphorylation of eEF2 (Scheetz *et al.* 2000). At least two outstanding review articles summarized the biochemical and molecular alterations associated with the roles of eEF2 phosphorylation during synaptic plasticity or memory formation (Sossin & Costa-Mattioli 2019; Taha *et al.* 2013). For this review, we focus on a discussion of functional neuronal phenotypes associated with genetic manipulation of eEF2K in rodents.

The majority of studies in the neuroscience field on genetic eEF2K manipulation are carried out in transgenic mice with global eEF2K knockout (eEF2K KO) originally developed by the laboratory of Alexey Ryazanov or Christopher Proud (Ryazanov 2002; Moore *et al.* 2015; Heise *et al.* 2017). As expected, phosphorylation of eEF2 is dramatically decreased in the eEF2K KO mice (Zimmermann *et al.* 2018; Heise *et al.* 2017). Thus, it is predicted that neuronal protein synthesis shall be increased in the eEF2K KO mice given the roles of eEF2 in mRNA translation described above. Using the non-radioactive assay SUnSET (surface sensing of translation), we found that overall de novo protein synthesis is increased in acute living hippocampal slices from the eEF2K KO mice (Zimmermann *et al.* 2018). Another study also using the SUnSET method, however, did not reveal significant alterations of general protein synthesis in cultured neurons from the eEF2K KO mice (Heise *et al.* 2017). Such findings indicate that protein synthesis might be differently regulated by the eEF2K/eEF2 signaling, possibly depending on different brain regions or subcellular locations (e.g. cell bodies or dendrites/synapses). It is worth mentioning that measurement of de novo protein synthesis for both studies was carried out under basal resting state. Regulation of protein synthesis via eEF2K and eEF2 phosphorylation might be further exemplified during intense neuronal activities (e.g. Induction of synaptic plasticity). Alternatively, the inconsistent findings on protein synthesis could be related to the limitation of the SUnSET method in precisely detecting specific pools of proteins. More comprehensive studies using advanced techniques such as those in cellular imaging combined with large-scale proteomics analysis shall shed light on this topic.

eEF2K KO mice are well characterized and they are “normal” as assessed by numerous basic measurements such as life span, body weight, food intake, gross brain morphology (Heise *et al.* 2017). Further, comprehensive behavioral studies have been performed by Heise and colleagues (Heise *et al.* 2017) to assess a broad range of cognitive functions in the eEF2K KO mice. Overall, adult eEF2K KO mice exhibit normal cognitive behaviors in most assays including open field (OF) and elevated plus maze (EPM) for testing locomotor activities and anxiety-like behavior; hidden-platform Morris water maze (MWM) for testing spatial learning/memory; novel object recognition (NOR) for testing long-term recognition memory; marble burying for testing repetitive and perseverative-like behavior; reversal MWM task for testing cognitive flexibility (Heise *et al.* 2017). Notably, we recently reported that age-related long-term recognition memory impairments in mice, assessed by

the NOR test, were alleviated in eEF2K KO mice (Gosrani *et al.* 2020). In comparison, age-related spatial learning and memory deficits evaluated by the MWM test were not affected by eEF2K knockout (Gosrani *et al.* 2020). Both the NOR (for assessing long-term cognitive function) and MWM tests are established as hippocampal-dependent behavioral assays. Meanwhile, other brain regions such as the prefrontal cortex are also involved (Akirav & Maroun 2006; Buzsáki & Moser 2013). Recent studies indicate that protein synthesis process in specific cell types (e.g. inhibitory or excitatory neurons) is associated with different neuronal phenotypes (Sharma *et al.* 2020; Shrestha *et al.* 2020a; Shrestha *et al.* 2020b). Together with the above discussion on the SUnSET findings, it would be important for future studies to elucidate potential distinct roles of eEF2 phosphorylation in the regulation of cognitive function that are associated with different brain regions and/or neurons.

