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PERK REGULATES CALCIUM DYNAMICS
IN COGNITION AND NEURODEGENERATIVE DISEASE

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by
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ABSTRACT

PERK (EIF2AK3), an ER-resident eIF2α kinase, is well known for its role in eIF2α-dependent protein synthesis and translational control. Recent study in insulin-secreting β-cells revealed that PERK regulates Ca\textsuperscript{2+} dynamics in β-cells, which underlies glucose-stimulated insulin secretion and is protein synthesis-independent. In my thesis, motivated by PERK’s acute regulation of Ca\textsuperscript{2+} dynamics in β-cells, I explored if PERK also regulates Ca\textsuperscript{2+} dynamics in pyramidal neurons, and whether this regulation plays any physiological role in the central nervous system.

I found that acute PERK inhibition by the use of a highly specific PERK inhibitor reduced G\textsubscript{q} protein-coupled intracellular Ca\textsuperscript{2+} rise in cortical pyramidal neurons. Further experiments revealed that PERK inhibition increased IP\textsubscript{3} receptor mediated ER Ca\textsuperscript{2+} release, but decreased receptor-operated Ca\textsuperscript{2+} entry from extracellular space.

Working memory is a cognitive function that is independent of new protein synthesis, but heavily relies on Ca\textsuperscript{2+} dynamics. I identified working memory deficit in forebrain-specific Perk knockout and pharmacologically PERK-inhibited mice, suggesting that PERK’s acute regulation of Ca\textsuperscript{2+} dynamics may play a critical role in cognition.

Alzheimer’s disease is an age-related neurodegenerative disease characterized by abnormal aggregation of amyloid-β peptides in the cortex and hippocampus. PERK has been speculated to regulate Alzheimer’s disease progression by its eIF2α-dependent protein synthesis and translational control. To test this hypothesis, I ablated Perk in the forebrain of an Alzheimer’s disease mouse model. I found that knocking down PERK in the forebrain alleviated the Alzheimer’s disease mice’s behavior deficits, but did not affect the amyloid-β plaque density or the expression of amyloid precursor protein and amyloid-β peptides in their forebrain. In addition, acute PERK inhibition corrected the exaggerated G\textsubscript{q} protein-coupled Ca\textsuperscript{2+} signaling in primary
neurons cultured from the Alzheimer’s disease mice model. Considering the important role of Ca\(^{2+}\) signaling in Alzheimer’s disease progression, it is possible that PERK regulates the disease progression by Ca\(^{2+}\) modulation.

Taken together, in my thesis work, I discovered that PERK acutely regulates G\(_q\) protein-coupled Ca\(^{2+}\) dynamics in pyramidal neurons, and PERK’s modulation of Ca\(^{2+}\) dynamics may be directly connected to its regulation of working memory and its role in Alzheimer’s disease progression. My thesis work illustrates a novel role of PERK in the central nervous system that is eIF2α-independent, and suggests that PERK’s regulation of Ca\(^{2+}\) dynamics may play important physiological roles in the central nervous system.
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To my parents and my love.
Chapter 1

Introduction

1.1 The discovery of PERK and its role in learning, memory and Alzheimer’s disease

The discovery of PERK and its signaling pathways

Protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), a type I transmembrane endoplasmic reticulum (ER)-resident protein, was initially discovered as a mammalian eIF2α kinase abundantly expressed in the pancreas, and characterized as an eIF2α-dependent translation initiation inhibitor (1). In later studies, PERK was further discovered to be an immediate effector of the unfolded protein response in cultured cells, which occurs when there is an overload of unfolded ER client proteins (2,3). As an ER-resident protein kinase, PERK’s regulatory domain is located in the ER lumen and its catalytic domain is located in the cytoplasm. At steady state, PERK’s activation is inhibited by the binding of BiP (4), an ER-resident chaperone required for protein folding (5), and Ca²⁺ (6) to its regulatory domain. Under certain circumstances, such as overload of ER client proteins or depletion of ER Ca²⁺, dissociation of BiP or Ca²⁺ from its regulatory domain promotes its dimerization, and PERK is subsequently activated by trans-autophosphorylation on serine and threonine residues in its catalytic domain (1,7) (Fig. 1-1).
PERK’s activity is suppressed at steady state due to the binding of BiP and Ca\(^{2+}\) to its regulatory domain in the ER lumen. Under the unfolded proteins response or in response to ER Ca\(^{2+}\) depletion, BiP or Ca\(^{2+}\) dissociates from its regulatory domain, and PERK is activated by trans-autophosphorylation of the catalytic domain after dimerization.

So far, two substrates of PERK have been identified: the \(\alpha\) subunit of eukaryotic translation initiation factor-2 (eIF2\(\alpha\))(1) and NF-E2 related factor 2 (Nrf2) (8). PERK is well known for its role in eIF2\(\alpha\)-dependent protein synthesis and translational control. Upon activation, PERK phosphorylates eIF2\(\alpha\) at serine 51, which can subsequently modulate protein translation in two opposite ways: attenuation of global protein synthesis via inhibition of eIF2B mediated GTP exchange (3,9), and induction of translation of specific genes with upstream open reading frames including CREB2/ATF4 (10). The other substrate of PERK kinase activity, Nrf2, is a transcription factor that regulates detoxifying enzymes’ expression under oxidative stress (11). The identification of Nrf2 as a direct substrate of PERK (8) suggests that PERK can function in an eIF2\(\alpha\)-independent pathway.
PERK’s role in β-cell development and function

Although PERK is ubiquitously expressed in various tissues in rodents, it is most abundantly expressed in the pancreas (1), where it plays a vital role in β-cell development and function (12-14). Thus a brief review of PERK’s role in β-cells will facilitate the understanding of PERK’s role in other cell types including neurons. In 2002, loss of function *Perk* knockout mice were generated. These mice display severe permanent neonatal diabetes due to low pancreatic and serum insulin (12,15). This rapid loss of insulin-secreting β-cell mass before the onset of hyperglycemia in the neonatal *Perk* knockout mice combined with PERK’s known role in unfolded protein response led to the initial speculation that the onset of diabetes was due to β-cell apoptosis caused by unresolved ER stress. However, further studies failed to provide direct evidence supporting this hypothesis, as the expression of ER stress markers was not induced in *Perk*-deficient islets at the time when there was already a significant loss of β-cell mass (13). On the other hand, it was not only discovered that loss of β-cell mass in the *Perk*-deficient pancreas stems from impaired β-cell proliferation and differentiation during the neonatal stage (13), but also that PERK is required for normal β-cell function including proinsulin trafficking and glucose stimulated insulin secretion (13,14). The observation of several distinct β-cell defects in the *Perk*-deficient mice suggests that PERK plays a fundamental role in pancreas development and β-cell function.

While PERK’s roles in β-cell development and function are easily observed, the molecular mechanisms mediating PERK’s roles in β-cell remain elusive, since the onset of several β-cell defects in genetic *Perk* knockout mice makes it undetermined if these defects are solely PERK-dependent or there is a causal relationship between genotype and phenotype. To answer this question, PERK has to be acutely ablated in β-cells in order to study its immediate effect on β-cell function. The recent development of a highly specific and potent inhibitor of PERK, GSK2606414, which inhibits PERK’s enzymatic activity by competing for the ATP binding
domain, enables us to study PERK’s immediate effect on β-cell function (16). The treatment of β-cell lines with PERK inhibitor recapitulates the β-cell dysfunctions observed in genetically Perk-ablated cells, including impaired proinsulin trafficking and insulin secretion, indicating that PERK does play a critical role in β-cell function (17). Moreover, it was discovered that PERK regulates intracellular Ca\(^{2+}\) dynamics in concert with calcineurin in β-cells (17). Acute PERK inhibition impairs store-operated Ca\(^{2+}\) influx and secretagogue-stimulated ER Ca\(^{2+}\) reuptake mediated by SERCA pump. Both of these functions are calcineurin-dependent, and are essential Ca\(^{2+}\) signals underlying stimulated-insulin secretion. Although the molecular mechanism behind PERK/calcineurin’s regulation of store-operated Ca\(^{2+}\) influx is still unknown, PERK has been shown to regulate SERCA pump activity via its regulation of calcineurin-calnexin pathway (17).

**Fig. 1-2 PERK regulates Ca\(^{2+}\) dynamics in insulin-secreting β cells**

In insulin-secreting β-cells, PERK positively regulates the activities of store-operated Ca\(^{2+}\) channels (SOCC) and SERCA pump in a calcineurin (CN)-dependent pathway. While it is still unclear how PERK regulates SOCC through CN, PERK’s regulation of SERCA pump is likely to be mediated through CN-calnexin (CNX) pathway. At steady state, the binding of CNX with SERCA prevents it from activation. PERK-dependent activation of CN dephosphorylates CNX, which releases CNX from SERCA and results in SERCA activation.
PERK’s role in learning, memory and Alzheimer’s disease

Compared to its well-studied role in pancreas development and function, the study of PERK’s role in brain development and cognition started only a few years ago. Autosomal-recessive Perk mutations are the cause of Wolcott-Rallison Syndrome in humans, a rare genetic disease characterized by multiple developmental defects including: permanent neonatal diabetes, osteopenia, growth retardation and recurrent hepatitis, most of which are recapitulated in Perk knockout mice (12,18). In addition to above clinical manifestations, some of the Wolcott-Rallison Syndrome patients were diagnosed with mental retardation (18,19), suggesting the possible role of PERK in brain development and cognitive function.

As an eIF2α-dependent protein translation regulator, the activation of PERK phosphorylates eIF2α, which can subsequently repress global protein synthesis (3), while simultaneously induce the translation of specific genes including CREB2/ATF4 (10). Since both de novo protein synthesis and CREB2 are key regulators of long-term memory storage, the study of PERK in memory and cognition has been mainly focused on protein synthesis-dependent cognitive functions. So far it has been shown that PERK is required for memory flexibility and group 1 metabotropic glutamate receptor-dependent long-term depression (20,21). Considering PERK’s role in eIF2α-dependent protein translational control, it has been hypothesized that PERK’s regulation of these cognitive functions is also eIF2α-dependent. However, genetic reduction of eIF2α phosphorylation by single allele mutation of eIF2α phosphorylation site (22), or knockdown of other eIF2α kinases, such as GCN2(23) and PKR(24), lowers the threshold for late phase long-term potentiation and facilitates long-term memory storage, a phenotype that is absent in forebrain-specific Perk knockout mice (20,21). Thus, it is very likely that PERK imparts additional regulation on cognition that is eIF2α-independent.

In addition to the study of PERK in cognition, functional studies of PERK have also been carried out in the development of Alzheimer’s disease, a neurodegenerative disease characterized
by the abnormal aggregation of amyloid \( \beta \) peptides in the brain. These studies were motivated by PERK’s known function in protein translational control, as well as the observation of elevated phosphorylation of PERK and its substrate eIF2\( \alpha \) in brain tissues from Alzheimer’s disease patients (25,26) and Alzheimer’s disease mice models (27,28). Results of the mice studies show that genetic Perk knockdown or reduced PERK expression in brain alleviates the disease related long-term memory deficits and amyloid \( \beta \) peptide expression in two Alzheimer’s disease mice models (27,28). However, no evidence has been reported suggesting that PERK alleviates the disease progression by protein translational control. And the molecular mechanisms behind its role in Alzheimer’s disease progression remain controversial and unclear.

Motivated by the discovery that PERK regulates \( \text{Ca}^{2+} \) dynamics in insulin-secreting \( \beta \) cells in an eIF2\( \alpha \)-independent pathway (17), I explored the role of PERK in modulating \( \text{Ca}^{2+} \) dynamics in pyramidal neurons, and also the physiological roles of PERK’s regulation of \( \text{Ca}^{2+} \) signals in working memory and Alzheimer’s disease. To facilitate the understanding of my thesis work, this introduction covers brief reviews of the following three topics: 1) Different forms of memories including working memory (section 1.2); 2) Alzheimer’s disease and its related hypothesis (section 1.3); 3) \( G_q \) protein-coupled intracellular signaling cascades and the roles of \( G_q \) protein-coupled intracellular \( \text{Ca}^{2+} \) mobilization in working memory and Alzheimer’s disease (section 1.4).
1.2 Working memory, short-term memory and long-term memory

Depending on content, memories can be classified into two categories: conscious declarative memory, the ability to “recollect everyday events and factual knowledge” (29), and unconscious procedural memory, the ability to retrieve “the knowledge acquired during skill learning” (30). Depending on duration, memories can also be divided into short-term memory, which develops within a few seconds or minutes and lasts for hours, and long-term memory, which is defined as the memory lasts for more than twenty-four hours (31). Depending on their nature, memories are classified into transient memory, which includes working memory, and archival memory, which includes short-term memory and long-term memory (32). It is important to note that working memory, short-term memory and long-term memory are encoded by three distinct neural systems. Working memory serves as an on-line system to maintain information temporarily for task execution, whereas short- and long-term memories serve as off-line systems to preserve the information for later use.

How our brain encodes working memory was first illustrated by Fuster and his coworkers, as they observed sustained firing of neurons in the prefrontal cortex during the delayed response task in rhesus monkeys (33). It is now widely accepted that working memory relies on the electrical activity of neurons to temporarily store information. The molecular basis underlying working memory has been extensively studied. Accumulating evidence suggests that $G_q$-coupled intracellular signaling cascade plays a prominent role in working memory: reducing $G_q$ signaling pathway activity via depletion of $G_q$-protein-coupled receptor-specific neurotransmitters such as dopamine (34), blocking $G_q$ protein-coupled receptors including muscarinic acetylcholine and metabotropic glutamate receptors (35-38), or directly inhibiting $G_q$/phospholipase C (PLC) activity by infusion of PLC inhibitor (39) all lead to the impairment of working memory in animals. How the $G_q$-protein-coupled signaling pathway regulates working memory capacity will be talked in more detail in the third section of this chapter.
Working memory mainly relies on the electrical activity of neurons and these memories only persist under persistent neuronal firing. Conversely, short-term and long-term memories require modifications on synaptic plasticity in order to preserve the information for a longer period of time. Specifically, long-term memory has been shown to require protein synthesis-dependent memory consolidation. The first piece of evidence supporting this conclusion came from Flexber and his coworkers’ study with the use of a protein synthesis inhibitor, puromycin. They showed that intracerebral administration of puromycin after a training session impaired mice’s long-term memory in a Y-maze shock avoidance task (40). Considerable studies performed soon after studying the effect of protein synthesis inhibition on long-term memory with the use of protein synthesis inhibitors strongly supported the hypothesis (41). However, questions as to how newly synthesized protein affects long-term memory and the localization of newly synthesized protein pools were still not understood then. In 1996, Kang and Schuman presented the first evidence suggesting local protein synthesis existed in order to fulfill the immediate protein need for neurotrophin induced synaptic plasticity (42). It is now widely accepted that local protein synthesis plays a critical role in long-term synaptic plasticity (43).

The encoding of short-term memory does not require new protein synthesis(44). Instead it depends on the post-translational modifications of existing proteins mediated by intracellular secondary messengers to modulate synaptic plasticity (45). In invertebrates, it has been shown that serotonin mediated intracellular cyclic AMP increase plays a critical role in short-term memory formation via continued cyclic AMP-dependent protein kinase activity (44). In mammals, it has also been shown that cyclic AMP-dependent protein kinase (PKA)’s activity is required for short-term memory formation (32).

Besides the general molecular basis underlying short-term and long-term memory, it is still debatable whether or not the two forms of memory are sequentially connected or parallel to each other. The idea that the two forms of memory are stepwise-linked was initially proposed by Hedd
and Gerard in their dual trace theories of memory, which stated that reverberating neural activity underlies short-term memory and the stabilization of it produces long-term memory (31). This theory soon gained support based on the finding that administration of protein synthesis inhibitor in goldfish did not affect their learning ability in shock avoidance task, but did impair their long-term memory (46). Similar studies performed in rodents obtained the same results; blocking protein synthesis either pharmaceutically or genetically disturbed mice’s long-term memory but preserved short-term memory (41,47). These studies underscored the importance of consolidation in the long-term memory and are consistent with the dual trace theories of memory. However, they don’t rule out the possibility that short-term memory and long-term memory are separate processes. One way to address the interdependence between them is to block short-term memory and study if long-term memory is impaired or not. Emptage and Carew showed that when blocking the short-term facilitation in Aplysia by the use of a non-specific serotonin antagonist, cyproheptadine, the long-term facilitation of a monosynaptic response was preserved (48). This is the first study suggesting long-term memory expression does not depend on short-term memory. However, there are some concern about the conclusion since monosynaptic response does not represent memory well (32).
1.3 Alzheimer’s disease

The pathogenesis of Alzheimer’s disease

Alzheimer’s disease (AD) is an age-related neurodegenerative disease affecting about 2% of the population in industrialized countries. Clinically, AD patients lose their memory and cognitive abilities. Pathologically, extracellular deposits of amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles are formed in the brain regions involved in learning and memory, which include cortex and hippocampus(49).

Aβ peptide is a 40-42 amino acid peptide generated by the proteolysis of amyloid precursor protein (APP), which is mediated by the enzyme activity of α-, β- and γ- secretases/secretase complex. While three enzymes have been identified with α-secretase activity: a disintegrin and metalloprotease (ADAM) 9, ADAM10 and ADAM17; BACE1 is considered as the major β-secretase; and γ-secretase complex is considered to be composed of four components: the catalytic component presenilin 1 or presenilin 2, and three essential cofactors including nicastrin, anterior pharynx defective 1 and presenilin enhancer 2 (50).

The proteolysis of APP occurs in two pathways: non-amyloidogenic pathway and amyloidogenic pathway (51). In the non-amyloidogenic pathway, APP is cleaved by α-secretase within the Aβ peptide sequence, which results in the generation of soluble APPα and a membrane anchored 83-amino acid ε-terminal fragment (C83). Since the cleavage of α-secretase happens within the Aβ peptide region, it prevents the generation of Aβ. In the amyloidogenic pathway, the cleavage of APP by β-secretase generates soluble APPβ and membrane anchored 99-amino acid ε-terminal fragment (C99). Subsequent cleavage of C99 by γ-secretase complex generates Aβ peptide and APP intracellular domain (AICD) (51) (Fig.1-2).

In addition to Aβ plaques, neurofibrillary tangles are another pathologic hallmark of Alzheimer’s disease. Neurofibrillary tangles consist of aggregated, aberrantly phosphorylated forms of the microtubule-associated protein Tau. In adult human brain, there are six major
isoforms of Tau generated from a single gene through alternative splicing. The primary function of Tau is to stabilize microtubules via binding, which is regulated by serine/threonine-directed phosphorylation. Tau fibrillization is believed to begin with the detachment of Tau from microtubules, which can be triggered by the change of phosphorylation status of Tau. The increased cytosol unbound Tau may aggregate into small nonfibrillar Tau deposits. The nonfibrillar Tau deposits subsequently go through structure transition and develop into pleated β-sheet containing structures, and eventually lead to the development of neurofibrillary tangles (52).