With elegant modification in some specific behavioral paradigms, Heise and colleagues revealed that the eEF2K KO mice exhibited impaired long-term trace fear conditioning, which is considered to be heavily dependent on the function of hippocampal Dentate Gyrus (DG) (Heise *et al.* 2017). Performance of the eEF2K KO mice assessed by regular fear conditioning protocols is largely normal (Heise *et al.* 2017). The researchers also performed immunohistochemical experiments to probe for phosphorylated eEF2 in the hippocampus and found higher levels of eEF2 phosphorylation in the area CA3 and DG compared to the staining in the area CA1 (Heise *et al.* 2017). Another study from the group of Kobi Rosenblum reported that cortical-dependent associate taste learning was impaired in a line of transgenic mice with eEF2K repression (Gildish *et al.* 2012).

In further pursuing DG-related roles of the eEF2K/eEF2 signaling in the regulation of cognition function, the Rosenblum group generated a “conditional” eEF2K knockout mice (eEF2K cKO) by injecting adeno-associated viruses (AAVs) containing CaMKII-Cre to the DG subregion of the eEF2K floxed mice (Taha *et al.* 2020). They reported that CaMKII-Cre-injected mice showed enhanced hippocampal neurogenesis. They further performed a series of behavioral tests to characterize cognitive phenotypes associated with the selective repression of eEF2K in DG. They found that knockdown of eEF2K in DG was able to enhance performance in multiple hippocampal-dependent behavioral assays including a weak protocol MWM, pattern separation of contextual fear memory, and spatial pattern completion (Taha *et al.* 2020). Furthermore, they showed in aged mice that the DG-specific knockdown of eEF2K improved contextual fear memory four weeks after training (Taha *et al.* 2020). To the best of my knowledge, this is the only report on studying cognitive effects in rodents with brain region-specific suppression of eEF2K. It would be important for future studies to determine the behavioral and synaptic phenotypes in transgenic mice with conditional eEF2K suppression in other brain regions. It is also important to explore the detailed mechanisms underlying the different behavioral phenotypes in these mice as compared with those in the global eEF2K KO mice.

Long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus are probably the most intensively studied forms of synaptic plasticity, coordination of both is considered as a key molecular mechanism underlying learning and memory (Malenka & Bear 2004). A previous study from the laboratory of Paul Worley reported that late LTP

induced by a strong electrical protocol (four trains high-frequency stimulation (HFS)) was facilitated in hippocampal slices from the eEF2K KO mice (Park *et al.* 2008). Meanwhile, we found that hippocampal late LTP induced by a weaker protocol (two trains HFS) was unaltered in the eEF2K KO mice (Zimmermann *et al.* 2018). The mechanisms underlying the different LTP performances remain unclear.

LTD is broadly divided into two categories: N-methyl-D-aspartate receptor (NMDAR)-dependent and metabotropic glutamate receptor (mGluR)-dependent LTD. NMDAR-dependent. LTD is usually induced by electrical low-frequency stimulation (LFS) and requires activation of NMDARs, while mGluR-dependent LTD requires activation of group 1 mGluRs (includes mGluR1 and 5) and is typically induced by DHPG [(RS)-3,5-dihydroxyphenylglycine], a selective agonist for group 1 mGluR (Malenka & Bear 2004; Lüscher & Huber 2010). Park and colleagues reported that NMDAR-dependent LTD induced by LFS protocol was unaltered in hippocampal slices from eEF2K KO mice. In comparison, mGluR-dependent LTD elicited by DHPG treatment was significantly impaired in hippocampal slices derived from the eEF2K KO mice (Park *et al.* 2008).

Compared to the multiple studies with suppression of eEF2K and eEF2 phosphorylation, there is only one report that I am aware of investigating neuronal functions in transgenic mice with overexpression of eEF2K (Im *et al.* 2009). Taking advantage of a Cre/loxP recombination system, Im and colleagues developed the hip-eEF-2K-tg mice in which the eEF2 gene was overexpressed specifically in the hippocampus, resulting in hyper-phosphorylation of eEF2 and consequently inhibition of general protein synthesis (Im *et al.* 2009). They further examined the memory phenotypes in the transgenic mice, mainly by using the regular fear conditioning test. The hip-eEF-2K-tg mice behaved normally in short-memory performance evaluated by both contextual and cued fear conditioning tasks. Examination of long-term memory revealed that the freezing response of the hip-eEF-2K-tg mice was impaired during contextual fear conditioning but unaltered during cued conditioning (Im *et al.* 2009). Further, late LTP was impaired in the hippocampus of the hip-eEF-2K-tg mice (Im *et al.* 2009). More comprehensive and systematic studies (both in vivo and in vitro) are needed to investigate neuronal phenotypes associated with elevated eEF2K activities and eEF2 hyper-phosphorylation.

Hyper-phosphorylation of eEF2 in Alzheimer's disease: A Double-edge Sword?

Alzheimer's disease (AD) is an age-related neurodegenerative disease and the most common form of dementia syndromes (Holtzman *et al.* 2011; Querfurth & LaFerla 2010; Association 2019; Roberson & Mucke 2006). AD has become a global threat for public health mainly due to the significant increase of the aging population. The two classical hallmarks of AD brain pathology are Aβ plaques and hyper-phosphorylated tau tangles. Meanwhile, multiple studies indicate that synaptic deficits are better correlated with clinical symptoms in AD patients, and synaptic failure might be a key and early event during AD pathogenesis (Selkoe 2002; Ma & Klann 2012; Teich *et al.* 2015). Despite decades of research, the detailed molecular signaling mechanisms underlying AD-associated cognitive impairments

and synaptic deficits remain elusive, hindering the identification of effective therapeutic strategies and novel diagnostic/prognostic biomarkers for the disease.

Studies from our and other groups described abnormal hyper-phosphorylation (Thr56) of eEF2 in post mortem brain tissues from human AD patients and hippocampus of multiple transgenic mouse models of AD including APP/PS1, 3xTg, and Tg19959 mice (Ma *et al.* 2014; Li *et al.* 2005; Jan *et al.* 2017; Beckelman *et al.* 2019). By doing immunohistochemical experiments in human AD brain samples, Jan and colleagues also revealed that the increase of eEF2 phosphorylation is more pronounced in the hippocampus compared to the staining in the cortex (Jan *et al.* 2017). Importantly, examination of human AD brain tissues showed significantly increased immunostaining of phospho (Thr56)-eEF2 in neurons (Jan *et al.* 2017; Beckelman *et al.* 2019). We also reported increased levels of eEF2 phosphorylation in the hippocampus of old mice (19–21 months) compared to mice of 3–5 months old (Yang *et al.* 2019). Interestingly, our study with human samples suggested that eEF2 hyper-phosphorylation could be specific to AD-related pathological processes since we did not observe the similarly elevated levels of eEF2 phosphorylation in post mortem brain samples from human patients with other neurodegenerative diseases such as frontotemporal dementia (FTD) and Lewy body dementia (LBD) (Beckelman *et al.* 2019).

Substantial evidence has indicated that energy metabolism dysregulation and chronic oxidative stress induced by excessive reactive oxygen species (ROS) play a central role in the pathogenesis of many neurodegenerative diseases including AD (Lin & Beal 2006; Ma & Klann 2014; Ma & Klann 2012; Massaad *et al.* 2009; Ma *et al.* 2011a; Dumont *et al.* 2009). Another related common feature of many neurodegenerative diseases is the accumulation of misfolded proteins, which induces the unfolded protein response (UPR) and significant cellular stress (Wek & Cavener 2007; Ma & Klann 2014). Numerous biological processes are stimulated to coordinate the stress response, one important component of which is inhibition of global protein synthesis through regulation of various translational factors including phosphorylation of initiation factor eIF2 α subunit (eIF2α) and elongation factor eEF2 (Wek *et al.* 2006; Wek 2018). The process of UPR involves activation of PERK (protein kinase RNA-like endoplasmic reticulum kinase), which phosphorylates eIF2α and results in inhibition of translation initiation (Wek 2018; Wek & Cavener 2007; Trinh & Klann 2013). On the other hand, AD-related oxidative stress and low energy state induce activation of AMPK, which in turn actives eEF2K and eEF2 phosphorylation, leading to inhibition of translation elongation (Ma *et al.* 2014; Vingtdeux *et al.* 2011; Wang *et al.* 2019) but see (Jan *et al.* 2017). Transient repression of global protein synthesis in response to cellular stress could be neuroprotective because it allows neurons to conserve energy resources (protein synthesis is a high energy-consuming process) meanwhile enhancing the translation of specific stress-related mRNAs, thus reconfiguring gene expression to cope with the cellular stress (Paschen *et al.* 2007; Ma & Klann 2014) (Fig. 1). Nevertheless, the severe and chronic cellular stress associated with pathological conditions in many neurodegenerative diseases (e.g. AD) could result in continuous hyper-phosphorylation of eEF2 (via activation of AMPK and eEF2K) and consequently long-lasting repression of general protein synthesis. An enhanced and prolonged eEF2 hyper-phosphorylation would eventually impair neuronal plasticity and cognitive function because integral mechanisms of de novo protein synthesis are essential for memory consolidation and maintenance of long-