![Fig. 1-3 Proteolysis of amyloid precursor protein](image)

In the non-amyloidogenic pathway, APP is cleaved by α-secretase within the Aβ peptide sequence, which results in the generation of soluble APPα and a membrane anchored 83-amino acid c-terminal fragment (C83). In the amyloidogenic pathway, the cleavage of APP by β-secretase generates soluble APPβ (sAPPβ) and membrane anchored 99-amino acid c-terminal fragment (C99). Subsequent cleavage of C99 by γ-secretase complex generates Aβ peptide and APP intracellular domain (AICD).
The amyloid hypothesis of Alzheimer’s disease

The Aβ peptides derived from Alzheimer’s disease and Down syndrome patients’ cerebrovascular amyloidosis were first purified and sequenced in 1980s, and it was discovered that both diseases share the same Aβ peptide (53,54). Shortly after this, Aβ peptide was identified as the core component of the senile plaques found in Alzheimer’s disease and Down syndrome patients (55). The amyloid hypothesis was proposed shortly after the above findings, which states that the cerebral accumulation of Aβ peptide is the primary driving force of Alzheimer’s disease related pathogenesis, including the development of intracellular neurofibrillary tangles and the loss of synapse and neurons (56).

The amyloid hypothesis is well supported by the genetic evidence, since the early-onset familial Alzheimer’s disease associated genes APP, presenilin 1 and presenilin 2, and the late-onset Alzheimer’s disease associated gene APOE, are closely correlated to APP metabolism (56). Moreover, several lines of evidence support the fundamental role of Aβ rather than Tau protein in the initiation of Alzheimer’s disease. First of all, mutations in tau have been identified as the cause of an autosomal dominantly inherited dementia named frontotemporal dementia with Parkinsonism, which is linked to chromosome 17(FTDP-17) (57,58). FTDP-17 is pathologically characterized by the deposition of neurofibrillary tangles in the brain, with no amyloid plaques observed (59), which suggests that the development of Aβ plaques is independent of neurofibrillary tangles formation in Alzheimer’s disease. Secondly, double mutant mice overexpressing mutant human APP (hAPP) and mutant human Tau (hTau) exhibit substantially enhanced neurofibrillary tangles compared to the single mutant hTau mice, while showing similar Aβ plaque formation compared to the single mutant hAPP mice (60), which suggests that the formation of Aβ plaques proceeds and positively influences the development of neurofibrillary tangles.
The amyloid hypothesis is suggested to be a possible cause of Alzheimer’s disease, but the molecular mechanism behind it remains to be determined. While numerous efforts have been made in the past thirty years exploring how amyloid accumulation may give rise to the disease progression, several observations made during this process present concerns regarding the hypothesis. Firstly, amyloid depositions have been observed in individuals without overt symptoms of dementia (61,62), indicating Aβ plaque does not necessarily correlate with cognitive impairment. Secondly, the level of soluble Aβ correlates better with the severity of Alzheimer’s disease compared to insoluble Aβ (62,63). Finally, several clinical trials targeting amyloid deposits by immunotherapeutic approaches have been conducted in the last decade (64-67). With one trial reported reduced amyloid deposits five years after immunization (65), none of the trials obtained efficacy in the antibody responder group. The repeated failures of clinical trials further raise the concern about the validation of the amyloid hypothesis.
The calcium hypothesis of Alzheimer’s disease

An alternative hypothesis of Alzheimer’s disease is the calcium hypothesis, which was initially proposed by Khachaturian in 1989 and states that disrupted calcium (Ca²⁺) homeostasis might be the reason that gives rise to both brain aging in the elderly and neurodegeneration in AD patients (68). As one of the most important second messengers in the central nervous system, Ca²⁺ mediates many neuronal processes, which include presynaptic neurotransmitter release, neuron excitability, cell apoptosis, gene expression and regulation of Ca²⁺-sensitive protein activities which are closely related to cognitive functions. In neuronal cells, there are two types of Ca²⁺ channels mediating Ca²⁺ release from ER: the inositol-1,4,5-triphosphate receptor (IP₃R) and the ryanodine receptor (RyR). It is also known that ER Ca²⁺ level is kept at relatively high level due to sarco-ER Ca²⁺ transport ATPase (SERCA) pump sitting on the ER membrane, which pumps Ca²⁺ from cytosol into ER (69).

Although no supporting evidence was provided when the hypothesis was first proposed, accumulating evidence in recent years suggests that disturbed Ca²⁺ homeostasis plays an important role in the progression of Alzheimer’s disease. Augmented IP₃R-mediated Ca²⁺ release was observed in the fibroblasts derived from asymptomatic members of Alzheimer’s disease family before the onset of any evident clinical symptoms (70). Enhanced IP₃R and RyR-dependent Ca²⁺ release were seen in brain slices from young Alzheimer’s disease transgenic mice with presenilin 1M146V knock-in when no histological change and cognitive deficits were observed yet (71,72). Taken together, the above results suggest that altered Ca²⁺ signaling might be a critical step in the pathogenesis of Alzheimer’s disease.

The mechanism underlying altered Ca²⁺ signaling in Alzheimer’s disease is still unclear, but emerging evidence suggested that enlarged ER Ca²⁺ pool might be the reason giving rise to augmented IP₃R and RyR-mediated Ca²⁺ release (73). The enlarged ER Ca²⁺ pool has been observed in hippocampal neurons from presenilin1 and presenilin2 double knockout mice and
Alzheimer’s disease transgenic mice with presenilin 1<sub>M146V</sub> knock-in (74), and it has been attributed to presenilin-1, an integral ER resident transmembrane protein with its major role identified as the catalytic core of the γ-secretase complex. Presenilin-1 is suggested to form ER Ca<sup>2+</sup> leak channel, thus its mutation is likely to cause ER Ca<sup>2+</sup> overfilling (75), although its role as the ER Ca<sup>2+</sup> leak channel remains controversial (76). Presenilin-1 has also been suggested to regulate SERCA pump activity, which in turn can cause enlarged ER Ca<sup>2+</sup> pool (77). Moreover, presenilin 1 may directly regulate the gating of IP<sub>3</sub>R and RyR (78,79). In terms of enhanced RyR mediated Ca<sup>2+</sup> release, it was suggested that it’s a complementary response to enlarged ER Ca<sup>2+</sup> pool caused by increased RyR expression, with no change observed in the expression of IP<sub>3</sub>R (74).

An important question to be addressed in the calcium hypothesis is how does enhanced ER Ca<sup>2+</sup> signaling regulate the neuropathogenesis and clinical symptoms of Alzheimer’s disease? It has been shown that augmented intracellular Ca<sup>2+</sup> release can enhance Aβ generation (78), increase Glycogen Synthase Kinase 3β-dependent Tau phosphorylation (80), activate CREB activity (81), a transcription factor which plays a critical role in memory formation, and modulate synaptic plasticity (82) (Fig. 1-3). Moreover, it has been demonstrated that genetic reduction of IP<sub>3</sub>R by 50% normalized the enhanced IP<sub>3</sub>R mediated Ca<sup>2+</sup> release in cortical and hippocampal neurons from Alzheimer’s disease transgenic mice with presenilin 1<sub>M146V</sub> knock-in, restored normal RyR activity, and more importantly, alleviated the amyloid deposition, Tau hyperphosphorylation and cognitive deficits of the diseased mice (83). Taken together, accumulating evidence indicated that enhanced IP<sub>3</sub>R mediated ER Ca<sup>2+</sup> release might be one of the reasons leading to presenilin-1 associated familial Alzheimer’s disease. With the repetitive failure of the clinical trials targeting amyloid deposition, IP<sub>3</sub>R-mediated ER Ca<sup>2+</sup> release might serve as a potential therapy target.
Fig. 1-4 Calcium hypothesis of Alzheimer’s disease

In the calcium hypothesis, it is speculated that the disrupted Ca\textsuperscript{2+} homeostasis may be the main reason that gives rise to neurodegeneration in Alzheimer’s disease. Augmented intracellular Ca\textsuperscript{2+} release has been observed in several Alzheimer’s disease mice models, which may be due to enlarged ER Ca\textsuperscript{2+} pool caused by presenilin-1 mutations. Augmented intracellular Ca\textsuperscript{2+} release may drive the neuropathogenesis of Alzheimer’s disease by enhanced Aβ generation, increased Glycogen Synthase Kinase 3β-dependent Tau phosphorylation, increased CREB activity and synaptic plasticity modulation.
1.4 The role of $G_q$ protein-coupled intracellular signaling cascade in working memory and Alzheimer’s disease

**G protein-coupled receptors**

G protein-coupled receptors (GPCR) are a large superfamily of membrane proteins, which convey signal transduction across cell membrane upon external ligand binding. Nearly 800 GPCRs in human and 1700 GPCRs in mice have been identified so far (84), and they mediate a large variety of physiological functions including olfactory function, organismal homeostasis, embryonic development, gonadal development and learning and memory (85,86). Based on phylogenetic analysis, human GPCRs are grouped into five main families: Rhodopsin, Secretin, Glutamate, Adhesion and Frizzled/Tas2 (87). All GPCRs share a similar structure: seven transmembrane-spanning $\alpha$ helices, an extracellular N terminus, an intracellular C terminus, and three interhelical loops on each side of the membrane (88). Despite their similar structures, there is a tremendous variety in the GPCR binding ligands, ranging from photons, ions, amino acids, nucleotides, hormones to neurotransmitters (89). Extracellular ligand binding brings a conformational change in the cytoplasmic domain of the GPCR (88), which activates GPCR-coupled G protein in the cytosol and initiates intracellular signaling cascades.

Compared to the GPCR superfamily, the heterotrimeric G protein family is relatively small. Heterotrimeric G protein consists of three subunits, $G_\alpha$, $G_\beta$ and $G_\gamma$, with the $G_\alpha$ subunit being a GTPase (90). In humans, there are 21 $G_\alpha$ subunits encoded by 16 genes, 6 $G_\beta$ subunits encoded by 5 genes, and 12 $G_\gamma$ subunits (88). Based on amino acid sequence similarity, $G_\alpha$ subunits are grouped into four classes, $G_\alpha_s$, $G_\alpha_i$, $G_\alpha_q$, $G_{12/13}$. G proteins are named after their $G_\alpha$ subunit.
The $G_q$ protein-coupled pathway and its modulation of neuron excitability

Similar to other subtypes of the G protein, $G_q$ protein is activated by extracellular ligand binding. The subsequent activation of $G_q$ in the cytosol activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP$_2$) resulting in the generation of inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). While the increased level of cytosol IP$_3$ induces intracellular Ca$^{2+}$ release via binding with IP$_3$ receptor, the activation of $G_q$ protein-coupled receptor and $G_q$/PLC cascade further induces receptor-operated Ca$^{2+}$ influx from extracellular space. Thus the activation of $G_q$ protein-coupled receptor leads to a substantial increase of intracellular Ca$^{2+}$ level (Fig. 1-4).

Neurons in the central nervous system express a variety of $G_q$ protein-coupled receptors including group 1 metabotropic glutamate receptor (mGluR1), muscarinic acetylcholine receptor (mAChR) subtype 1 and 3, and 5-Hydroxytryptamine receptor subtype 2A (5-HT$_{2A}$R) (91). The activation of $G_q$ protein-coupled receptors modulates neuron excitability by regulating a number of ion channels’ activity. Activation of $G_q$ pathway inhibits at least four K$^+$ currents, which include the slow Ca$^{2+}$ - dependent after-hyperpolarizing current ($I_{AHP}$) (92,93), the voltage-independent K$^+$ leak current ($I_{K+\text{ leak}}$)(94), the voltage sensitive M current ($I_M$) (95), and a voltage-dependent slowly-inactivating K$^+$ current ($I_{K+\text{ slow}}$) (96). It also suppresses L-type and N-type voltage-gated Ca$^{2+}$ channels (97,98). On the other hand, the activation of $G_q$ pathway induces a small conductance Ca$^{2+}$-dependent K$^+$ current ($I_{SK}$) (99), a long-lasting $G_q$ - dependent, Ca$^{2+}$-activated nonselective cationic current ($I_{can}$) (100), and a Ca$^{2+}$-independent nonselective cationic conductance (101). The modulation of a large percentage of ion channels’ activity is mediated by $G_q$/PLC activation-coupled intracellular second messenger change. For example, the suppression of KCNQ channel-dependent $I_M$ is mediated by $G_q$/PLC activation-coupled PIP$_2$ depletion (102), and the activation of $I_{SK}$ and $I_{CAN}$ is induced by $G_q$ protein-coupled intracellular Ca$^{2+}$ rise (99,100). In addition, $G_q$ protein also couples GPCRs to ion channels in a membrane-delimited
manner that is independent of cytosol second messengers. For example, both L-type and N-type voltage-gated Ca\(^{2+}\) channels have been suggested to be modulated by membrane-delimited pathway (103).

The overall effect of G\(_q\) protein-coupled receptors’ activation is an increase of postsynaptic neuron activity. The activation of both mGluR1 and mAChR has been shown to have a number of excitatory effects on postsynaptic pyramidal neurons in hippocampus, which include membrane depolarization, an increase in input resistance, and an increase in cell firing (93,104). These effects are attributed to the combined effect of activation of Ca\(^{2+}\)-activated and Ca\(^{2+}\)-independent nonselective cationic conductance, the inhibition of K\(^+\) currents, and the suppression of GABA inhibition mediated by G\(_q\) pathway activation (105-107).
**Fig. 1-5 G₉ protein-coupled intracellular signaling cascade**

G₉ is activated by extracellular ligand binding with G₉-coupled receptor on cell membrane. Upon activation, the Gα subunit dissociates from the Gβγ subunits. With Gβγ subunits anchored to cell membrane, Gα is released into the cytosol and activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂), resulting in the generation of inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). While the increased level of cytosol IP₃ induces intracellular Ca²⁺ release via binding with IP₃ receptor, the activation of G₉-coupled receptor and G₉/PLC cascade further induces receptor-operated Ca²⁺ influx from extracellular space.
The role of the Gq protein-coupled intracellular signaling cascade in working memory

Working memory is the cognitive capacity to actively and temporarily maintain information for the purpose of task execution (108). The molecular basis underlying working memory has been extensively studied, and several lines of evidence suggest Gq protein-coupled signaling pathway plays a prominent role in working memory. Reducing Gq pathway activity via depletion of neurotransmitters specific to Gq protein-coupled receptors (34), blockage of Gq protein-coupled receptors (35-38), or directly inhibiting Gq/phospholipase C (PLC) activity by infusion of PLC inhibitor (39) all lead to impairment of working memory in animals. Gq/PLC pathway regulates working memory in several ways. First of all, activation of Gq protein-coupled receptors, which include mGluR1 and mAChR, increases postsynaptic neuron excitability via ion channels gating, as discussed in the previous section. More specifically, activation of mGluR1 or mAChR has been shown to greatly enhance the action potential-induced neuron firing (109,110), which is considered as a neuronal correlate of working memory. Secondly, Gq/PLC coupled intracellular Ca2+ rise activates several Ca2+ dependent protein enzymes, including phosphatase calcineurin, kinase CaMKII and PKC, all of which have been shown to regulate working memory capacity (111).
The role of the $G_q$ protein-coupled intracellular signaling cascade in Alzheimer’s disease

The $G_q$ protein-coupled intracellular signaling cascade has been suggested to modulate Alzheimer’s disease progression in several ways. Firstly, cholinergic innervation deficits have been observed in the cerebral cortex and hippocampus of Alzheimer’s disease patients, and the degeneration of cholinergic neurons in basal forebrain and the associated loss of cholinergic innervation in cerebral cortex and other related brain region have been proposed to be the primary cause of the disease (112). The observed deficits include reduced choline uptake (113), reduced acetylcholine release (114), reduced activity of choline acetyltransferase (115,116), which is an enzyme responsible for acetylcholine synthesis, and selective degeneration of cholinergic neurons in the nucleus basalis of Meynert of basal forebrain (117), which was considered as the primary source of cholinergic innervation for cerebral cortex. Although the subsequent observation of absence of cholinergic innervation deficits in some Alzheimer’s disease patients, and lack of cognitive impairments in olivopontocerebellar atrophy patients with diminished choline acetyltransferase activity challenged the validity of the cholinergic hypothesis (112), it is still possible that cholinergic innervation plays a role in Alzheimer’s disease development.

Secondly, $G_q$ protein-coupled intracellular signaling cascade has been suggested to modulate Aβ generation. The activation of $G_q$ protein and subsequent activation of protein kinase C (PKC) shift the APP metabolism towards the $\alpha$-secretase mediated non-amyloidogenic pathway, and thus decrease the generation of Aβ peptides (50). Specifically, the activation of M1 and M3 $G_q$-coupled mAChRs has been shown to increase soluble APPα release in vitro, via a PKC-dependent pathway (118,119). The activation of $G_q$-coupled serotonin receptor 5-HT$_2A$R also increases soluble APPα release in vitro, but the effect is PKC independent (120). General stimulation of metabotropic glutamate receptors also accelerates the generation of soluble APPα (121), although the activation of mGluR1 increases the activity of both $\alpha$- and $\beta$-secretases, with the overall effect of a slight but significant increase of Aβ40 production (122).
Chapter 2

PERK regulates G_q protein-coupled intracellular Ca^{2+} dynamics

in primary cortical neurons

INTRODUCTION

Calcium (Ca^{2+}) serves as an important second messenger in the central nervous system, as it regulates various neuronal processes including presynaptic neurotransmitter release, synaptic plasticity, neuron excitability, and neuronal gene transcription (123). Initiators of intracellular Ca^{2+} rise in neurons include the G_q-protein coupled receptors, whose activation upon agonist binding leads to the activation of G_q/phospholipase C (PLC) pathway. Activated PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) resulting in the generation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). While the increased cytosol IP_3 induces internal Ca^{2+} release by binding with ER resident inositol-1,4,5-triphosphate receptor (IP_3R), the activation of G_q/PLC cascade further stimulates receptor-operated Ca^{2+} influx from extracellular space.