term synaptic plasticity (Richter & Klann 2009; Sossin & Costa-Mattioli 2019; Rosenberg *et al.* 2014) (Fig. 1). Interestingly, studies in cultured neurons and *C. elegans* demonstrated that inhibition of eEF2K activity was able to blunt the increased ROS induced by Aβ oligomers (Jan *et al.* 2017). Taken together, there may exist a retrospective relationship between the eEF2K signaling and cellular stress.

Repression of eEF2K as a therapeutic strategy for AD-associated synaptic failure and cognitive impairments: Promises and Caveats.

From a general view of translational medicine, there are several appealing features of targeting eEF2K as a potential therapeutic strategy for AD and other aging-related dementia syndromes. First, as described above, eEF2K is an unusual kinase that belongs to a very small group of kinases termed "α-kinases", whose catalytic domains are distinct from those in the CPKs (e.g. serine/threonine kinases and tyrosine kinases), which consists of the vast majority of the eukaryotic protein kinase superfamily (Drennan & Ryazanov 2004). Thus, compounds targeting eEF2K i.e. eEF2K inhibitors, if designed appropriately targeting the α-kinase-specific domains, are unlikely to affect activities of other CPKs that play important roles for a broad spectrum of biological processes. Second, multiple lines of studies suggested that eEF2K activity is not required for development or cell survival under physiological conditions. As discussed, transgenic mice with global homozygous knockout (or heterozygous knockdown) of the eEF2K gene appear normal in all measurements on basic biological functions during different stages of development (Ryazanov 2002; Heise *et al.* 2017; Gosrani *et al.* 2020; Beckelman *et al.* 2019). Wild type mice with the chronic treatment of various eEF2K inhibitors are also "normal" based on numerous measurements including life span, weight change, gross morphology of hippocampus, locomotor activities, and cognitive performance (Ma lab, unpublished data). Therefore, it is promising for future studies in a clinical setting that eEF2K inhibitors can be given safely without causing severe side effects (but see below discussion on the caveats of eEF2K inhibitors). The safety issue is critically important for patients with AD and related dementia syndromes who usually need to keep taking medicine over a long period. Third, there is a one-on-one relationship between eEF2K and eEF2 in that eEF2K is the only known upstream kinase for eEF2 and eEF2 is the only known substrate for eEF2K (phosphorylation at the Thr56 site) (Ryazanov & Davydova 1989; Proud 2015). Such specificity can further strengthen the selectivity and efficiency of the eEF2K inhibitors for therapeutic purposes.

To investigate the roles of eEF2K/eEF2 signaling dysregulation in AD pathophysiology, we genetically reduced AD-related eEF2 hyper-phosphorylation by crossing the eEF2K KO mice with two different lines of AD model mice. In AD mice with heterozygous knockdown of eEF2K, we found that AD-associated impairments of hippocampal de novo protein synthesis (assessed by SUnSET and analysis of the dendritic polyribosome assembly) were restored (Beckelman *et al.* 2019). Importantly, aging-related long-term memory deficits (assessed by multiple behavioral tasks) and synaptic plasticity (including LTP and mGluR-LTD) impairments in both AD model mice were significantly improved with genetic suppression of eEF2K (Beckelman *et al.* 2019; Yang *et al.* 2021) (Fig. 2). Notably, neither brain Aβ deposit nor tau phosphorylation in AD model mice was affected

with genetic reduction of eEF2K, adding another line of evidence supporting potential therapeutic strategies through targeting mechanisms independent of Aβ or tau (Beckelman *et al.* 2019; Pimplikar *et al.* 2010; Kametani & Hasegawa 2018).