The neurons in the central nervous system express a variety of G_q protein-coupled receptors including the M1 and M3 muscarinic acetylcholine receptors (mACHR), the group 1 metabotropic glutamate receptor (mGluR1), and the bradykinin-2 receptor (91,124). G_q protein-coupled intracellular Ca^{2+} dynamics has been shown to play prominent roles in a number of neuronal processes. For example, in pyramidal neurons, G_q protein-coupled intracellular Ca^{2+} rise induced by mACHR or mGluR1 activation is required for the induction of Ca^{2+}-activated nonselective cationic current (I_{CAN}) (100,125), which is considered the ionic mechanism underlying persistent neuronal firing essential for working memory. In cerebellar Purkinje cells, mGluR1-induced postsynaptic Ca^{2+} rise in dendritic spines has been shown to be essential for long-term synaptic depression (126).
PERK, an eIF2α kinase well known for its role in eIF2α-dependent protein synthesis and translational control, has been shown to modulate protein synthesis-dependent cognitive functions including learning and memory flexibility (20), and long-term depression mediated by mGluR1 (21). On the other hand, it is recently discovered that in β-cells, PERK acutely regulates Ca^{2+} dynamics underlying glucose-stimulated insulin secretion, which is independent on protein synthesis (17). Considering the critical role of Ca^{2+} signaling in synaptic transmission and neuronal plasticity, I hypothesized that PERK may also regulate Ca^{2+} dynamics in pyramidal neurons. I show herein that acute PERK inhibition impairs G_{q} protein-coupled intracellular Ca^{2+} rise. More specifically, PERK inhibition enhances IP_{3}R mediated ER Ca^{2+} release, but suppresses receptor-operated external Ca^{2+} influx. G_{q} protein-coupled intracellular Ca^{2+} rise is also impaired in genetic Perk knockout neurons. Taken together, my findings suggest that PERK regulates G_{q} protein-coupled Ca^{2+} dynamics in pyramidal neurons.
MATERIALS AND METHODS

Reagents

PERK inhibitor GSK2606414 was a kind gift from Jeffery M. Axten and Rakesh Kumar, GlaxoSmithKline, Collegeville, PA. (S)-3,5-Dihydroxyphenylglycine (DHPG) and thapsigargin were purchased from Tocris, bradykinin acetate salt was purchased from Sigma, carbachol was purchased from EMD Millipore, and Bt3-Ins(1, 4, 5)P3/AM (IP3-AM) was purchased from SiChem.

Primary neuronal culture

Mouse primary cortical neurons were prepared as previously described(127). Briefly, the cerebral cortex was isolated from day 0 pups and dissociated in 5ml 0.05% trypsin-EDTA with DNase1 for 30 min at 37 °C(5% CO2). The tissue was then washed twice with HBSS containing 10% FBS, and mechanically triturated in 2ml neuronal medium with DNase1. 8ml fresh neuronal medium was added after trituration and the cells were collected by centrifugation at 120Xg for 5 min. The cells were re-suspended in fresh neuronal medium and plated on glial-coated 12mm glass coverslips in a 24-well plate at the density around 150,000 cells per well. Total medium was changed on the 1st day in vitro (DIV) and 50% of the medium was changed on DIV 3 and DIV 5. Genetic Perk knockout neurons were prepared from Perk-floxed Nestin-Cre mice in the same way, and the genotype of each pup was determined later. The cells were maintained in MEM based neuronal medium containing 5% FBS (GEMINI Bio-Products), 2% B27 (Invitrogen), 1mM L-Glutamine (Gibco), 20mM D-Glucose, 2µM Cytosine Arabinoside (AraC), 40 units/ml penicillin, 40µg/ml streptomycin and 100ng/ml Amphotericin B, and final pH was adjusted to 7.4 with NaHCO3 (100mg/500ml). Total medium was changed on DIV 1 and 50% of the medium was changed on DIV 3 and DIV 5. DIV 14-19 neurons were used for Ca2+ imaging experiments and immunocytochemistry.
Intracellular Ca\(^{2+}\) measurements and Ca\(^{2+}\) imaging data analysis

Intracellular Ca\(^{2+}\) levels were measured using the ratiometric Ca\(^{2+}\) probe Fura-2 AM (Molecular Probes). Briefly, coverslips seeded with neurons at DIV 14-19 were incubated in bath solution with 2µM Fura-2-AM for 30 min at room temperature in the dark. Coverslips were then transferred to fresh bath for 15 min to allow the cleavage of AM esters by cellular esterases. After dye loading, the coverslips were put into a perfusion chamber mounted on Nikon TE-200-S inverted microscope with Xenon arc lamp as the fluorescence excitation source. Ratios of images with the fluorescent emission signal excited at 340nm over 380nm were obtained using an excitation filter wheel (340nm/380nm, Chroma Technology) and UV-2A filter cube (Nikon). Images were collected every 5 sec using a 20X objective and a cooled charge-couple device (CCD) camera. SimplePCI imaging software was used for the control of filter wheel and collection of data. Tyrode’s solution (123mM NaCl, 30mM Glucose, 25mM HEPES, 5mM KCl, 2mM CaCl\(_2\) and 1mM MgCl\(_2\)) mimicking cerebrospinal fluid was used as bath solution, and final pH was adjusted to 7.4 before every experiment. Cells were perfused with bath solution at the constant rate of 1 drop/2 sec during Ca\(^{2+}\) imaging process, and this rate was also used for the application of different drugs as described in figure legends. Triangular shaped pyramidal neurons were selected for imaging and the soma was selected as region of interest. Sister coverslips from 2-3 independent cultures were used for each experiment, and pooled data was analyzed.

Ca\(^{2+}\) imaging measurements were analyzed by calculating the area under the curve (AUC). Due to the inherent variation of primary neuron culture, a small percentage of neurons displayed high Ca\(^{2+}\) transients, which obscured the drug-stimulated intracellular Ca\(^{2+}\) rise. For this reason, basal Ca\(^{2+}\) transients over 100 sec were analyzed, and those with AUC >2 (<5%) were excluded from final analysis.
**Synaptoneurosome isolation**

Synaptoneuroosomes were isolated as previously described (128). Briefly, mouse prefrontal cortex was isolated and homogenized mechanically in 1.1 ml ice-cold synaptoneurosome buffer (100mM HEPES, 1mM EDTA, 2mM EGTA, 0.5mM DTT, PH 7.0, with 1X protease inhibitor and 1X phosphatase inhibitor cocktails) using a polypropylene pestle. 100ul of the homogenate was set aside for western blot analysis and the rest was diluted with 7ml ice-cold synaptoneurosome buffer and gently sonicated. The sonicated homogenate was then filtered through two layers of pre-wetted 100µm pore nylon net filters followed by filtration through one layer of pre-wetted 5µm pore hydrophilic membrane. The resulting filtrate was centrifuged at 1000Xg for 10 min and the pellet corresponding to synaptoneurosome was collected.

**Western blot analysis**

To determine PERK knockdown efficiency in genetic Perk knockout neurons, mouse cerebral cortex was isolated from day 0 Perk-floxed Nestin-Cre pups, and homogenized mechanically in ice-cold synaptoneurosome buffer using a polypropylene pestle. Protein lysates from synaptoneurosome and brain tissue were prepared using RIPA buffer with 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Samples were denatured by boiling in 2X Laemmli buffer for 5 min. The following primary antibodies were used in western blot analysis: anti-PERK produced in rabbit (1:500, Cell Signaling, #3192), anti-β-actin produced in mouse (1:1000, GenScript, A00702), anti-synaptotagmin produced in mouse (1:1000, gift from Dr. Yingwei Mao, Penn State University), anti-NMDAR 2B produced in rabbit (1:200, Santa Cruz, sc-9057), anti-CREB-1 produced in rabbit (1:200, Santa Cruz, sc-58), anti-Ribophorin 1 produced in goat (1:200, Santa Cruz, sc-12164). All the primary antibodies were applied overnight at 4°C. Appropriate secondary antibodies conjugated with horseradish peroxidase were applied at the concentration of 1:1000 for 1 hour at room temperature. The chemiluminescence signal was generated using Pierce ECL 2 western blotting substrate kit (Thermo Scientific) and detected by
Typhoon FLA 7000 (GE Healthcare). The signal was quantified using the ImageQuant TL software (GE Healthcare).

**Synapse formation analysis**

Immuncytotoxic detection of the presynaptic marker Synapsin 1 and dendritic marker MAP2 was used to visualize synapses. Neurons seeded on glial-coated coverslips were fixed with 4% paraformaldehyde, 4% sucrose in PBS for 15 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS for 10 min, and blocked with 5% horse serum (Invitrogen) in PBS containing 0.1% Triton X-100 for 1 hour. The cells were then incubated with the mixture of following primary antibodies overnight at 4°C: mouse anti-Synapsin 1 (Chemicon), rabbit anti-MAP2 (Millipore). Appropriate secondary antibodies conjugated with Alexa Fluor 546 or 488 (Molecular Probes) were applied for 1 hour. The coverslips were mounted using the anti-fade reagent with DAPI (Molecular Probes). The fluorescence images were captured at 40X using Nikon Eclipse E1000 and SPOT 5.1 software. The fluorescence intensities were kept consistent for image capture to allow for comparison between genotypes.

To compare synapse formation density between wild-type and Perk knockout neurons, Synapsin 1 positive puncta in contact with MAP2 labeled dendrites within middle basal dendrites were counted, and the dendrite length was measured using ImageJ.
RESULTS

GSK2606414 serves as a potent and specific inhibitor of PERK enzyme activity

To acutely inhibit PERK enzyme activity in primary cortical neurons, I took advantage of a highly specific inhibitor of PERK, GSK2606414 (PERKi), which acts by competing for the ATP binding domain in the catalytic cite (16). To verify the effectiveness of PERKi in vitro, primary cortical neurons were pretreated with 500nM PERKi for 15 min followed by activation of PERK with 100nM thapsigargin for 30 min. Thapsigargin is a non-reversible SERCA pump inhibitor (129), that activates the PERK kinase activity by causing ER calcium depletion (2). 15 min-pretreatment of 500nM PERKi inhibited thapsigargin-induced PERK activation and eIF2α phosphorylation (Fig. 2-1), demonstrating that the PERKi effectively inhibits PERK’s activity in primary neurons.

Fig. 2-1 GSK2606414 serves as a specific and potent inhibitor of PERK enzyme activity

Western blot analysis showing PERK inhibitor (PI) pretreatment prevented thapsigargin (TG)-induced phosphorylation of PERK and its substrate eIF2α in primary cortical neurons (one-way ANOVA followed by Bonferroni’s post-hoc test, * p<0.05, ** p<0.01). Cells were pretreated with 500nM PI or DMSO for 15 min followed by co-treatment with or without 100nM TG for 30 min. The phosphorylation level of PERK is shown by p-PERK blot, and the shifted band in PERK blot due to the dimerization and auto-phosphorylation of PERK after its activation. The experiment was repeated for 3 times.
**PERK is expressed in the synaptoneurosomes**

At the gross subcellular level PERK has been shown to be expressed in both cell body and dendrites (21). To determine if PERK is also present in the proximate region of the synapse, synaptoneurosomes containing presynaptic and postsynaptic vesicularized components (128) were isolated from the prefrontal cortex. Western blot analysis confirmed that the synaptoneurosome fraction was enriched of a presynaptic marker synaptotagmin, a postsynaptic marker NMDAR 2B, and clear of a nuclear marker CREB-1 (Fig. 2-2A), which suggests that the synaptoneurosome preparation was successful. Moreover, PERK’s signal was detected in the synaptoneurosome fraction (Fig. 2-2B). To exclude the possibility that PERK’s signal was due to rough ER contamination from soma, the ratio of PERK’s expression in synaptoneurosome over prefrontal cortex homogenate fraction was determined, and was compared to that of a rough ER marker, Ribophorin 1, which is predominantly expressed in soma (130). The significantly higher expression ratio of PERK compared to that of Ribophorin 1 suggests that PERK is expressed in the synaptoneurosomes.
Fig. 2-2 PERK is expressed in synaptoneurosome

A. Representative western blot on the left shows the expression of fraction markers in the homogenate and synaptoneurosome isolated from mouse prefrontal cortex. (H: homogenate; S: synaptoneurosome). The protein quantification on the right represents pooled data from both genotypes. (n=6; * p<0.05, *** p<0.001, two-tailed student’s t-Test)

B. Representative western blot on the left shows the expression of PERK and rough ER marker Ribophorin 1 in homogenate and synaptoneurosome collected from wild-type mice’s prefrontal cortex. The quantification on the right represents the ratio of each protein’s expression in synaptoneurosome over homogenate fraction after actin normalization. (n=3; * p<0.05, two-tailed student’s t-Test)
Acute PERK inhibition impairs $G_q$ protein-coupled intracellular Ca$^{2+}$ rise in primary cortical neurons

To examine if acute PERK inhibition impairs $G_q$ protein-coupled intracellular Ca$^{2+}$ ([Ca$^{2+}$]) rise, I used Fura-2 AM to visualize [Ca$^{2+}$]$_i$ dynamics in primary neurons. I first tested if PERKi impacts cholinergic activation induced [Ca$^{2+}$]$_i$ rise by treating neurons with an acetylcholine receptor agonist, carbachol, and found that 15 min 500nM PERKi pretreatment significantly reduced 250µM carbachol-induced [Ca$^{2+}$]$_i$ rise (Fig. 2-3A). Carbachol activates both muscarinic acetylcholine and nicotinic acetylcholine receptors with the former being a $G_q$ protein-coupled receptor, and the latter a Ca$^{2+}$-permeable ionotropic receptor (131). Thus the carbachol-induced [Ca$^{2+}$]$_i$ rise can be attributed to the stimulation of either receptor. To further examine how PERK inhibition impacts $G_q$ protein-coupled intracellular Ca$^{2+}$ signaling, I tested another $G_q$ protein-coupled receptor, mGluR1, by the use of its specific agonist, DHPG. Again, acute PERK inhibition significantly suppressed 50µM DHPG induced [Ca$^{2+}$]$_i$ rise (Fig. 2-3B). I also tested a third $G_q$ protein-coupled receptor, the bradykinin-2 receptor, using its specific agonist, bradykinin, and found that 1µM bradykinin induced [Ca$^{2+}$]$_i$ increase was almost abolished by PERKi treatment (Fig. 2-3C). That PERK inhibition is able to block cytosolic Ca$^{2+}$ influx stimulated by three different $G_q$ protein-coupled receptors argues strongly that PERKi impacts the $G_q$/PLC pathway at a point downstream of receptor binding.
Fig. 2-3 $G_q$ protein-coupled intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) rise is impaired by acute PERK inhibition

A. $[Ca^{2+}]_i$ of primary neurons in response to 250µM carbachol treatment.
   (DMSO n=21, PI n=17; ***p<0.001, two-tailed student’s t-Test)
B. $[Ca^{2+}]_i$ of primary neurons in response to 50µM DHPG treatment
   (DMSO n=36, PI n=57; ***p<0.001, two-tailed student’s t-Test)
C. $[Ca^{2+}]_i$ of primary neurons in response to 1µM bradykinin treatment
   (DMSO n=25, PI n=37; ***p<0.001, two-tailed student’s t-Test)

In all of the experiments above, cells were pretreated with 500nM PI or DMSO for 15 min before recording. In the representative graph on the left, each $Ca^{2+}$ trace represents the average of 6-11 neurons that were imaged from the same coverslip. Basal $Ca^{2+}$ oscillation over 100 sec before treatment and drug-stimulated $[Ca^{2+}]_i$ rise over 200 sec were quantified by calculating the area under the curve (AUC). Final analysis is presented as AUC/100 sec and shown in the bar graph on the right.
IP$_3$-AM induced $[\text{Ca}^{2+}]_i$ rise is impaired by acute PERK inhibition

Receptor-simulated activation of the G$_q$/PLC pathway produces IP$_3$ and increases $[\text{Ca}^{2+}]_i$ by induction of IP$_3$ receptor mediated ER Ca$^{2+}$ release and receptor-operated Ca$^{2+}$ influx. To determine if PERKi affects PLC activity or a downstream Ca$^{2+}$ channel, IP$_3$-AM induced $[\text{Ca}^{2+}]_i$ rise was examined in PERK-inhibited neurons and DMSO controls. IP$_3$-AM is a cell-permeable derivative of IP$_3$, which diffuses into cytosol and activates IP$_3$ receptor after the AM moiety is removed by cellular esterases. IP$_3$-AM induced a delayed but sustained $[\text{Ca}^{2+}]_i$ rise in DMSO controls (Fig. 2-4). The delayed response likely reflects the time required to remove the AM moiety by cellular esterases. Acute PERK inhibition substantially suppressed IP$_3$-AM induced $[\text{Ca}^{2+}]_i$ rise which does not require PLC activity, indicating that the likely target for PERKi is a downstream Ca$^{2+}$ channel rather than PLC.

![Graph showing IP$_3$-AM induced $[\text{Ca}^{2+}]_i$ rise in DMSO and PERK-inhibited neurons](image)

**Fig. 2-4** IP$_3$-AM induced intracellular Ca$^{2+}$ ($[\text{Ca}^{2+}]_i$) rise is impaired by acute PERK inhibition

$[\text{Ca}^{2+}]_i$ of primary cortical neurons in response to 1µM IP$_3$-AM treatment.