What are exactly the molecular mechanisms underlying the improvement of synaptic and cognitive phenotypes in AD mice with eEF2K suppression? Given the critical role of eEF2K/eEF2 signaling in controlling translation elongation, and in light of the evidence showing deficits of protein synthesis capacity in AD (Ma *et al.* 2013), the parsimonious answer for the above question would be enhancement of protein synthesis. However, this will instantly bring another outstanding question: what are the particular proteins (whose synthesis is facilitated by suppression of eEF2K and eEF2 phosphorylation) that are responsible for the rescuing phenotypes? We tried addressing this question (to a certain extent) by performing mass spectrometry (MS)-based proteomics analysis in brain samples of AD mice with genetic or pharmacological reduction of eEF2K (Beckelman *et al.* 2019) (Ma lab, unpublished data). The proteomics data showed multiple proteins whose expression levels were dysregulated (both increased and decreased) in AD mice and restored with eEF2K inhibition. Further functional analysis using bioinformatics tools revealed proteins that were involved in synaptic function, cytoskeletal dynamics, mitochondrial function, calcium buffering, and cell adhesion (Beckelman *et al.* 2019)(Ma lab, unpublished data). Future in-depth investigation at the functional level would help provide insights into the exact roles of these proteins in AD pathogenesis. Moreover, it should be cautioned that most if not all proteomic studies in the AD field are designed to examine overall protein profiling alterations and did not really differentiate “newly synthesized proteins”, which are critical for the maintenance of long-term forms of memory and plasticity (Alberini 2008; Sutton & Schuman 2006; Klann & Dever 2004).

Decades of research in identifying particular “memory proteins” or “plasticity-related proteins (PRPs)” have not yielded consensus among neuroscientists (Okuda *et al.* 2020). Here we propose an alternative concept that the “general” protein synthesis upregulation per se could be the main mechanism for improvement of AD pathophysiology with eEF2K inhibition. This concept is also supported by previous studies demonstrating that the boost of general protein synthesis through regulation on the initiation phase of protein synthesis, either genetically or pharmacologically, resulted in the improvement of multiple aspects of AD pathophysiology (Ma *et al.* 2013; Yang *et al.* 2016; Oliveira *et al.* 2021).

Through electrophysiology studies, Heise and colleagues found that the synaptic activities mediated by the GABAergic inhibitory neurons, which contribute to the tonic inhibition in the hippocampus, were significantly upregulated in hippocampal slices derived from the eEF2K KO mice (Heise *et al.* 2017). Dysfunctional GABAergic neurons and reduced tonic inhibition is one of the most frequent causes of brain epileptic/seizure activities (Pavlov & Walker 2013). In line with the ex vivo electrophysiological data, the authors further uncovered with in vivo studies that the eEF2K KO mice were more resistant to seizure activities induced by pentylenetetrazol (PTZ) (Heise *et al.* 2017). Interestingly, multiple lines of evidence suggest a link between epileptic seizure-like activities and the development of AD pathophysiology (Palop & Mucke 2016).

Besides AD, aberrant eEF2K activity has been linked to several other human diseases including cardiovascular disease and cancer (Liu & Proud 2016). Development of eEF2K inhibitors was initially motivated by the concept that targeting aberrant protein synthesis associated with eEF2K signaling dysregulation might be a viable anti-cancer strategy (Liu & Proud 2016). NH125 is among the first identified small molecule antagonists of eEF2K through studies in cancer cell lines (Arora *et al.* 2003). We have shown in ex vivo studies that Aβ-induced LTP failure in mouse hippocampal slices was reserved by treatment of NH125 (Ma *et al.* 2014). Another study showed that in vivo NH125 treatment was able to reduce eEF2 hyper-phosphorylation and seizure phenotype in an epilepsy mouse model (Heise *et al.* 2017). It needs to be cautioned that two studies conducted in non-neuronal cell lines argued the specificity and inhibition potency of NH125 on eEF2K, as well as the actual effects on eEF2 phosphorylation (Chen *et al.* 2011; Devkota *et al.* 2012). Investigation on potential eEF2K-independent mechanisms underlying the beneficial effects of NH125 on electrophysiological and cognitive phenotypes in neuronal disorders warrants further studies.