(DMSO n=48, PI n=48; *** p<0.001, two-tailed student’s t-Test)

Cells were pretreated with 500nM PI or DMSO for 15 min before recording. In the representative graph on the left, each Ca$^{2+}$ trace represents the average of 12-14 neurons that were imaged from the same coverslip. Basal Ca$^{2+}$ oscillation over 100 sec before treatment and IP$_3$-AM-stimulated $[\text{Ca}^{2+}]_i$, rise over 600 sec were quantified by calculating the area under the curve (AUC). Final analysis is presented as AUC/100 sec and shown in the bar graph on the right.
**Acute PERK inhibition increases IP$_3$ receptor mediated ER Ca$^{2+}$ release**

Two sources of Ca$^{2+}$ influx contribute to G$_q$ protein-coupled [Ca$^{2+}$]$_i$ increase: IP$_3$R mediated ER Ca$^{2+}$ release and receptor-operated Ca$^{2+}$ entry (ROCE) from the extracellular medium. To study PERKi’s effect on internal Ca$^{2+}$ release, I measured [Ca$^{2+}$]$_i$ rise upon carbachol treatment in the absence of extracellular Ca$^{2+}$, to exclude any contribution from nicotinic acetylcholine receptor or receptor-operated Ca$^{2+}$ channel (ROCC)-dependent Ca$^{2+}$ influx. Cells were perfused with Ca$^{2+}$-free bath for 100 sec before stimulated with 250µM carbachol. Carbachol treatment in Ca$^{2+}$-free bath triggered a transient and small [Ca$^{2+}$]$_i$, increase due to Ca$^{2+}$ release from intracellular stores, which was significantly higher in PERK-inhibited neurons (Fig. 2-5A). The experiment was repeated using 50µM DHPG to simulate mGluR1 and similar result was obtained (Fig. 2-5B). Taken together, these results suggest that acute PERK inhibition increases IP$_3$R mediated ER Ca$^{2+}$ release.
Fig. 2-5 Acute PERK inhibition increases IP3 receptor mediated ER Ca2+ release

A. [Ca2+]i of primary cortical neurons in response to 250µM carbachol treatment in Ca2+ free bath.
(DMSO n=29, PI=26; * p<0.05, two-tailed student’s t-Test)

B. [Ca2+]i of primary cortical neurons in response to 50µM DHPG treatment in Ca2+ free bath.
(DMSO n=33, PI=39; * p<0.05, two-tailed student’s t-Test)

In both experiments, cells were pretreated with 500nM PI or DMSO for 15 min before recording. Drug treatment started 100 sec after Ca2+ free bath perfusion. In the representative graph on the left, each Ca2+ trace represents the average of 8-12 neurons that were imaged from the same coverslip. Basal Ca2+ oscillation over 100 sec before treatment and drug-stimulated [Ca2+]i rise were quantified by calculating the area under the curve (AUC), and shown in the bar graph on the right.
Acute PERK inhibition impairs receptor-operated Ca\(^{2+}\) entry, but not store-operated Ca\(^{2+}\) entry

The observation that acute PERK inhibition impairs G\(_q\) protein-coupled [Ca\(^{2+}\)]\(_i\), mobilization and increases IP\(_3\)-R-dependent ER Ca\(^{2+}\) release suggests that ROCE is impaired as a result of PERKi treatment. To test this hypothesis, DHPG stimulated ROCE was examined in PERK-inhibited neurons and DMSO controls after ER Ca\(^{2+}\) depletion by the use of a SERCA pump inhibitor, thapsigargin (129). The pretreatment with thapsigargin caused a rapid and irreversible depletion of ER Ca\(^{2+}\). Upon DHPG stimulation, the rise of [Ca\(^{2+}\)] in ER Ca\(^{2+}\) depleted-neurons was largely contributed by ROCC-dependent extracellular Ca\(^{2+}\) influx. PERKi treatment significantly reduced DHPG induced [Ca\(^{2+}\)]\(_i\) rise in ER Ca\(^{2+}\) depleted-neurons, indicating that ROCC-dependent extracellular Ca\(^{2+}\) influx is impaired upon PERK inhibition (Fig. 2-6A).

Store-operated Ca\(^{2+}\) entry (SOCE) refers to cytosol Ca\(^{2+}\) influx mediated by cell membrane Ca\(^{2+}\) channels triggered by ER Ca\(^{2+}\) store depletion. Since ROCE and SOCE are two closely related processes, and store depletion is an integral component of ROCE, we next examined PERKi’s effect on SOCE in primary cortical neurons. Cells were treated with 1\(\mu\)M thapsigargin in Ca\(^{2+}\)-free bath for 300 sec to deplete ER Ca\(^{2+}\) and activate store-operated Ca\(^{2+}\) channels (SOCC). Subsequent reintroduction of 2mM Ca\(^{2+}\) into the bath elicited a sustained [Ca\(^{2+}\)]\(_i\) elevation, reflecting SOCC mediated Ca\(^{2+}\) influx. No difference was observed between PERK-inhibited neurons and DMSO controls (Fig. 2-6B), suggesting that acute PERK inhibition does not affect SOCE. Previous studies have shown that thapsigargin induced SOCE in pyramidal neurons is L-type voltage-gated Ca\(^{2+}\) channel (VGCC)-independent (132), thus L-type VGCC inhibitor was not included in the bath.
Fig. 2-6 Acute PERK inhibition impairs receptor-operated $\text{Ca}^{2+}$ entry, but not store-operated $\text{Ca}^{2+}$ entry

A. [Ca$^{2+}$], of thapsigargin (TG) pretreated primary neurons in response to 50µM DHPG treatment. Cells were pretreated with 500 nM PI or DMSO for 15 min before recording, and perfused with 1µM TG for 300 sec before 50µM DHPG treatment. In the representative graph on the left, each $\text{Ca}^{2+}$ trace represents the average of 8-9 neurons that were imaged from the same coverslip. Basal $\text{Ca}^{2+}$ oscillation over 100 sec before treatment and DHPG-stimulated [Ca$^{2+}$] rise over 500 sec were quantified by calculating the area under the curve (AUC). Final analysis is presented as AUC/100 sec and shown in the bar graph on the right (DMSO n=37, PI n=35; *** p<0.001, two-tailed student’s t-Test).

B. Store-operated $\text{Ca}^{2+}$ entry in primary cortical neurons. Cells were pretreated with 500nM PI or DMSO for 15 min before recording, and perfused with 1µM TG in $\text{Ca}^{2+}$ free bath for 300 sec before reintroduction of 2mM $\text{Ca}^{2+}$. In the representative graph on the left, each $\text{Ca}^{2+}$ trace represents the average of 9-12 neurons that were imaged from the same coverslip. Store-operated $\text{Ca}^{2+}$ entry over 500 sec was quantified by calculating the area under the curve (AUC). Final analysis is presented as AUC/100 sec and shown in the bar graph on the right (DMSO n=45, PI n=36; n.s. not significant, two-tailed student’s t-Test).
Acute PERK inhibition does not affect VGCC dependent Ca\(^{2+}\) influx

Previously it has been shown that acute PERK inhibition impairs VGCC-dependent Ca\(^{2+}\) influx in \(\beta\) cells. To examine if PERK also regulates VGCC-dependent Ca\(^{2+}\) influx in primary neurons, 34mM KCl induced Ca\(^{2+}\) influx was examined in PERK-inhibited neurons and DMSO controls, and no significant difference was observed between treatments (Fig. 2-7).

**Fig. 2-7** 34mM KCl induced VGCC-dependent intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) rise is not impaired by acute PERK inhibition

[Ca\(^{2+}\)]\(_i\) of primary neurons in response to 34mM KCl treatment.

Cells were pretreated with 500nM PI or DMSO for 15 min before recording. In the representative graph on the left, each Ca\(^{2+}\) trace represents the average of 11-15 neurons that were imaged from the same coverslip. Basal Ca\(^{2+}\) oscillation over 100 sec before treatment and 34mM KCl-stimulated [Ca\(^{2+}\)]\(_i\) rise over 300 sec were quantified by calculating the area under the curve (AUC). Final analysis is presented as AUC/100 sec and shown in the bar graph on the right. (DMSO n=43, PI n=36; n.s. non-significant, two-tailed student’s t-Test)
**Gq protein-coupled [Ca^{2+}], rise is impaired in genetic Perk knockout primary cortical neurons**

To investigate if the impaired Gq protein-coupled [Ca^{2+}], mobilization could be mimicked by genetic ablation of Perk, primary cortical neurons from brain-specific Perk KO (BrPKO) mice were examined. BrPKO mice were generated by crossing Perk-floxed mice (12) with the transgenic Nestin-Cre mice strain (133), which enables widespread deletion of the loxP-flanked Perk gene sequence in neurons and glial cells during embryonic stage (134,135). Western blot analysis confirmed almost complete knockdown of PERK in the cerebral cortex of BrPKO Nestin-Cre Perk-floxed mice at postnatal day 0 (Fig. 2-8A). With the concern that knockdown of PERK may affect neuronal differentiation and synapse formation *in vitro*, synapse density was examined in BrPKO and wild-type primary cortical neurons by double immunofluorescence staining of the presynaptic marker Synapsin 1 and the dendritic marker MAP2 prior to examining their Gq protein-coupled [Ca^{2+}], rise. No significant difference was observed in synapse density between genotypes (Fig. 2-8B). To determine if Gq protein-coupled [Ca^{2+}], mobilization is impaired in BrPKO primary cortical neurons, mGluR1 agonist DHPG was applied, and significantly smaller DHPG-stimulated [Ca^{2+}], rise was observed in BrPKO neurons (Fig. 2-8C), which is consistent with the pharmacological PERK inhibition results.
Fig. 2-8 $G_q$ protein-coupled intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) mobilization is impaired in genetic Perk knockout primary cortical neurons

A. Western blot analysis confirmed almost complete knockdown of PERK in the cerebral cortex of BrPKO mice at postnatal day 0 (BrPKO: Nestin-Cre Perk-floxed; *** $p<0.001$, two-tailed student’s t-Test).

B. No difference in synapse density was observed between WT and BrPKO primary cortical neurons. Representative image on the left shows the immunofluorescent staining of Synapsin 1(red) and MAP2 (green) in primary cortical neurons. Synapse density quantification in the bar graph on the right represents
pooled data from 3 mice per genotype (5 neurons were randomly picked for synapse density quantification per animal, n=15 for each genotype; WT and BrPKO neurons were cultured from the same litter; n.s. not significant, two-tailed student’s t-Test).

C. DHPG stimulated [Ca^{2+}]_{i} rise is impaired in genetic Perk KO primary neurons. In the representative graph on the left, each Ca^{2+} trace represents the average of 8-10 neurons that were imaged from the same coverslip. Basal Ca^{2+} oscillation over 100 sec before treatment and DHPG-stimulated [Ca^{2+}]_{i} rise over 200 sec were quantified by calculating the area under the curve (AUC). Final analysis is presented as AUC/100 sec and shown in the bar graph on the right (WT n=44, BrPKO n=34; *** p<0.001, two-tailed student’s t-Test).
DISCUSSION

Although earlier studies have demonstrated that PERK plays an important role in regulating cognitive functions including behavior flexibility (20) and mGluR1-dependent long-term depression (21), the underlying mechanisms remain unknown. Previously it has been shown that PERK regulates Ca\textsuperscript{2+} dynamics in electrically excitable pancreatic \(\beta\) cells (17), suggesting that PERK may also regulate Ca\textsuperscript{2+} dynamics in neurons. Neuronal cytosolic Ca\textsuperscript{2+} rise is contributed by two major Ca\textsuperscript{2+} sources: internal Ca\textsuperscript{2+} release mediated by ER-resident IP\textsubscript{3}R or Ryanodine receptor, and external Ca\textsuperscript{2+} influx mediated by voltage-dependent Ca\textsuperscript{2+} channel, ionotropic glutamate receptor, nicotinic acetylcholine receptor, or TRPCs (136). PERK’s subcellular localization in the soma, dendrites and synaptoneurosomes suggests the possibility that it plays multiple roles in Ca\textsuperscript{2+} channel regulation. Moreover, its localization within ER membrane and primary spatial expression in soma and dendrites are functionally important for its regulation of ER-resident IP\textsubscript{3}R, and potential regulation of TRPCs, which are localized mainly in soma and dendrites (137-139).

In this study, I investigated the role of PERK in G\textsubscript{q} protein-coupled [Ca\textsuperscript{2+}], mobilization in primary cortical neurons, and identified it as a negative regulator of IP\textsubscript{3}R-dependent ER Ca\textsuperscript{2+} release and a positive regulator of receptor-operated Ca\textsuperscript{2+} entry. My finding that inhibition of PERK alters Ca\textsuperscript{2+} dynamics within a few minutes after inhibitor application is inconsistent with the hypothesis that these effects are mediated by changes in protein translation. Moreover, it is unlikely that these observations are due to off-target effects because genetic ablation of Perk mimicked the impaired G\textsubscript{q} protein-coupled [Ca\textsuperscript{2+}], mobilization observed in pharmacologically PERK-inhibited neurons.

How then does PERK regulate these processes? I speculate that PERK’s regulation of IP\textsubscript{3}R-dependent ER Ca\textsuperscript{2+} release is mediated by the regulation of calcineurin, a Ca\textsuperscript{2+}/calmodulin-dependent protein phosphatase that negatively regulates IP\textsubscript{3}R (140,141). PERK and calcineurin
have been shown to physically interact, which impacts their individual enzymatic activities (142). Moreover, in pancreatic insulin-secreting β-cells, PERK positively regulates calcineurin activity and calcineurin is a downstream mediator of PERK’s action on Ca\(^{2+}\)-dependent insulin secretion (17). These results led me to speculate that PERK might negatively regulate IP\(_3\)R activity through its positive regulation of calcineurin in pyramidal neurons.

For G\(_q/PLC\) coupled ROCE, the family of TRPC channels form nonselective receptor-operated Ca\(^{2+}\) channels (143). A number of intracellular signals generated downstream of G\(_q/PLC\) pathway have been shown to activate TRPCs, which includes increased PLC activity, generation of DAG and internal Ca\(^{2+}\) store depletion (143). Among them, DAG is the only identified second messenger that directly gates TRPC activity. DAG has been shown to activate TRPC3/6/7 channels (144,145) while inhibiting TRPC5 channel activity (146). Since PERK has an intrinsic DAG kinase activity of converting DAG into phosphatidic acid (147), it is possible that PERK regulates TRPC activity by modulating intramembrane DAG levels. In addition, it is also possible that PERK regulates ROCE via its interaction with calcineurin. In neuronal PC12D cells, it has been shown that calcineurin is recruited to the TRPC6 centered multiprotein complex induced by M1 mAChR activation, and it is essential for TRPC6 dephosphorylation and M1 mAChR dissociation from the complex, suggesting that calcineurin might play a regulatory role in receptor-operated TRPC6 activity (148).

Receptor-operated and stored-operated Ca\(^{2+}\) entries are closely related: store depletion is an integral component of ROCE, and TRPCs have been suggested to be the Ca\(^{2+}\) channels involved in both processes. Although almost all of the TRPCs can be activated by store depletion (149-156), there is accumulating evidence suggesting that the regulation of TRPC3/6/7 (144,145,157) and TRPC4/5 (158,159) activities can also be store depletion-independent. My observation that acute PERK inhibition impairs ROCE but not SOCE suggests that PERK’s regulation of ROCE might be independent of internal Ca\(^{2+}\) release.
Considering PERK’s role in eIF2α-dependent protein synthesis and translational control, it has been hypothesized that PERK’s regulation over memory flexibility and mGluR1-dependent long-term depression is eIF2α-dependent (20,21). However, genetic reduction of eIF2α phosphorylation by single allele phosphorylation site mutation of eIF2α (22), or knockdown of other eIF2α kinases GCN2 (23) and PKR (24), lowers the threshold for late phase long-term potentiation and facilitates long-term memory storage, a phenotype that is absent in forebrain-specific Perk knockout mice (20,21). Thus, it is very likely that PERK imparts additional regulation on cognition that is eIF2α-independent. This study’s discovery of PERK-dependent regulation of Gq protein-coupled Ca\textsuperscript{2+} dynamics in primary cortical neurons supports the above hypothesis. Further studies are required to elucidate the specific pathways that underlie PERK’s regulation of intracellular Ca\textsuperscript{2+} dynamics.

As an eIF2α kinase, how did PERK evolve to be a modulator of Gq protein-coupled Ca\textsuperscript{2+} dynamics in pyramidal neurons? I speculate that during early vertebrate evolution, PERK first played an eIF2α-dependent role in CNS. Given its localization on the ER, which is the major organelle for intracellular Ca\textsuperscript{2+} storage, and its regulation by ER/cytosolic Ca\textsuperscript{2+} (2,17), the constant interaction with Ca\textsuperscript{2+} may have provided PERK the opportunity to evolve an additional function to regulate intracellular Ca\textsuperscript{2+} dynamics through mechanism independent of eIF2a and protein translation. The fact that PERK is activated by ER Ca\textsuperscript{2+} depletion (2), and the discoveries of PERK being a negative regulator of IP\textsubscript{3}R and a positive regulator of ROCC shown herein, fit well into this hypothesis: when ER Ca\textsuperscript{2+} stores are depleted under physiological responses such as activation of Gq protein-coupled receptor, PERK is activated due to Ca\textsuperscript{2+} dissociation from its regulatory domain in the ER, and it subsequently replenishes ER Ca\textsuperscript{2+} by inhibiting IP\textsubscript{3}R mediated ER Ca\textsuperscript{2+} release and activating ROCE (Fig. 2-9).
Fig. 2-9 Proposed model for PERK’s regulation of $G_q$ protein-coupled $Ca^{2+}$ dynamics in pyramidal neurons

Upon extracellular ligand binding, $G_q$ protein-coupled receptor is activated, which subsequently activates $G_q/PLC$. Activated PLC hydrolyzes PIP$_2$ into IP$_3$ and DAG. Increased cytosol IP$_3$ induces ER $Ca^{2+}$ depletion by binding with ER-resident IP$_3$R, which may activate PERK due to $Ca^{2+}$ dissociation from its regulatory domain in the ER. Activated PERK may then restore ER $Ca^{2+}$ level by inhibiting IP$_3$R mediated ER $Ca^{2+}$ release and activating receptor-operated $Ca^{2+}$ entry.
Chapter 3
PERK Regulates Working Memory and Protein Synthesis-Dependent Memory Flexibility

INTRODUCTION

Working memory is the cognitive capacity to actively and temporarily maintain information for the purpose of task execution (108). The dorsolateral prefrontal cortex in primates, which is homologous to the medium prefrontal cortex in rodents (160,161), is essential for working memory as evidenced by lesion studies (162), electrophysiological recordings (33) and brain imaging (163,164). At the cellular level, sustained neuronal firing was observed during the delay period of working memory, which is now considered an important neuronal correlate of working memory (165). The molecular mechanisms underlying working memory have been studied extensively in recent years, and it has been shown that intracellular Ca\textsuperscript{2+} signaling as stimulated by muscarinic acetylcholine or metabotropic glutamate receptor (mGluR) is critical for working memory (35-38,125,166).

PERK, an eIF2\textalpha{} kinase, is well known for its role in eIF2\textalpha{}-dependent protein synthesis and translational control. Upon activation PERK phosphorylates the \textalpha{} subunit of the translation initiation factor eIF2, which can subsequently modulate protein translation in two opposite ways: repression of global protein synthesis (3), and induction of translation of specific genes including CREB2/ATF4 (10). Since both \textit{de novo} protein synthesis and CREB2 are key regulators of long-term memory storage (41,167), PERK’s role in protein synthesis-dependent cognition has been comprehensively studied, where it has been shown that PERK is required for normal flexibility in learning and memory (20), and mGluR-dependent long-term depression (21).