In addition to NH125, several other small molecule compounds are shown to inhibit eEF2K activities such as A-484954 and Jan-384 (Liu & Proud 2016; Kenney *et al.* 2016). Very few studies have been performed to test the effects of these inhibitors on AD-related abnormalities, especially at the functional level. The compound A-484954 is considered to be more potent and selective than NH125 based on studies from cell culture (Chen *et al.* 2011). Research from our laboratory revealed that treatment with A-484954 (in two different AD model mice) was able to rescue AD-associated cognitive dysfunction assessed by multiple behavioral assays including MWM and NOR. Similar to the results from the genetic manipulation, the therapeutic effects of the eEF2K inhibitor appear to be independent of effects on brain Aβ and tau pathology. Furthermore, A-484954 treatment alleviated AD-associated defects in dendritic spine morphology, postsynaptic density formation, protein synthesis, and dendritic polyribosome assembly (Ma lab, unpublished data). Our proof-of-principle study suggests translational implication of inhibiting eEF2K for AD and related dementia syndromes.

There are some caveats in proposing eEF2K inhibitors as a potential therapy for AD and related dementia syndromes. First, as indicated in the discussion above, short-term activation of eEF2K and eEF2 phosphorylation might be an important mechanism that is actually beneficial for neurons to cope with cellular stress (Fig. 1). Further, too much or too little protein synthesis can both lead to neuronal diseases with impairments of synaptic and cognitive functions (Tahmasebi *et al.* 2018; Scheper *et al.* 2007; Rosenberg *et al.* 2014). It is thus critical to optimize the treatment paradigm (dose, duration, etc.) to “restore” (but not over-suppress) levels of eEF2 phosphorylation and the machinery for mRNA translation. Second, overall there is a lack of understanding of the 3-dimensional structure and binding mechanisms of the current eEF2K inhibitors (e.g. A-484594), which hampers the development of eEF2K inhibitors with optimal selectivity and potency (Liu & Proud 2016; Wang *et al.* 2015). It is urgent to identify more selective and potent antagonists of eEF2K, which would be a useful tool to help investigate the roles of eEF2 phosphorylation in cognitive function and neuronal plasticity.

Conclusions and Future Perspectives

Regulation of eEF2K activity and eEF2 phosphorylation is involved in the process of memory formation and synaptic plasticity, as evidenced in ample research studies from various model systems. A number of recent studies have also indicated that targeting dysregulation of eEF2K/eEF2 signaling could be a viable therapeutic strategy for Alzheimer's disease and related dementia syndromes. Meanwhile, the use of the global eEF2K knockout model makes it challenging to tease out peripheral effects of eEF2 de-phosphorylation. For a more detailed understanding of the roles of eEF2K/eEF2 signaling in the central nervous system under physiological or pathological conditions (e.g. AD), it is critical to develop advanced model systems with region-specific manipulation (up- or down-regulation) of eEF2K and eEF2 phosphorylation. Given all the arguments around the compounds currently available for inhibition of eEF2K, there is also an urgent need for the development of better eEF2K inhibitors to facilitate both basic and translational research related to the eEF2K signaling. Finally, there are plenty of studies indicating a pivotal role of eEF2K signaling and related protein synthesis regulation in tumorigenesis (Liu & Proud 2016). Interestingly, previous studies in the human population using epidemiologic approaches suggest that there might be an inverse relationship between the incidence of AD and cancer (Driver *et al.* 2012; Musicco *et al.* 2013; Realmuto *et al.* 2012). It would an intriguing topic for future studies to elucidate whether dysregulation of the eEF2K/eEF2 signaling represents one of the common pathological mechanisms at the molecular level contributing to the development of both cancer and AD.