Besides its role in cognition, the function of PERK has been most extensively studied in the pancreatic insulin-secreting \textbeta{}-cells where it regulates cell proliferation, proinsulin trafficking through the secretory pathways, and insulin secretion (13,14). Unexpectedly the mechanism of
PERK-dependent regulation of insulin secretion was found to be independent of eIF2α phosphorylation and protein synthesis. By acutely inhibiting PERK kinase activity using a newly available pharmacological inhibitor, it was discovered that PERK regulates Ca\(^{2+}\) dynamics in β-cells (17), which underlies glucose-stimulated insulin secretion. Considering the critical role of Ca\(^{2+}\) signaling in learning and memory, I hypothesized that PERK might regulate working memory, which is independent of new protein synthesis, but is largely driven by Ca\(^{2+}\) dynamics. I show herein that PERK regulates working memory. Moreover, pharmacological PERK inhibition in wild-type mice mimics the memory flexibility impairment observed in genetic Perk knockout mice. These findings illustrate a novel role of PERK in cognitive function, and suggest that PERK regulates both Ca\(^{2+}\) dynamics-dependent working memory and protein synthesis-dependent memory flexibility.
MATERIALS AND METHODS

Mouse strains and genotyping

*BrPerk* KO mice were generated by crossing *Perk*-floxed mice (12) with *αCaMKII-Cre* mice T29-1 strain (168) in C57BL/6J background. Adult *BrPerk* KO mice and their wild-type littermates aged 3-7 months old were used in the behavior experiments to ensure the maximum knockdown of PERK in the forebrain (169). Wild-type mice used in the pharmacological PERK inhibition experiments were 3 month old, and in C57BL/6J background (purchased from the Jackson laboratory). All the animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Penn State University (IACUC# 43379).

Brain tissue collection

To compare PERK knockdown efficiency in different brain regions, *BrPerk* KO mice and their wild-type littermates were euthanized by CO$_2$ inhalation in accordance with the IACUC protocol approved by Penn State University. Different brain regions including prefrontal cortex, hippocampus and cerebellum were isolated for western blot analysis.

Western blot analysis

Protein lysates from different brain regions and whole cells were prepared using RIPA buffer with 1X protease inhibitor and 1X phosphatase inhibitor cocktails from Sigma. Denatured protein samples were generated by boiling in 2X Laemmli buffer for 5 min. NuPAGE 4-12% Bis-Tris Midi Gel (Thermo Fisher Scientific) was used for electrophoresis. To enable the comparison of PERK knockdown efficiency in different brain regions, protein quantification was performed on protein lysates from brain tissue using Peirce BCA protein assay kit (Thermo scientifc, # 23227), and 50µg protein per sample was loaded for western blot. The following primary antibodies were used in western blot analysis: anti-PERK produced in rabbit (1:500, Cell Signaling, #3192), anti-p-PERK produced in rabbit (1:500, Cell Signaling, #3179), anti-eIF2α
[pS^{52}] produced in rabbit (1:1000, Invitrogen, 44728G), anti-α-tubulin produced in mouse (1:1000, Sigma, T5168).

**Gavage administration of PERK inhibitor GSK2606414**

GSK2606414 was suspended in vehicle (0.5% HPMC+0.01% tween-80 in water with pH 4.0) at the concentration of 15mg/ml. Mice were gavaged with GSK2606414 at the dosage of 150mg/kg body weight or equivalent amount of vehicle during the experiment.

**Spontaneous alternation Y-maze task**

Spontaneous alternation Y-maze task was performed as described (170). Each mouse was put in the middle of a symmetrical Y maze with three identical arms (arms 35cm long X 16cm high) and given free access to the arms during an 8 min period. The test was video-recorded and the results including percentage alternation and number of arms entered were blindly analyzed later. The first 2 min were for habituation and the subsequent 6 min were analyzed for the sequence of entrance. An entry was recorded only when all of the animal’s limbs were within the arm. Percentage alternation was calculated as follows: the number of triads containing entries into three arms/maximum possible alternations (total number of arms entered-2) X 100. The chance level for the alternation rate is 22.2% (171).

**Reference memory test using radial-arm water maze**

Reference memory was measured in radial-arm water maze, which was prepared similar to what has been previously described(172). A circular pool with an interior diameter of 92 cm and a depth of 63 cm was used. Six identical, black, plastic 60° bended, V-shaped inserts with 45 cm in height were put into the pool to form six arms (30 cm long X 10 cm wide). The pool was filled with water made opaque by mixing with non-toxic white paint so that the platform was invisible. Visual cues were put on the conjunction of every other V-shaped insert to help orient the mouse.

Each mouse was given 8 trials per day to locate a hidden escape platform placed at the end of a fixed arm. In each trial, the animal was released from a starting arm, and given 60 s to locate
the hidden platform. Once the animal got onto the platform, it was immediately taken out as a positive reward. The platform stayed in the same arm during the experiment, but the starting arm was changed every trial to avoid forming the swimming pattern. In the first 12 trials of training, the platform was altered between visible and invisible to accelerate the initial training process. The number of errors (number of wrong arms entered before getting onto the platform) was manually recorded throughout the experiment. Wild-type animals typically take 4 to 5 days to meet the criterion that they make less than 1 error on average in a trial block (4 trials per block) before locating the platform.

**The delay match-to-place task using radial-arm water maze**

We performed the delay match-to-place task in the same radial-arm water maze described above. The test was consisted of two days, day 1 for training and day 2 for testing. On each day, there were five sessions for each mouse. A session consisted of a location trial and a match trial, which both lasted 120 s. In the location trial, a hidden platform was placed at the end of one arm and the animal was released from the starting arm. If the mouse successfully got onto the platform, it would be quickly taken out as a positive reward. If the mouse failed to locate the platform within the time, it would be guided onto the platform and placed on it for 10 s before being taken out. 5 s after the location trial, the mouse was released again from the starting arm to start the match trial, with the platform staying in the same location. The escape latency (time taken to get onto the platform) and the number of errors (number of wrong arms entered before getting onto the platform) were manually recorded throughout the experiment. The position of the platform was changed between sessions, while the starting arm remained the same (If the six arms were labeled as A-F clockwise, the platform was placed in different arms with the order of B-D-F-C-E, with A being the starting arm).
**Y-water maze reversal task**

Y-water maze reversal task was performed as previously described [17]. A symmetrical Y-maze with three identical arms (arms 35 cm long X 16cm high) was used and water was made opaque with non-toxic white paint to make the escape platform invisible. The experiment was consisted of 2 days, with training session on day 1 and test and reversal session on day 2. During the training session, each mouse was trained in 10-20 trials to locate a hidden escape platform placed at the end of one arm, with 60 s allowed in each trial. The starting arm remained the same during the experiment. Mice were considered passing the training once they made 5 successful trials in a row (a success is defined as the animal being able to get onto the platform at its first choice within 1 min). 24 hours later, the mice were returned to Y-water maze and tested for their long-term memory in 2-3 trials, with the platform staying in the same arm. Those who made all correct arm choices in the test trials were selected for the reversal session. Reversal session was carried out right after the test, with the escape platform switched to the opposing arm, and the mice’s ability to locate the new position of the platform was measured. The results for all sessions were manually recorded.

**Fear extinction and fear conditioning test**

Both fear extinction and fear conditioning tests were carried out in the pre-pulse inhibition box from Panlab.

In fear extinction test, the mice were fear conditioned on day 0 by 5 pairs of auditory conditional stimulus (85dB white noise lasting for 30 s) and unconditional stimulus (0.65mA foot-shock for 2 s) in context A (the chamber was cleaned with 1% acetic acid solution and the light was turned on in both chamber and test room). There was a 1 min interval between each pair of stimuli. Fear extinction was performed 24hrs after fear conditioning in an altered environment context B (the chamber was cleaned previously with 70% ethanol, both chamber light and test room light were turned off with red fluorescent lights on as the replacement). During the
extinction session, animals received 15 consecutive presentations of auditory conditional stimulus (85dB white noise lasting for 30 s) without foot-shock everyday for 2-3 days. There was a 1 min-interval between each auditory stimulus.

In fear conditioning test, the mice were fear conditioned on day 0 by 2 pairs of auditory conditional stimulus (85dB white noise lasting for 30 s) and unconditional stimulus (0.65mA foot-shock for 2 s) in context A. 1 min interval was set up between each pair. 24 hrs later, the mice were placed back into context A for 5 min with freezing rate recorded. No stimulus was presented during the contextual fear conditioning. The mice were returned back to the holding room immediately after the test. After the contextual fear conditioning which took about for 2 hrs, the chamber environment was changed to context B and the mice were brought back to the test room again for auditory fear conditioning, in which the animals were presented with 3 min of conditional stimulus (85dB white noise).

For both experiments, the freezing rate was recorded by the equipment and analyzed after the experiment.
RESULTS

Forebrain-specific Perk knockout mice are impaired in spontaneous alternation Y-maze task

To study the function of PERK in learning and memory, forebrain-specific Perk knockout (BrPerk KO) mice were generated by crossing αCamKII-Cre with Perk-floxed mice previously generated in our lab (12). The expression of Cre recombinase in the αCaMKII-Cre strain occurs primarily in postnatal forebrain peaking at 3 months old (169). I therefore restricted the studies to adult BrPerk KO mice at least 3 months old. Previously it has been reported that Cre recombinase is expressed in both cortex and hippocampus in the αCaMKII-Cre strain (173,174). Western blot analysis of homogenates of different brain regions confirmed that the expression of PERK was substantially knocked down in the prefrontal cortex (Fig. 3-1A), which is the major brain region involved in working memory and memory flexibility in rodents (108,175). Significant knockdown of PERK was also observed in the hippocampus but not in the cerebellum (Fig. 3-1A). The small amount of residual PERK seen in BrPerk KO mice forebrain was likely due to PERK’s expression in glia and in non-pyramidal neurons, and to incomplete deletion of PERK in pyramidal neurons. The generation of BrPerk KO mice enabled me to specifically look at the function of PERK in the forebrain at adult stage without disturbing PERK’s function in other tissues or in earlier neurodevelopment.

As a specific form of memory, working memory lasts for only several seconds and does not require new protein synthesis. To determine if PERK is required for working memory, BrPerk KO mice were subjected to the spontaneous alternation Y-maze task, which is a spatial working memory test that is based on the willingness of rodents to explore a new environment (176). In this task, an animal must remember the arm it had visited previously in order to alternate the arm entry in the next trial. Thus the alternation rate is an indicator of the animal’s spatial working memory capacity. BrPerk KO mice exhibited significantly lower alternation rate when compared
to their wild-type littermates, but did not differ in the total number of arms entered (Fig. 3-1B). These results suggest that BrPerk KO mice are impaired in spatial working memory, but exhibit similar levels of locomotion and exploratory behavior.

![Western blot analysis showing substantial knockdown of PERK in the prefrontal cortex (PFC) of BrPerk KO mice. PERK was also significantly knocked down in the hippocampus (HIP) but not in the cerebellum (CER). (BKO: BrPerk KO; *** p<0.001, * p<0.05, n.s. not significant, two-tailed student’s t-Test; n=5 for each genotype).](image)

![Spontaneous alternation Y-maze task. BrPerk KO mice exhibited lower alternation rate compared to the wild-type littermates, indicating their poor spatial working memory (WT n=15, BrPerk KO n=22; ** p<0.01, two-tailed student’s t-Test). No difference was observed in total number of arms entered (p=0.70, n.s. not significant, two-tailed student’s t-Test), indicating similar motor ability and curiosity for the environment between two genotypes. Impaired spontaneous alternation was observed in BrPerk KO group in both genders (Fig. S11). The figures represent pooled results of both genders.](image)

**Fig. 3-1 BrPerk KO mice are impaired in spontaneous alternation Y-maze task**

(A) Western blot analysis showing substantial knockdown of PERK in the prefrontal cortex (PFC) of BrPerk KO mice. PERK was also significantly knocked down in the hippocampus (HIP) but not in the cerebellum (CER). (BKO: BrPerk KO; *** p<0.001, * p<0.05, n.s. not significant, two-tailed student’s t-Test; n=5 for each genotype).

(B) Spontaneous alternation Y-maze task. BrPerk KO mice exhibited lower alternation rate compared to the wild-type littermates, indicating their poor spatial working memory (WT n=15, BrPerk KO n=22; ** p<0.01, two-tailed student’s t-Test). No difference was observed in total number of arms entered (p=0.70, n.s. not significant, two-tailed student’s t-Test), indicating similar motor ability and curiosity for the environment between two genotypes. Impaired spontaneous alternation was observed in BrPerk KO group in both genders (Fig. S11). The figures represent pooled results of both genders.
**BrPerk KO mice are impaired in the delay match-to-place task**

To further examine the working memory capacity in BrPerk KO mice, the BrPerk KO mice were challenged with a more complex working memory test: the delay match-to-place task using radial-arm water maze. Successful accomplishment of this task requires good spatial reference memory and working memory. Previously it has been shown that BrPerk KO mice possess normal hippocampus-dependent spatial reference memory when tested in Morris water maze (20). I repeated the spatial reference memory test in radial-arm water maze, and confirmed that there is no difference between BrPerk KO mice and their wild-type littermates (Fig. 3-2A). After the reference memory test, BrPerk KO mice were subjected to a delay match-to-place test in radial-arm water maze. In this test, the mouse was given five sessions each day, with the location of the hidden platform changed between successive sessions. Each session consisted of two trials, a location trial followed by a match trial. A mouse with intact working memory would remember the location of the hidden platform in the location trial, and use visual cues to orient itself, thus displaying substantial savings of escape latency in the match trial (39). The BrPerk KO mice made more errors and required longer escape latency in locating the platform in match trials compared to their wild-type littermates (Fig. 3-2B), suggesting their impairment in working memory.
Fig. 3-2 *BrPerk* KO mice are impaired in the delay match-to-place working memory task

(A) Spatial reference memory test in radial-arm water maze. *BrPerk* KO mice exhibited normal reference memory in a 4 day reference platform task in radial-arm water maze (WT n=5, *BrPerk* KO n=6). Each mouse was given 8 trials per day during the 4 day task. A trial block represents the average number of errors of 4 trials. Only male mice were used in the experiment.

(B) The delay match-to-place task in radial-arm water maze. *BrPerk* KO mice made more errors and required longer escape latency than their wild-type littermates to locate the hidden platform in the match trial (WT n=16, *BrPerk* KO n=20; *** p<0.001, ** p<0.01, two-tailed student’s t-Test). Impaired spatial working memory was observed in *BrPerk* KO group in both genders (Fig. S12). The figures represent pooled results of both genders.
Pharmacologically PERK-inhibited mice are impaired in spontaneous alternation Y-maze task

To investigate if a similar behavioral impairment would occur following pharmacological inhibition of PERK activity, I systematically treated the wild-type mice with a newly developed PERK inhibitor (PERKi), GSK2606414. GSK2606414 is a highly specific inhibitor of PERK, which acts by competing for the ATP binding domain in the catalytic site (16), and has been proven to be effective in acutely inhibiting PERK’s activity in cultured cells (16). Previously it has been shown that 500nM PERKi pretreatment for 15 min sufficiently abolished thapsigargin induced PERK activation and eIF2α phosphorylation in primary neurons (Fig. 2-1A), suggesting that PERKi works effectively in acutely inhibiting PERK’s activity in vitro. Due to the low level of PERK activation in mice brain at steady state (preliminary data), I was unable to validate the efficacy of PERKi in vivo by western blot analysis. On the other hand, the efficacy of PERKi in vivo has been validated by others using liquid chromatography-tandem mass spectrometry measurements, and it was demonstrated that orally administered GSK2606414 penetrates the blood-brain barrier in mice (177).

Oral gavage is the only effective means of administering the PERKi, however oral gavage results in acute stress (178) and short-term elevation of corticosterone levels (179). To relieve gavage-induced stress, mice were acclimated to gavage administration by dosing with vehicle daily for 5 days prior to the administration of the inhibitor. After gavage acclimation, the animals were administered with 2 doses of PERKi or vehicle, and their working memory was assessed in spontaneous alternation Y-maze task 8 hours after the 2nd dose of PERKi (Fig. 3-3A). The selection of 8 hours allowed dissipation of gavage-induced stress while retaining sufficient inhibitor levels in circulation (preliminary data). Since saturating concentrations of GSK2606414 in mice brains have been reported 14 hours after 2 doses of compound administration at the concentration of 50mg/kg (177), a sufficient amount of the PERKi should be present in the brain.
8 hours after 2 doses of GSK2606414 administration at the concentration of 150mg/kg. The PERK-inhibited mice exhibited a lower alternation rate compared to the vehicle controls (Fig. 3-3B), suggesting the impairment of spatial working memory. Similar to genetic Perk ablation, no difference was seen in the total number of arms entered between the PERKi and vehicle groups. However, the mice that received gavage treatment showed significantly less total arm entries when compared to the mice with no gavage treatment (number of arms entered in Fig 3-1B and Fig 3-3B were compared; p<0.001, one-way ANOVA followed by Bonferroni’s post-hoc test), which suggests the possible impairment in animal performance due to the residual gavage-induced stress.

Fig. 3-3 WT mice with short-term PERK inhibition are impaired in Y-maze spontaneous alternation

(A) Schematic of PERK inhibitor treatment in spontaneous alternation Y-maze task.

(B) Spontaneous alternation Y-maze task. Short-term PERK-inhibited mice showed a lower alteration rate compared to vehicle group (Vehicle n=17, PERKi n=18; *** p<0.001, two-tailed student’s t-Test). No difference was observed in the total number of arms entered (n.s. not significant, two-tailed student’s t-Test).
Pharmacological PERK inhibition mimics fear extinction impairment in BrPerk KO mice

Previous studies reported that BrPerk KO mice are impaired in protein synthesis-dependent memory flexibility, as shown by Y-water maze reversal task and fear extinction task (20). I tested the memory flexibility of the BrPerk KO using the Y-water maze reversal task and confirmed this result (Fig. 3-4). To examine whether pharmacological PERK inhibition could mimic the memory flexibility impairments observed in genetic Perk knockout mice, I measured fear extinction memory in wild-type mice orally administered with PERKi. I chose to test fear extinction memory because this task require less precise cognition compared to Y-water maze reversal task, and thus is less likely to be confounded by gavage-induced stress.

Fear extinction memory was measured in wild-type mice which received short-term or long-term PERKi treatment. In the short-term PERKi treatment group, the mice were first acclimated to gavage for 5 days by daily vehicle administration, followed by 2 doses of PERKi or vehicle before the behavioral test (Fig. 3-3A), and one dose daily during the course of the experiment. Short-term PERK inhibition did not affect fear conditioning (Fig. 3-5A). However, whereas the vehicle group started to exhibit a progressive decline of freezing rate with repeated exposure to CS on the 2nd and 3rd of extinction session, the freezing rate of PERKi group remained elevated (Figs 3-5B and 3-5C). This result mimics the extinction impairment observed previously in BrPerk KO mice. It is noteworthy that neither group exhibited significant fear extinction on day 1. This was likely the result of gavage-induced stress, because in our initial trials, when mice were not first acclimated to gavage, neither vehicle nor PERKi group showed significant decline in the freezing rate over 2 days of extinction training (Fig. 3-6).