Acknowledgements

This work was supported by National Institutes of Health grants R01 AG055581, R01 AG056622, and R01 AG073823 (T.M.), the Alzheimer's Association grant NIRG-15-362799 (T.M.), the BrightFocus Foundation grant A2017457S (T.M.). I apologize for authors whose publications relevant to this topic were not included due to the space limit.

List of Abbreviations:

AD	Alzheimer's disease
ADRD	Alzheimer's disease and related dementias
AMPK	AMP-activated protein kinase
α-kinases	alpha-kinases or atypical kinases
AKT	Protein kinase B
BDNF	brain-derived neurotrophic factor
CPKs	conventional protein kinases
CaMKIII	Calmodulin-dependent protein kinase III
DG	dentate gyrus
eEF2	eukaryotic elongation factor 2

eEF2K	eukaryotic elongation factor 2 kinase
eIF2α	eukaryotic initiation factor 2 alpha
ERK	extracellular signal-regulated kinase
LTP	long-term potentiation
LTD	long-term depression
mTORC1	mammalian target of rapamycin complex 1
MWM	Morris water maze
NOR	novel object recognition
PERK	protein kinase RNA-like endoplasmic reticulum kinase
ROS	reactive oxygen species
SUnSET	surface sensing of translation

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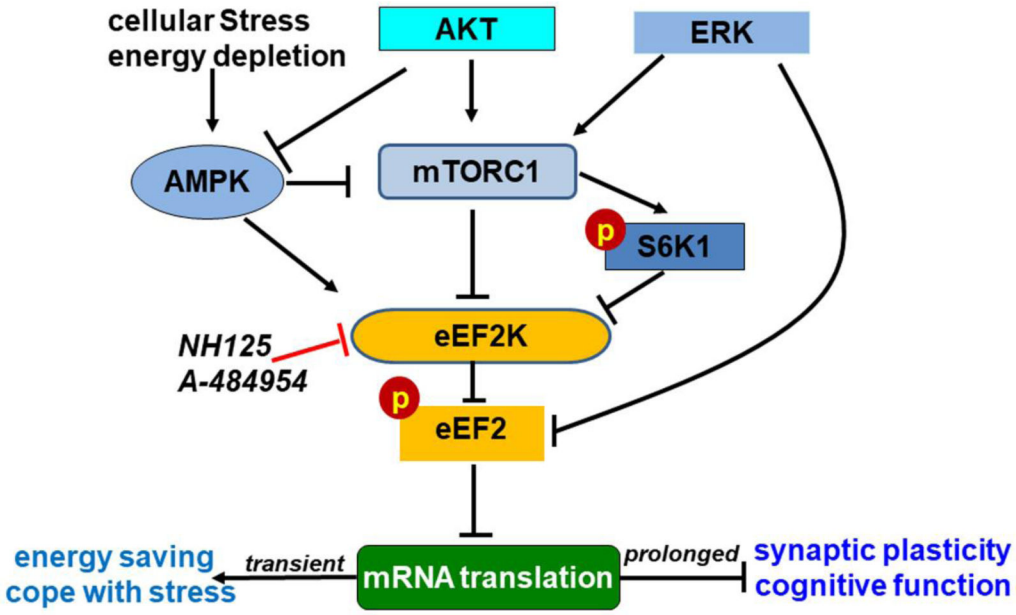

Fig.1.

Schematic model that depicts the upstream regulators and downstream effectors of eEF2K. Small molecule eEF2K antagonists (NH125 and A-484594) currently available are indicated. Arrows denote activation and blunted lines indicate inhibition. AKT: Protein kinase B; ERK: extracellular signal-regulated kinase; AMPK: AMP-activated protein kinase; mTORC1: mammalian target of rapamycin complex 1; eEF2K: eukaryotic elongation factor 2 kinase; eEF2: eukaryotic elongation factor 2.

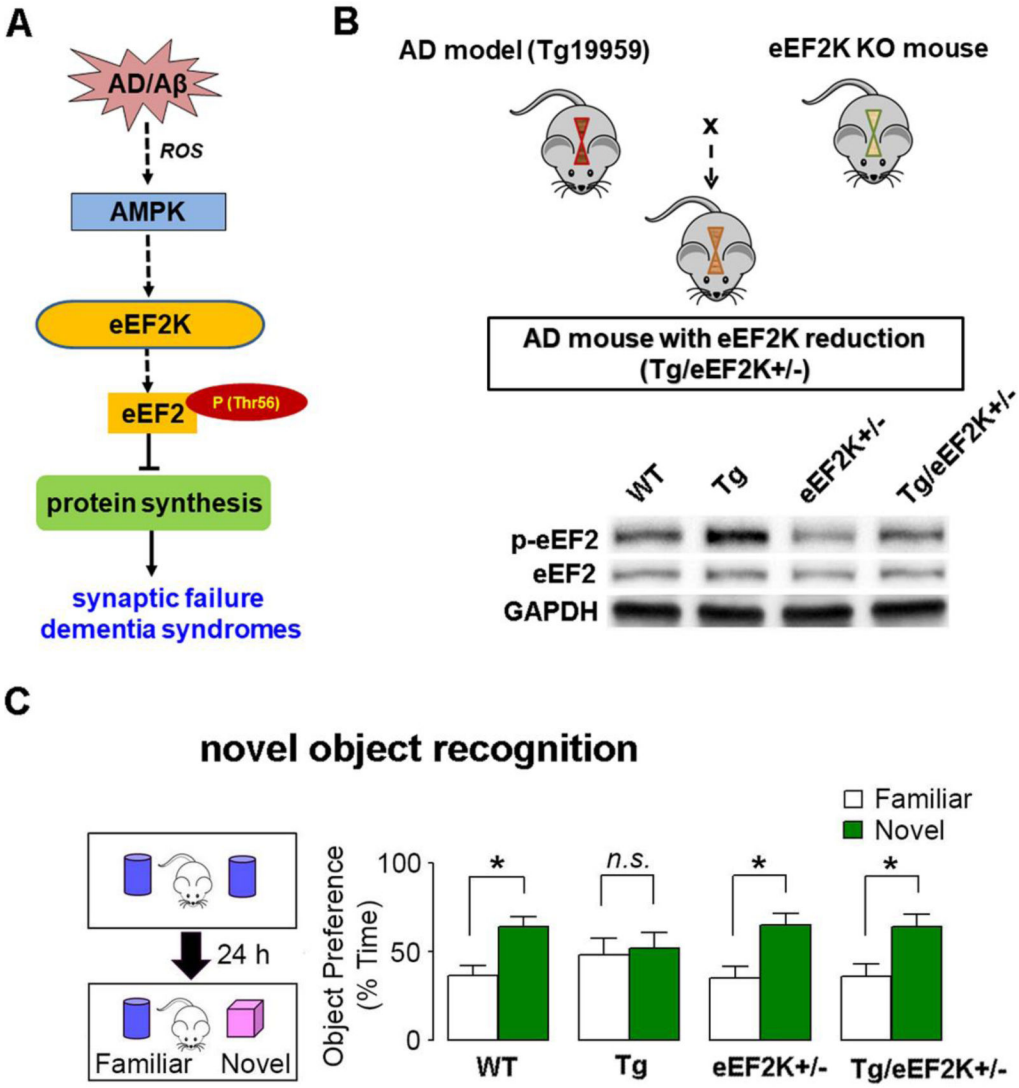

Fig. 2.

Repression of eEF2K activity as a potential therapeutic strategy for Alzheimer's disease. (a) A working model describing the link between dysregulation of eEF2K signaling and AD-associated synaptic failure and dementia syndromes. (b) Cartoon diagram describing a genetic approach to suppress AD-associated eEF2 hyper-phosphorylation. Representative western blot images showed restoration of eEF2 phosphorylation in the hippocampus of Tg19959 AD model mice. Adapted from Beckelman, et al., *JCI*, 2019. (c) Genetic suppression of eEF2K and eEF2 phosphorylation alleviated cognitive impairments displayed in AD model mice. Results from novel object recognition (NOR) behavioral assay were shown. Adapted from Beckelman, et al., *JCI*, 2019. Please also refer to the main text for details. AD: Alzheimer's disease; AMPK: AMP-activated protein kinase; eEF2K: eukaryotic elongation factor 2 kinase; eEF2: eukaryotic elongation factor 2. KO: knockout.