In the long-term PERKi treatment group, mice were orally administered with inhibitor or vehicle for 2 weeks prior to the behavioral test. The mice in this experiment were not acclimated to gavage because of long-term gavage administration. As with the short-term PERK inhibition, no difference in fear conditioning was observed between treatments (Fig. 3-7A). With longer
inhibitor treatment, while both groups exhibited significant fear extinction on day 1, PERK-inhibited mice exhibited delayed extinction over the 2 days (Figs 3-7B and 3-7C).

![Graph showing the percentage of correct arm choice over trial blocks for BrPerk KO mice](image)

**Fig. 3-4 BrPerk KO mice exhibit impaired memory flexibility in Y-maze reversal task**

*BrPerk* KO mice exhibited normal spatial learning in the training session on day 1, and normal long-term memory in the test session on day 2 (WT: 4 out of 4 passed the test; *BrPerk* KO: 4 out of 5 passed the test). In the reversal session on day 2, when the platform was switched to the opposing arm, *BrPerk* KO mice exhibited impaired memory flexibility, as illustrated by the lower percentage of correct arm choice/block (WT n=4; *BrPerk* KO n=4; ** p<0.01. two-tailed student’s t-Test; 4 trials were included per block in the training and reversal session, 2 trials were performed in the test session). A retraining trial was performed between the third and fourth trial block in the reversal session, the mice who never located the platform in its new location were guided onto it.
Fig. 3-5 WT mice with short-term PERK inhibitor gavage administration are impaired in fear extinction memory

(A) Fear conditioning on day 0. No difference was observed between treatments.

(B) Fear extinction over 3 days (3 CS presentations/block; Vehicle, n=6; PERKi, n=5; * p<0.05, two-tailed student’s t-Test). PERK-inhibited mice exhibited a higher freezing rate over 3 days’ extinction session (p<0.001, non-parametric paired sign test). Only male mice were used in the experiment.

(C) Average freezing rate over 3 days’ extinction session. Short-term PERK-inhibited mice exhibited a higher average freezing rate on the 2nd and 3rd day of extinction session, indicating their impairment in fear extinction (* p<0.05, two-tailed student’s t-Test).
Fig. 3-6 Mice with acute gavage administration do not exhibit fear extinction during 2 day extinction training

For mice received acute gavage administration without gavage acclimation, while both vehicle and PERKi group acquired conditioned fear on day 0, neither group exhibited reduced freezing rate over the 2 day extinction training (Vehicle n=10; PERKi n=9).
Fig. 3-7 WT mice with long-term PERK inhibitor gavage administration are impaired in fear extinction memory

(A) Fear conditioning on day 0. No difference was observed between treatments.

(B) Fear extinction over 2 days (3 CS presentations/block; Vehicle n=10, PERKi n=8, * p<0.05, *** p<0.001, two-tailed student’s t-Test). Long-term PERK-inhibited mice exhibited delayed fear extinction over the 2 days (p<0.01, non-parametric paired sign test). Only male mice were used in the experiment.

(C) Average freezing rate over 2 days” extinction session. PERK-inhibited mice exhibited a higher average freezing rate over 2 days’ extinction session, indicating their impairment in fear extinction (* p<0.05, two-tailed student’s t-Test).
Contextual and auditory fear conditioning in *BrPerk* KO mice

While I was investigating whether the *BrPerk* KO mice behavior phenotypes could be mimicked in the acutely PERK-inhibited mice, I also explored if *BrPerk* KO mice are impaired in fear conditioning, a memory process that involves both long-term memory and fear related memory. Preliminary data suggested that there is no significant difference in contextual conditioning or auditory conditioning between *BrPerk* KO mice and their wild-type littermates (Fig. 3-8). However, considering the small number of replicates in the wild-type group, it is possible that current number of replicates is not sufficient to resolve significance. The experiment needs to be repeated with 8-10 replicates included in each genotype to draw a solid conclusion.

**Fig. 3-8 Performance of *BrPerk* KO mice and wild-type littermates in contextual and auditory fear conditioning**

A. Percentage freezing of *BrPerk* KO and wild-type mice in contextual fear conditioning.

(WT n=4; BrPerk KO n=8; p=0.1, student’s t-Test)

B. Percentage freezing of *BrPerk* KO and wild-type mice in auditory fear conditioning.

(WT n=4; BrPerk KO n=8; p=0.069, student’s t-Test)
DISCUSSION

Previous study of PERK’s function in the brain has focused on protein synthesis-dependent cognitive abilities where it has been demonstrated that PERK is required for memory flexibility (20) and normal expression of mGluR-dependent long-term depression (21) via eIF2α-dependent protein translational control. Two other eIF2α kinases, GCN2 and PKR, have also been shown to be involved in protein synthesis-dependent memory regulation (23,24). Motivated by the recent discovery that PERK acutely regulates Ca\textsuperscript{2+} dynamics in β-cells (17) and primary cortical neurons (Chapter 2), which is independent of phosphorylation of eIF2α and protein translational control, I explored the possibility that PERK may regulate Ca\textsuperscript{2+} dynamics-dependent working memory. I found that BrPerk KO mice are impaired in working memory, as shown by spontaneous alternation Y-maze task and the delay match-to-place task. Since over the course of development the genetic Perk knockout mice may have acquired a compensatory mechanism that could potentially confound the interpretation of underlying mechanisms, I examined the effects of pharmacologically ablating PERK activity in the brain of wild-type mice by oral administration of a specific and potent PERK inhibitor. PERK-inhibited mice exhibited a significant impairment in working memory in the spontaneous alternation Y-maze task. Moreover, oral administration of PERKi mimics the fear extinction deficit observed in forebrain-specific Perk knockout mice. Together, these results suggest that the behavior phenotypes observed in BrPerk KO mice are attributable to the absence of PERK rather than to a developmental compensatory response.

Spontaneous alternation Y-maze task and the delay match-to-place task in radial-arm water maze were used to test spatial working memory in this study. Y-maze spontaneous alternation occurs because of rodents’ natural exploratory behavior, which causes them to alternate spontaneously in the Y-maze, at a rate significantly higher than that could be due to chance (176). Although the neuronal mechanisms underlying spontaneous alternation are still unclear, this test has been widely used to measure spatial working memory, because it does not require excessive
animal handling and measures subject’s locomotion in the meantime (180). In addition to spontaneous alternation Y-maze task, I also subjected BrPerk KO mice to the delay match-to-place task. The delay match-to-place task is a robust behavioral test, but it is also a more complex test for measuring spatial working memory. Since the initial introduction by Morris and coworkers (181), modifications have been made to simplify the procedure without reducing the maze’s complexity (39,182). I performed the delay match-to-place task in radial-arm water maze, which preserves the spatial complexity of the Morris water tank, but simplifies scoring procedure with the benefits of a defined swim path.

The neuronal mechanisms underlying PERK’s regulation of working memory is unknown. I speculate that PERK regulates working memory by modulating \( G_q \) protein receptor-coupled intracellular \( \text{Ca}^{2+} \) dynamics in pyramidal neurons. As shown in chapter 2, acute PERK inhibition suppresses \( G_q \) protein receptor-coupled \([\text{Ca}^{2+}]_i\), rise by inhibiting receptor-operated \( \text{Ca}^{2+} \) influx in primary cortical neurons. Meanwhile, \( G_q \) protein receptor-coupled \([\text{Ca}^{2+}]_i\), rise has been suggested to regulate working memory in several ways. Firstly, the induced \([\text{Ca}^{2+}]_i\), rise is known to activate several \( \text{Ca}^{2+} \)-dependent protein enzymes, including the phosphatase calcineurin, and the kinases CaMKII and PKC, all of which have been shown to regulate working memory capacity (111). Secondly, the \( I_{\text{CAN}} \), which is identified as the ionic mechanism underlying neuronal persistent firing (125), is \( G_q \) protein and \( \text{Ca}^{2+} \)-dependent (100). Finally, \( G_q \) protein-coupled \([\text{Ca}^{2+}]_i\), rise has direct effects on intrinsic neuronal excitability. It has been demonstrated that pharmacological activation of mGluR1 in prefrontal cortex pyramidal neurons triggers a biphasic electrical response-SK channel-dependent neuronal hyperpolarization followed by TRPC-dependent neuronal depolarization, and the amplitude of both are regulated by the extent of \([\text{Ca}^{2+}]_i\), rise (166,183).

Fear extinction is a form of inhibitory learning, a process by which a pre-conditioned fear response is extinguished after repeated exposure to neutral conditioned stimulus without the
aversive stimulus. Previously it has been shown that BrPerk KO mice are impaired in fear extinction, which is associated with reduced eIF2α-dependent ATF4 expression (20). Here I showed that pharmacological PERK inhibition mimics the fear extinction impairment observed in BrPerk KO mice, which suggests that the behavior phenotype observed in BrPerk KO mice is attributable to the absence of PERK, and confirms PERK’s role in regulating memory flexibility in addition to working memory.

Although different molecular mechanisms have been suggested underlying working memory and memory flexibility, both cognitive functions are considered as core components of executive function, regulated in different sub-regions in prefrontal cortex, but modulated by similar neurotransmitters (108,175). Thus, the possibility remains that the working memory and memory flexibility deficits observed in BrPerk mice may be due to an impairment in a common pathway that is normally regulated by PERK.

Based on cell culture experiments, PERK was initially identified as an effector of unfolded protein and ER stress response (1-3) and from studies it was speculated that the function of PERK in whole organisms was to temporarily repress protein synthesis in order to relieve the client protein load in the ER. However subsequent studies in Perk KO mice, which display an array of dysfunctions, failed to find evidence in support of this hypothesis (13,184). Rather our lab found that PERK plays important developmental and physiological regulatory functions that are unrelated to stress throughout the body (13,14,184,185). In this study, I investigated PERK’s role in cognition, and showed for the first time that PERK regulates new protein-synthesis independent working memory. This discovery reveals a novel physiological role of PERK in cognitive function, which is unrelated to stress responses. Moreover, eIF2α dephosphorylation has been shown to be critical for late phase long-term potentiation (L-LTP) elicitation and long-term memory (LTM) storage, as genetic reduction of eIF2α phosphorylation by knockdown of eIF2α kinases GCN2 (23) or PKR (24), or single allele phosphorylation site mutation of eIF2α
(Serine to Alanine at phosphorylation site Ser51) (22) lowers the threshold for L-LTP and facilitates LTM, while pharmacological promotion of eIF2α phosphorylation impairs L-LTP and LTM (22). On the other hand, forebrain-specific Perk knockout mice exhibit normal LTP and LTM (20,21), but impaired memory flexibility (20). The significant phenotype difference between Perk knockout mice and other mice models with reduced eIF2α phosphorylation suggest that PERK may possess additional regulation of cognition that is eIF2α-independent. The discovery of PERK’s regulation over Ca²⁺ dynamics-dependent working memory supports the above hypothesis. Further study is needed to elucidate the specific pathways that participate in PERK’s regulation of the different cognitive functions.
Chapter 4

PERK regulates Alzheimer’s disease progression by modulating intracellular Ca\(^{2+}\) dynamics

INTRODUCTION

Alzheimer’s disease is an age-related neurodegenerative disease affecting about 2% of the population in industrialized countries. Clinically, Alzheimer’s disease patients lose their memory and cognitive abilities. Pathologically, extracellular deposits of amyloid β (Aβ) plaques and intracellular neurofibrillary tangles are formed in the brain regions involved in learning and memory, which include cortex and hippocampus (49).

Several hypotheses have been proposed for the etiology of Alzheimer’s disease. Among them, the amyloid hypothesis, which suggests that the accumulation of Aβ plaques is the primary cause driving the disease development, is the one that gains most attention. Despite the extensive genetic, neuropathological evidence supporting this hypothesis, there have been some controversies regarding the validity of it, including the detection of amyloid plaques in humans without dementia (186) and the failure of clinical trials targeting the Aβ deposition (187).

An alternative hypothesis of Alzheimer’s disease development is the calcium hypothesis, which states that the disrupted calcium (Ca\(^{2+}\)) homeostasis plays a leading role in the disease progression (68). Although there was no experimental support for the hypothesis when it was first proposed by Khachaturian in 1989 (68), accumulating evidence was obtained soon after. Enhanced inositol-1,4,5-triphosphate receptor (IP\(_3\)R)-mediated Ca\(^{2+}\) release was observed in the fibroblasts derived from asymptomatic members from Alzheimer’s disease family (70). Augmented IP\(_3\)R and Ryanodine receptor (RyR)-dependent Ca\(^{2+}\) release was observed in the brain slices from young Alzheimer’s disease transgenic mice before any obvious neuropathology (71,83). Taken together, these evidences suggest that altered intracellular Ca\(^{2+}\) signaling may play an important role in Alzheimer’s disease progression.
PERK, an eIF2α-dependent regulator of protein synthesis and translation, is activated in the cortex and hippocampus of Alzheimer’s disease patients (25). Given the fact that many neurodegenerative diseases including Alzheimer’s disease are characterized by disease-specific protein aggregation, it is speculated that PERK may regulate Alzheimer’s disease progression via its protein translational control. Indeed, it has been shown that reducing PERK expression rescues long-term memory deficits in APP-PS1 mice and decreases forebrain Aβ expression (27,28). However, no direct evidence has been provided suggesting that PERK regulates amyloid β generation via protein translational control. In addition to PERK’s role as an eIF2α-dependent regulator of protein translation, I discovered that PERK acutely regulates Gq protein-coupled intracellular Ca2+ dynamics in pyramidal neurons (chapter 2). Considering the role of Ca2+ dynamics in Alzheimer’s disease progression, I hypothesized that PERK may regulate the disease progression via Ca2+ signal regulation. To test this hypothesis, Perk was genetically ablated in the forebrain of 5XFAD transgenic mice. Knocking down PERK in the forebrain alleviated 5XFAD mice behavior deficits, but did not affect Aβ plaque density or the expression of APP and Aβ in the forebrain. In addition, acute PERK inhibition corrects the exaggerated Ca2+ signaling in 5XFAD primary cortical neurons. Taken together, it is suggested that PERK may regulate Alzheimer’s disease progression by modulating Gq protein-coupled intracellular Ca2+ dynamics in pyramidal neurons.
MATERIALS AND METHODS

Mouse strain

A transgenic Alzheimer’s disease mouse model which overexpresses mutant human amyloid precursor protein (APP695) with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) Familial Alzheimer’s Disease (FAD) mutations and mutant human presenilin-1 (PS1) harboring two FAD mutations, M146L and L286V (5XFAD) was used in this study(170). The ablation of Perk in the forebrain of 5XFAD mice was achieved by crossing transgenic 5XFAD mice with αCaMKII-Cre Perk-floxed mice. 5XFAD αCaMKII-Cre Perk-floxed (5XFAD BrPerk KO) mice and their littermates including 5XFAD Perk-floxed (5XFAD), αCaMKII-Cre Perk-floxed (BrPerk KO) and Perk-floxed (wild-type) mice were used in the study.

Spontaneous alternation Y maze task

Spontaneous alternation Y-maze task was performed as described in chapter 3.

Radial arm water maze learning and memory task

The radial-arm water maze learning and memory task was carried out similar to the reference memory test using radial-arm water maze as described in chapter 3.

Perfusion

Mice were anesthetized using 2.5% avertin at the dose of 15ml/kg body weight, and were confirmed in deep anesthesia by the sign of no pinch reflex. During perfusion, chest above the diaphragm was quickly opened to expose the heart, and the perfusion needle was inserted into the tip of left ventricle. While turning on the perfusion pump, a cut was made on right atrium immediately to allow blood to flow out. The animal was perfused with phosphate buffered saline (PBS) for a few min until the liver turned pale. The perfusion solution was then switched to PBS containing 1X protease inhibitor and 1X phosphatase inhibitor cocktails for 3 min. After the perfusion, half of the brain was kept at -80°C for western blot analysis; the other half was
trimmed into smaller pieces and post-fixed in 10% neutral buffered formalin solution (HT5014, Sigma) for cryosection.

**Western blot analysis**

Protein lysates from brain tissue were prepared using RIPA buffer with 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Denatured protein samples were obtained by boiling in 2X Laemmli buffer for 5 min. The following primary antibodies were used in western blot analysis: anti-6E10 produced in mouse (1:1000, Covance, SIG-09320), anti-α-tubulin produced in mouse (1:1000, Sigma, T5168).

**Cryosection**

The perfused brain tissue was kept in 10% neutral buffered formalin solution at 4°C for 1 day, and stored in PBS at 4°C for up to 1 week. The day before cryosection, the post-fixed brain tissue was transferred to 30% sucrose in PBS overnight for cryoprotection. On the day of cryosection, the brain tissue was embedded into cryomatrix and sectioned at the thickness of 30µm. The brain slices were kept in 24-well plates in PBS sequentially for Thioflavine-S staining.

**Thioflavine-S Staining and Aβ plaque quantification**

Extracellular Aβ plaques were stained with Thioflavine-S (a kindly gift from Dr. Gong Chen’s lab) at the concentration of 2µg/ml in PBS for 20 min. Pictures were taken at the emission wavelength of 550nm. Density of Aβ plaques was determined by the number of plaques within a defined area divided by the size of the area. Both the number of plaques and the size of the area were determined by Image J.

**Primary neuronal culture**

The primary neuron culture was performed as described in chapter 2.

**Intracellular Ca^{2+} measurements**

Intracellular Ca^{2+} measurements were performed as described in chapter 2.
Gavage administration of PERK inhibitor GSK2606414

GSK2606414 and vehicle were prepared in the same way as described in chapter 3. Two month-old 5XFAD mice were gavaged with GSK2606414 or equivalent amount of vehicle at the dose of 150mg/kg daily for 2 months, with one week on and one week off.
RESULTS

Ablating *Perk* in the forebrain alleviates 5XFAD mice’s deficits in spatial working memory and long-term memory

To determine if PERK impacts the progression of Alzheimer’s disease, I genetically ablated *Perk* in the forebrain of 5XFAD mice by crossing them with *BrPerk* KO mice. Transgenic 5XFAD mice accumulate Aβ plaques quickly in the forebrain, and begin to show plaques in the cortex and hippocampus at the age of 4 months old (170). However, they do not exhibit memory impairments until the age of 5-6 month old, when they start to show deficits in spatial working memory and long-term memory (27,170). To examine if ablating *Perk* alleviates 5XFAD mice working memory deficits, 6 month-old 5XFAD *BrPerk* KO mice and their littermates (with 3 genotypes) were subjected to spontaneous alternation Y-maze task, which is a spatial working memory task based on the instinct of rodents to explore a new environment (176). 5XFAD mice exhibited significantly less alternation rate compared to their wild-type littermates, indicating their impairment in working memory (Fig. 4-1A). 5XFAD *BrPerk* KO mice showed a borderline higher alternation rate compared to 5XFAD mice, which suggests that knocking down PERK in the forebrain alleviates 5XFAD mice working memory deficits (Fig. 4-1A). Since PERK is required for working memory (as illustrated in chapter 2), *BrPerk* KO mice also exhibited impaired spatial working memory. No significant difference was observed in the total number of arms entered, suggesting similar locomotion and exploratory behavior among four genotypes.

In addition to working memory deficits, 5XFAD mice also exhibit impaired hippocampus-dependent long-term memory (27). To test if ablation of *Perk* rescues long-term memory impairment in 5XFAD mice, 5XFAD *BrPerk* KO mice and their littermates were challenged with the radial arm water maze learning and memory task. This task is similar to Morris water maze, with the exception that the swimming path is defined using a symmetric six-arm maze, so that the learning process is accelerated and the performance measurement is simplified. The test was
performed on 3 successive days, with 8 trials per day. During each trial, the animal was released from a starting arm to locate a hidden platform placed at the end of a fixed arm. While the platform stayed in the same place during the experiment, the starting arm was changed every trial to avoid forming the swimming pattern. The number of errors before locating the platform was manually recorded throughout the experiment. While all groups of mice exhibited comparative learning ability in first 2 days’ training, as shown by reduced number of errors made before locating the platform with the increase of trial number, the 5XFAD mice displayed moderate long-term memory deficits on day 3, and the deficits were not observed in the 5XFAD BrPerk group, suggesting that ablating Perk in the forebrain rescues 5XFAD mice’s mild long-term memory deficits (Fig. 4-1B). Consistent with previous finding (20), BrPerk KO mice displayed similar long-term memory compared to the wild-type controls. Taken together, the above results demonstrate that ablation of Perk in the forebrain of 5XFAD mice alleviates their memory deficits, suggesting that PERK plays an important role in the progression of Alzheimer’s disease.
Fig. 4-1 Ablating Perk in the forebrain alleviates 5XFAD mice’s deficits in spatial working memory and long-term memory

A. Y-maze spontaneous alteration. The impaired working memory in 5XFAD mice, as shown by significantly reduced alternation rate compared to wild-type littermates, was alleviated in 5XFAD BrPerk KO mice (WT n=5; BrPerk KO n=8; 5XFAD n=7; 5XFAD BrPerk KO n=9; One-way ANOVA, p<0.05; * p<0.05, two-tailed student’s t-Test).

B. Radial arm water maze learning and memory task. The modest long-term memory deficit in 5XFAD mice, as shown by more errors made in the last trial block compared to wild-type littermates, was alleviated in 5XFAD BrPerk KO mice (WT n=9; BrPerk KO n=9; 5XFAD n=5; 5XFAD BrPerk KO n=7; * p<0.05, two-tailed student’s t-Test). The number of errors in trial block 6 is shown in the bar graph on the right.
The alleviated memory deficits in 5XFAD BrPerk KO mice is not due to changes in the amyloidogenic pathway

The abnormal accumulation of Aβ peptides in the brain has been suggested to be detrimental to synaptic plasticity and cognitive function. As an eIF2α-dependent regulator of protein synthesis and translation, PERK is speculated to regulate Aβ production in an eIF2α-dependent pathway. To investigate if PERK is involved in the amyloidogenic pathway, the expression of amyloid precursor protein and Aβ peptides were analyzed in the brain of 5XFAD and 5XFAD BrPerk KO mice. No significant difference was observed between two genotypes (Fig. 4-2). Moreover, the density of Aβ plaques, which are the extracellular aggregation of secreted Aβ peptides, was determined and compared between 5XFAD and 5XFAD BrPerk KO mice. No significant difference was observed in either cortex or dentate gyrus between two genotypes (Fig. 4-3). Taken together, these results suggest that PERK is not involved in the amyloidogenic pathway.

Fig. 4-2 No difference was observed in brain expression of amyloid precursor protein (APP) and Aβ peptides between 5XFAD and 5XFAD BrPerk KO mice.

Representative western blot on the left shows the expression of APP and Aβ peptides in the brain of 8-10 months-old 5XFAD, 5XFAD BrPerk KO mice. Little signal of APP and Aβ was detected in the wild-type controls. No difference was observed in brain expression of APP and Aβ between 5XFAD and 5XFAD BrPerk KO mice (n=6 for each genotype; n.s. not significant, two-tailed student’s t-Test).
A. Representative picture showing the accumulation of $\text{A\beta}$ plaques stained by Thioflavin-S in 5XFAD and 5XFAD BrPerk KO mice. No signal was detected in wild-type mice brain slice, which suggested the specificity of Thioflavin-S against A\beta plaque.

B. No significant difference in cortical $\text{A\beta}$ plaque density was observed between 5XFAD and 5XFAD BrPerk KO mice over different ages (4.5 month: n=4 for both genotypes; 6.5 month: n=3 for both genotypes; 12 month: n=4 for 5XFAD; n=3 for 5XFAD BrPerk KO; n.s. not significant, two-tailed student’s t-Test).

C. No significant difference in $\text{A\beta}$ plaque density in dentate gyrus was observed between 5XFAD and 5XFAD BrPerk KO mice over different ages (4.5 month: n=4 for both genotypes; 6.5 month: n=3 for both genotypes; 12 month: n=4 for 5XFAD; n=3 for 5XFAD BrPerk KO; n.s. not significant, two-tailed student’s t-Test).
**Acute PERK inhibition rescues the exaggerated Ca\(^{2+}\) signaling in 5XFAD primary neurons**

Growing evidence suggests that altered Ca\(^{2+}\) signaling plays a role in Alzheimer’s disease progression by modulating neuronal excitability (71) and A\(\beta\) peptide generation (188,189). Since exaggerated IP\(_3\)R-mediated Ca\(^{2+}\) release has been observed in primary neurons from APP-PS1 transgenic mice (71,83), and PERK is required for \(G_\text{q}\) protein-coupled receptor-operated Ca\(^{2+}\) entry from extracellular space, I hypothesized that inhibiting PERK’s activity may correct the exaggerated Ca\(^{2+}\) signaling in APP-PS1 primary neurons. Primary cortical neurons were cultured from 5XFAD transgenic mice, and group1 metabotropic glutamate receptor (mGluR1)-coupled intracellular Ca\(^{2+}\) rise was examined by the use of its specific agonist, DHPG. Consistent with previous findings (71,83), enhanced DHPG-stimulated intracellular Ca\(^{2+}\) rise was observed in 5XFAD primary cortical neurons (Fig. 4-4A). Moreover, acute PERK inhibition by the use of a specific and potent PERK inhibitor (PERKi) GSK2606414 corrects the exaggerated Ca\(^{2+}\) rise induced by DHPG (Fig. 4-4B), suggesting that acute PERK inhibition rescues the altered Ca\(^{2+}\) signaling in 5XFAD primary neurons.
Fig. 4-4 Acute PERKi treatment corrects the exaggerated mGluR1-coupled intracellular Ca\(^{2+}\) rise in 5XFAD primary neurons

A. Exaggerated intracellular Ca\(^{2+}\) rise upon 50 μM DHPG treatment was observed in 5XFAD primary neurons (WT n=9; 5XFAD n=15; n.s. not significant, **) \(p<0.01\), two-tailed student’s t-Test). No difference was observed in basal Ca\(^{2+}\) oscillation between genotypes (n.s. not significant, two-tailed student’s t-Test).

B. Preliminary data suggests that acute PERKi treatment corrected the exaggerated 50μM DHPG-stimulated intracellular Ca\(^{2+}\) rise in 5XFAD primary neurons (WT DMSO n=7; 5XFAD DMSO n=5; WT PERKi n=4; 5XFAD PERKi n=13; One-way ANOVA, \(p<0.05\)). No difference was observed in basal Ca\(^{2+}\) oscillation among groups (One-way ANOVA, \(p>0.05\)).
Oral PERKi treatment reduces extracellular Aβ plaque accumulation in the cortex of 5XFAD mice

An important pathology of Alzheimer’s disease is the accumulation of Aβ plaques, which could be caused by exaggerated Ca\(^{2+}\) signaling due to increased Aβ peptide production and secretion (188,189). To test if pharmacologic PERK inhibition has an effect on Aβ plaque formation \textit{in vivo}, 5XFAD mice were orally administered with PERKi at the dose of 150mg/kg daily for 2 months at the age of 2 months old, with one week on and one week off. PERKi treatment significantly reduced extracellular Aβ plaques density in the cortex of 5XFAD mice (Fig. 4-5), suggesting that PERK inhibition plays an important role in Aβ plaque deposition.

Fig. 4-5 Oral PERKi treatment reduced extracellular Aβ plaques accumulation in the cortex of 5XFAD mice

5XFAD mice were gavaged with PERKi at the age of 2 months old. The treatment was given with one week on and one week off for 2 months.

A. PERKi treatment significantly reduced cortical Aβ plaque density in 5XFAD mice (Vehicle n=5, P.I. n=6; * p<0.05 two-tailed student’s t-Test).

B. No significant difference was observed in Aβ plaque density in dentate gyrus between treatments (Vehicle n=5, P.I. n=6; n.s. not significant, two-tailed student’s t-Test).
DISCUSSION

Growing evidence suggests that PERK is involved in Alzheimer’s disease progression, as elevated phosphorylation of PERK and its downstream substrate eIF2α have been observed in the brain of Alzheimer’s disease patients (25,26) and Alzheimer’s disease mouse model (27). As an important effector in unfolded protein response (2), it has been speculated that PERK is activated in Alzheimer’s disease due to overload of APP in the ER. Upon activation, PERK phosphorylates its substrate, eIF2α, which can subsequently regulate protein translation in two opposite ways: suppression of global protein synthesis (3), and induction of translation of certain mRNAs with upstream open reading frames including β-site APP cleaving enzyme-1 (BACE1) (190), which is a major β-secretase required for Aβ generation (191). Thus ablation of Perk may modulate Aβ generation by derepression of global protein synthesis or repression of BACE1 translation.

To study PERK’s role in the progression of Alzheimer’s disease, I genetically ablated Perk in the forebrain of a 5XFAD transgenic mouse model. Ablation of Perk rescued memory deficits in 5XFAD mice at the age of 6-8 months old, which suggests that PERK indeed plays a role in the disease progression. However, no difference was observed in the expression of APP, Aβ, or Aβ plaque density between 5XFAD BrPerk KO and 5XFAD mice, indicating that PERK does not modulate the amyloidogenic pathway.

In addition to PERK’s role as an eIF2α-dependent regulator of protein synthesis and translation, I’ve shown that PERK acutely regulates Gq protein-coupled intracellular Ca²⁺ dynamics in primary cortical neurons (chapter 2). Considering the important role of Ca²⁺ signaling in Alzheimer’s disease progression, it is possible that PERK modulates the disease progression by Ca²⁺ dynamics regulation. Significantly enhanced mGluR1-coupled intracellular Ca²⁺ rise was observed in 5XFAD primary cortical neurons. Moreover, acute PERK inhibition corrected the exaggerated Ca²⁺ signaling. To test if PERK inhibition affects Aβ plaque deposition in vivo, I treated 5XFAD mice with PERKi by oral administration. Two months PERKi oral
administration significantly reduced Aβ plaque density in the cortex of 5XFAD mice, which suggests that PERK inhibition affects Aβ plaque deposition in vivo.

Although pharmacologic PERK inhibition significantly reduced cortical Aβ plaque loading in 5XFAD mice, genetic Perk ablation did not affect Aβ plaque deposition. The difference here is not likely due to the way of PERK repression, since genetic ablation or reduction of Perk has been shown to decrease Aβ expression in APP-PS1 mice in other studies (27,28). I speculate that the difference between pharmacologic PERK inhibition and genetic Perk ablation’s effect on Aβ plaque deposition is due to the time of PERK repression. It has been demonstrated in both Alzheimer’s disease mice models and clinical trials that the intervention has to be performed in the early stage of the disease in order to have effects on neuropathology (66,192,193). In Tao et al’s study (28), although they used the same αCaMKII-Cre strain as I did to drive Perk ablation, they used an APP-PS1 mouse model with only 3 familial Alzheimer’s disease mutations, and thus their APP-PS1 mice develop amyloid pathology and memory deficits at a much slower rate compared to the 5XFAD transgenic mice. In Devi et al’s study (27), the expression of PERK was reduced to 50% early in the embryonic stage by crossing 5XFAD transgenic mice with Perk+/- mice, while in my study the knockdown of PERK occurs primarily in postnatal stage and peaks at 3 months of age due to the use of αCaMKII-Cre.

Although both Devi and Tao’s studies reported rescued long-term memory deficits, reduced PERK-eIF2α signaling, reduced ATF4 and Aβ expression, and increased expression of an Aβ-degrading enzyme, Neprilysin, in APP-PS1 mice models with genetic ablation or reduction of Perk (27,28), the molecular mechanisms behind remain controversial and unclear. The increased expression of Neprilysin certainly explained reduced Aβ expression, but there is no evidence suggesting that this was caused by reduced activity of PERK-eIF2α signaling pathway. In addition, the change of BACE1 expression in APP-PS1 mice with genetic ablation or reduction of Perk remains controversial. Tao et al reported on difference in BACE1 expression between
3XFAD BrPerk KO and 3XFAD mice (28). On the other hand, Devi et al reported reduced BACE1 expression in 5XFAD Perk +/- mice when compared to 5XFAD Perk +/- mice (27).

How then does PERK’s regulation of Gq protein-coupled intracellular Ca\(^{2+}\) dynamics regulate the neuropathogenesis and clinical symptoms of Alzheimer’s disease? It has been shown that augmented intracellular Ca\(^{2+}\) release can enhance Aβ generation (78), increase Glycogen Synthase Kinase 3β-dependent Tau phosphorylation (80), activate CREB activity (81), a transcription factor which plays a critical role in memory formation, and modulate synaptic plasticity (82). Moreover, genetic reduction of IP\(_3\)R by 50% normalized the enhanced IP\(_3\)R-mediated Ca\(^{2+}\) release in cortical and hippocampal neurons from Alzheimer’s disease transgenic mice with presenilin 1\(_{M146V}\) knock-in, restored normal RyR activity, and more importantly, alleviated the amyloid deposition, Tau hyperphosphorylation and cognitive deficits of the diseased mice (83). Thus, it is very likely that ablation of Perk alleviates the disease progression by correcting the exaggerated intracellular Ca\(^{2+}\) rise.

In this study, I examined PERK’s role in the development of Alzheimer’s disease, and presented evidence suggesting that PERK may regulate Alzheimer’s disease progression by modulating intracellular Ca\(^{2+}\) dynamics. My study illustrates that PERK may regulate Alzheimer’s disease progression in an eIF2α-independent pathway, and that the altered Ca\(^{2+}\) homeostasis may be the primary cause driving the progression of Alzheimer’s disease.
Chapter 5

Discussion

The discovery of PERK as a modulator of G_q protein-coupled Ca^{2+} dynamics illustrates a novel role of PERK in the central nervous system that is eIF2α-independent

Motivated by the discovery that PERK acutely regulates Ca^{2+} dynamics in insulin-secreting β-cells (17), I have been intrigued if PERK also regulates Ca^{2+} signals in neurons, and whether this regulation plays any physiological role in mammalian cognitive function and neurodegenerative disease. In my thesis, I showed for the first time that PERK acutely regulates G_q protein-coupled intracellular Ca^{2+} rise in primary cortical neurons. The finding that inhibition of PERK alters Ca^{2+} dynamics within a few minutes after inhibitor application suggests that this regulation is unlikely to be mediated by protein translation. To explore if PERK’s regulation of Ca^{2+} dynamics plays any physiological role in cognitive function, I performed a series of behavioral tests on forebrain-specific Perk knockout (BrPerk KO) mice, in which Perk is specifically knocked out in the pyramidal neurons of mice forebrain, and discovered that PERK regulates working memory, a cognitive function that is independent of new protein synthesis, but is largely driven by Ca^{2+} dynamics. Pharmacological PERK inhibition mimics the working memory impairment observed in BrPerk KO mice, suggesting that the working memory impairment observed in BrPerk KO mice is PERK-dependent. To investigate PERK’s role in the progression of Alzheimer’s disease, Perk was specifically knocked out in an Alzheimer’s disease mouse model with five familial Alzheimer’s disease mutations, and it was discovered that knocking down PERK in the forebrain of 5XFAD (5XFAD BrPerk KO) alleviated the disease mice behavioral deficits. While no difference was observed in the amyloidogenesis pathway in 5XFAD BrPerk KO mice compared to their 5XFAD littermates, it was discovered that acute PERK inhibition in 5XFAD primary cortical neurons corrected the exaggerated Ca^{2+} signaling, which has been suggested to drive Aβ pathogenesis (78). Taken together, my study demonstrates
that PERK regulates $G_q$ protein-coupled intracellular Ca$^{2+}$ rise in pyramidal neurons in an eIF2$\alpha$-independent pathway, and this regulation may play important physiological role in mammalian cognitive function and Alzheimer’s disease progression.

PERK is well known for its role in eIF2$\alpha$-dependent protein synthesis and translational control. For this reason, it has been hypothesized that PERK regulates memory flexibility and group 1 metabotropic glutamate receptor (mGluR 1)-dependent long-term depression in an eIF2$\alpha$-dependent pathway (20,21). Upon activation PERK phosphorylates eIF2$\alpha$, which can subsequently repress global protein synthesis (3), but induces the translation of specific genes including CREB2/ATF4 (10). Considering its role in global protein synthesis regulation, PERK’s effect on de novo protein synthesis at steady state or under DHPG stimulation has been assessed in mice prefrontal cortex (20) and hippocampus (21), respectively, and a minimal effect was observed in either case. On the other hand, it was shown that 70% repression of global protein synthesis in vivo by low-dose protein synthesis inhibitor anisomycin did not affect protein synthesis-dependent memory consolidation in mice (194). Taken together, the above results argue against the hypothesis that PERK regulates memory flexibility and mGluR 1-dependent long-term depression via global protein synthesis regulation.

In addition to general protein synthesis repression, increased phospho-eIF2$\alpha$ level can specifically induce the translation of CREB2/ATF4, which is a transcription factor that regulates long-term memory storage by negatively regulating CREB (167). Decreased expression of phospho-eIF2$\alpha$ and ATF4 was observed together with reduced PERK expression in the prefrontal cortex and hippocampus of BrPerk KO mice (20,21), and thus the reduced PERK/eIF2$\alpha$/ATF4 signaling has been hypothesized to be the molecular mechanism underlying the impaired memory flexibility (20) and mGluR 1-dependent long-term depression (21) in BrPerk KO mice.

In mammals, there are three other eIF2$\alpha$ kinases in addition to PERK: HRI (heme-regulated inhibitor), PKR (double-stranded RNA activated protein kinase), and GCN2 (general control non-
derepressible-2) (195,196). These four eIF2α kinases share a similar kinase domain, phosphorylate eIF2α on serine 51, but are activated by different cellular stressors (Fig. 5-1). With the exception of HRI, all of the eIF2α kinases are abundantly expressed in mammalian brain (197). If impaired PERK/eIF2α/ATF4 signaling pathway is the main cause underlying the protein synthesis-dependent behavior deficits observed in BrPerk KO mice, it is reasonable to speculate that similar behavior deficits would be observed in mice with reduced phospho-eIF2α in brain, which could be caused by knockdown of other eIF2α kinases or phosphorylation site-mutation of eIF2α. The roles of PERK, GCN2, PKR and eIF2α in protein-synthesis dependent synaptic plasticity and cognition have been investigated in BrPerk KO, Gcn2−/−, Pkr−/−, and eIF2α+/s51A mice, and the phenotypes in synaptic plasticity and behavior are summarized in Table 5-1. BrPerk KO mice possess hippocampus-dependent normal long-term potentiation, and exhibit normal long-term memory in Morris water maze task. However, both Gcn2−/− and Pkr−/− mice exhibit lowered threshold for late-phase long-term potentiation and correspondingly show enhanced long-term memory under weak training in Morris water maze task. This inconsistency may be derived from GCN2 and PKR’s functions independent of eIF2α, then eIF2α+/s51A mice are expected to display similar phenotypes compared to BrPerk KO mice. However, there is little overlap in synaptic plasticity and behavior phenotypes between the two strains. For example, at the level of synaptic plasticity, BrPerk KO mice exhibit normal long-term potentiation, but eIF2α+/s51A mice display a lower threshold for long-term potentiation. At the behavioral level, while BrPerk KO mice show normal long-term memory but impaired memory flexibility, which includes impaired extinction memory, eIF2α+/s51A mice exhibit enhanced long-term memory and extinction memory. The inconsistent phenotypes observed between BrPerk KO and eIF2α+/s51A mice could be due to the temporal difference in genetic modifications, since the knockdown of PERK occurs postnatally in BrPerk KO mice, whereas in eIF2α+/s51A mice, the serine 51 site-
mutation is constitutive. However, it is more likely that PERK imparts additional regulation of cognitive function that is eIF2α-independent.

The identification of PERK as a regulator of Gq protein-coupled intracellular Ca\textsuperscript{2+} dynamics in primary cortical neurons illustrates a novel role of PERK in central nervous system that is eIF2α-independent. Moreover, the findings of PERK as a modulator of Ca\textsuperscript{2+}-dependent working memory, and its role in Alzheimer’s disease progression possibly via Ca\textsuperscript{2+} dynamics regulation suggest that PERK’s regulation of Gq protein-coupled intracellular Ca\textsuperscript{2+} signaling may play important physiological roles in the central nervous system.

Fig. 5-1 Mammalian eIF2α kinases and their activation by different cellular stressors

(Figure generated by Dr. Douglas R. Cavener)
<table>
<thead>
<tr>
<th>Gene and KO region</th>
<th>Synaptic plasticity</th>
<th>Behavior deficits</th>
<th>Proposed pathway and Ref.</th>
</tr>
</thead>
</table>
| Forebrain specific *Perk* KO | • Normal basal synaptic transmission  
• Normal E-LTP and L-LTP  
• Enhanced mGluR-LTD  
• Normal NMDAR-LTD | • Impaired pre-pulse inhibition  
• Enhanced vertical locomotor activity  
• Enhanced preference for the familiar object in the novel object recognition task  
• Impaired spatial reversal learning in Y water maze reversal task  
• Impaired extinction memory | PERK/eIF2α/ATF4  
(20,21) |
| Global *Gcn2* KO | • Lowered threshold for L-LTP  
• Not sustained L-LTP  
• LTD unaltered | • Impaired contextual fear conditioning but intact auditory fear conditioning  
• Enhanced performance in MWM under weak training protocol; impaired performance in MWM under strong training protocol | GCN2/eIF2α/ATF4  
(23) |
| Global *Pkr* KO | • Neuronal hyperactivity  
• Reduced inhibitory synaptic transmission  
• Lowered threshold for L-LTP  
• LTP unaltered | • Enhanced performance in MWM under weak training protocol  
• Enhanced contextual, auditory fear conditioning and contextual fear extinction | PKR/IFN-γ  
(24) |
| Global eIF2α^{s51A} | • Lowered threshold for L-LTP  
• LTP unaltered | • Enhanced performance in MWM under weak and strong protocols  
• Enhanced performance in both contextual and auditory fear conditioning  
• Enhanced extinction memory | (22) |

**Table 5-1 Summary of synaptic plasticity and behavioral phenotypes observed in *BrPerk* KO, *Gcn2* KO, *Pkr* KO and eIF2α^{s51A} mice**

**Abbreviations**

E-LTP: early-phase long-term potentiation  
L-LTP: late-phase long-term potentiation  
LTD: long-term depression  
MWM: Morris water maze
The possible mechanisms underlying PERK’s regulation of Ca\(^{2+}\) dynamics in primary cortical neurons

The identification of PERK’s role in regulating \(G_q\)-protein-coupled Ca\(^{2+}\) dynamics, specifically as being a negative regulator of IP\(_3\)R-dependent ER Ca\(^{2+}\) release and a positive regulator of receptor-operated Ca\(^{2+}\) entry (ROCE), leads to another question: how then does PERK regulate these processes? I speculate that PERK’s regulation of IP\(_3\)R-dependent ER Ca\(^{2+}\) release is mediated by the regulation of calcineurin, a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase that negatively regulates IP\(_3\)R (140,141). PERK and calcineurin have been shown to physically interact, which impacts their individual enzymatic activities (142). Moreover, in pancreatic insulin-secreting β-cells, PERK positively regulates calcineurin activity and calcineurin is a downstream mediator of PERK’s action on Ca\(^{2+}\)-dependent insulin secretion (17). These results led to the speculation that PERK might negatively regulate IP\(_3\)R activity through its positive regulation of calcineurin in cortical neurons.

For the \(G_q/PLC\)-coupled ROCE, the family of TRPC channels form nonselective receptor-operated Ca\(^{2+}\) channels (143). A number of intracellular signals generated downstream of \(G_q/PLC\) pathway have been shown to activate TRPCs, which includes increased PLC activity, generation of DAG, and internal Ca\(^{2+}\) store depletion (143). The observation that acute PERK inhibition impairs receptor-operated, but not store-operated Ca\(^{2+}\) entry (SOCE) in primary cortical neurons suggests that PERK’s regulation of ROCE might be independent of internal Ca\(^{2+}\) release. So far diacylglycerol (DAG) is the only identified second messenger that directly gates TRPC activity. DAG has been shown to activate TRPC3/6/7 channels while inhibiting TRPC5 channel activity (144-146). Since PERK has an intrinsic DAG kinase activity of converting DAG into phosphatidic acid (147), it is possible that PERK regulates TRPCs activity by modulating intramembrane DAG levels. In addition, it is also possible that PERK regulates ROCE via its interaction with calcineurin. Growing evidence suggests calcineurin is involved in ROCE
regulation. In neuronal PC12D cells, it has been shown that calcineurin is recruited to the TRPC6 centered multiprotein complex upon M1 mAChR activation. This recruitment is essential for TRPC6 dephosphorylation and M1 mAChR dissociation from the complex, suggesting that calcineurin might play a regulatory role in receptor-operated TRPC6 activity (148).

Fig. 5-2 Proposed signaling pathways for PERK’s regulation of G<sub>q</sub> protein-coupled Ca<sup>2+</sup> dynamics in primary cortical neurons

Stimulation of G<sub>q</sub> protein-coupled receptor induces IP<sub>3</sub>R-mediated ER Ca<sup>2+</sup> release followed by the receptor-operated extracellular Ca<sup>2+</sup> entry. PERK may act as a negative regulator of IP<sub>3</sub>R via its positive regulation of calcineurin, a known IP<sub>3</sub>R suppressor. PERK’s positive regulation of ROC may be mediated through its DAG kinase activity, or calcineurin, which has been shown to modulate the activity of some TRPCs.
**PERK may modulate working memory by regulating G_q protein-coupled Ca^{2+} dynamics**

G_q protein-coupled intracellular signaling pathway plays a prominent role in working memory. Reducing G_q pathway activity by depletion of neurotransmitters specific to G_q protein-coupled receptors (34), blockage of G_q protein-coupled receptors (35-38), or direct inhibition of G_q/phospholipase C (PLC) activity by infusion of PLC inhibitor (39) all lead to impaired working memory in animals. Activation of G_q/PLC generates several intracellular second messengers, and among them, the substantial increase of intracellular Ca^{2+} ([Ca^{2+}]_{i}) level has been suggested to regulate working memory in several ways. Firstly, the induced [Ca^{2+}]_{i} rise is known to activate several Ca^{2+}-dependent protein enzymes, including phosphatase calcineurin, kinase CaMKII and PKC, all of which have been shown to regulate working memory capacity(111). Secondly, the Ca^{2+}-activated nonselective cationic current (I_{CAN}), which is identified as the ionic mechanism underlying neuronal persistent activity (125), is G protein- and Ca^{2+}-dependent(100). Finally, G_q protein-coupled [Ca^{2+}]_{i} rise has direct effects on intrinsic neuronal excitability. It has been demonstrated that pharmacological activation of mGluR 1 in pyramidal neurons triggers a biphasic electrical response: SK channel-dependent neuronal hyperpolarization followed by TRPC-dependent neuronal depolarization, and the amplitude of both are regulated by the extent of [Ca^{2+}]_{i} rise (166,183).

Considering the importance of G_q protein-coupled Ca^{2+} dynamics in working memory, I speculate that PERK regulates working memory by modulating G_q protein-coupled [Ca^{2+}]_{i} signaling in pyramidal neurons. However, current data is not sufficient to establish the causal relationship. To fully establish this causal relationship, a series of experiments need to be performed. First of all, it is important to identify PERK’s effector that mediates G_q protein-coupled extracellular Ca^{2+} influx. Since the family of TRPC channels form nonselective receptor-operated Ca^{2+} channels (143) underlying ROCE, they are good candidates. HEK293 cells will serve as a good system for the study, because they have endogenous expression of G_q/PLC but do
not express TRPCs (159). The investigation of PERK’s potential regulatory effects on TRPCs-mediating Ca\(^{2+}\) influx can be carried out in HEK293 cells overexpressing individual TRPC protein. To further determine PERK’s effector, ablation studies are required to investigate if genetic and/or pharmacological suppression of any potential candidate protein can mimic the impaired G\(_q\) protein-coupled extracellular Ca\(^{2+}\) influx phenotype observed in PERK-inhibited pyramidal neurons.

If PERK’s effector that mediates G\(_q\) protein-coupled extracellular Ca\(^{2+}\) influx can be successfully identified, then it will allow the further investigation of whether PERK’s regulation of G\(_q\) protein-coupled Ca\(^{2+}\) dynamics is directly connected to its modulation of working memory. The connection can be established if overexpression of the effector in pyramidal neurons can rescue the impaired working memory in BrPerk KO mice.

Persistent neuronal firing is considered as the neuronal correlate of working memory, and thus it is speculated that metabotropic glutamate/muscarinic acetylcholine receptor-mediated persistent neuronal firing is impaired in BrPerk KO mice. It is necessary to investigate if persistent neuronal firing is impaired in BrPerk KO mice, and also whether overexpression of PERK’s effector can rescue the impairment. The rescue of deficits at the electrophysiological level will certainly add strength to the statement that PERK’s regulation of G\(_q\) protein-coupled Ca\(^{2+}\) dynamics and its modulation of working memory are directly connected.

However, it is of note that even if the PERK’s effector is identified and a direct connection is established, it is still not clear if the working memory deficits in BrPerk KO mice is due to reduced G\(_q\) protein-coupled intracellular Ca\(^{2+}\) rise, the impaired TRPC mediated I\(_{\text{CAN}}\), or both. TRPCs have been suggested to function as the Ca\(^{2+}\) channels underlying ROCE, and they are also identified as the ionic channels mediating I\(_{\text{CAN}}\), which is essential for neuronal persistent firing underlying working memory. It will be necessary to investigate the candidate TRPC’s effect on
G_{\text{q}} protein-coupled intracellular Ca^{2+} dynamics and I_{\text{CAN}} in vivo, respectively, to delineate which factor plays a major role in PERK’s regulation of working memory.
The paradox of impaired working memory in BrPerk KO and 5XFAD mice, but alleviated working memory deficits in 5XFAD BrPerk KO mice

In my thesis, I have shown that PERK is required for working memory. It has also been demonstrated that in 5XFAD transgenic mice, working memory is significantly impaired (170). Therefore it is reasonable to expect a more significant working memory impairment in 5XFAD mice with Perk specifically knocked out in the forebrain (5xFAD BrPerk KO). However, the result is opposite: 5xFAD BrPerk KO mice possess a better working memory compared to BrPerk KO and 5XFAD mice. How can we explain this paradox?

Understanding the mechanisms underlying the working memory deficits in BrPerk KO mice, and 5XFAD mice individually may help to solve the puzzle. As mentioned above, the working memory deficits in BrPerk KO mice could be due to impaired Gq protein-coupled \([\text{Ca}^{2+}]_i\) rise. On the other hand, primary neurons from 5XFAD mice exhibit exaggerated Gq protein-coupled \([\text{Ca}^{2+}]_i\) rise. One explanation for the paradox is that maintaining \([\text{Ca}^{2+}]_i\) within a certain range is critical for animal’s working memory, and thus abnormally high or low \([\text{Ca}^{2+}]_i\) is detrimental. In fact it has been proposed that there is an inverted U relationship between intracellular \(\text{Ca}^{2+}\) level and working memory (111). Several \(\text{Ca}^{2+}\)-dependent protein enzymes, including phosphatase calcineurin, kinase CaMKII and PKC, have been shown to regulate working memory capacity (111). At relatively low \([\text{Ca}^{2+}]_i\) level, calcineurin is activated which facilitates working memory. When \([\text{Ca}^{2+}]_i\) rises to a certain level that is sufficient to activate protein kinases, CaMKII and PKC are activated, which inhibit working memory by antagonizing substrate dephosphorylation (111). Thus, it is very likely that in 5xFAD BrPerk KO mice, reduced PERK expression in 5xFAD mice forebrain corrects its exaggerated Gq protein-coupled \([\text{Ca}^{2+}]_i\) rise, and thus rescues the working memory impairment in 5XFAD mice.
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7-methyl-5-{1-[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine (GSK2606414), a potent and selective first-in-class inhibitor of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). *Journal of medicinal chemistry* 55, 7193-7207


Appendix A

PTZ-induced seizure response in BrPerk KO mice

Wolcott-Rallison Syndrome is a rare genetic disease caused by autosomal-recessive Perk mutation. In addition to the common clinical manifestations including permanent neonatal diabetes, osteopenia and growth retardation, some of the WRS patients are reported to develop spontaneous seizure(19,198), suggesting the possible involvement of PERK in epilepsy genesis. While no spontaneous seizure was observed in the BrPerk KO mice colony where the mice were kept at a relative young age (usually within 6 month old), occurrence of spontaneous seizure associated with BrPerk KO mice was observed in the 5XFAD BrPerk KO mice colony (Table S-1), where mice were kept till a much older age (around 1.5 years old). Although Fisher’s exact test suggested that there is no significant difference in spontaneous seizure occurrence between BrPerk KO mice and their wild-type littermates (p=0.099), it is possible that BrPerk KO mice might have a lower seizure threshold to the convulsant drug.

PTZ is an antagonist of GABA_\text{A} receptor(199), whose administration will cause seizure response due to increased neuron network excitability. PTZ-induced seizure behavior is dose-dependent, and it is classified into six stages in this study, as shown in Table S-2. BrPerk KO mice and their wild-type littermates were intraperitoneally administered with PTZ at the dosage of 50mg/kg for male and 42.5mg/kg for female, respectively, and the epileptiform behavior was recorded and analyzed. No difference was observed in PTZ-induced seizure behavior between genotypes in either gender (Fig. S1).

To gauge the severity of seizure response at the molecular level, the expression of c Fos, which is strongly induced in dentate gyrus after generalized tonic-clonic seizure(200), was used as a read-out of seizure severity. Dentate gyrus was isolated from male BrPerk KO mice and their
wild-type littermates 1 hour after PTZ injection. As expected, PTZ injection significantly induced c Fos expression in dentate gyrus compared to saline treatment (Fig. S2). Moreover, c Fos was induced to a higher fold in BrPerk KO mice compared to their wild-type littermates, suggesting that BrPerk KO mice are more sensitive to PTZ treatment (Fig. S2).

The aberrant hippocampal neurogenesis has been reported to contribute to epilepsy development due to increased excitatory activity(201). Since PERK has been shown to be involved in cell proliferation control(13), I explored if PERK is involved in neurogenesis, which will affect the epilepsy development. Hippocampal neurogenesis was studied by bromodeoxyuridine (BrdU) incorporation assay. No difference was observed in neurogenesis rate or survival rate of proliferating neuronal precursor cells between BrPerk KO and wild-type mice (Fig. S3A). In addition, preliminary data suggested that there is no difference in ECT-induced neurogenesis between genotypes (Fig. S3B), though more replicates are needed for a solid conclusion.

In this study, motived by the observation of spontaneous seizure in aged BrPerk KO mice and the report of seizure occurrence in some of the Wolcott-Rallison Syndrome patients, I studied if PERK is involved in PTZ-induced seizure development. While no difference was observed in PTZ-induced epileptiform behaviors between BrPerk KO mice and their wild-type littermates, BrPerk KO mice exhibited a higher fold of c Fos induction in dentate gyrus after PTZ injection. The above results suggest that BrPerk KO mice might be more susceptible to PTZ treatment, though it does not result in a more severe epileptiform behavior.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of mice observed seizure at least once</th>
<th>Total number of mice</th>
<th>Percentage of seizure occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3</td>
<td>25</td>
<td>12.0%</td>
</tr>
<tr>
<td>BrPerk KO</td>
<td>9</td>
<td>33</td>
<td>27.3%</td>
</tr>
<tr>
<td>5XFAD</td>
<td>2</td>
<td>21</td>
<td>9.5%</td>
</tr>
</tbody>
</table>

Table S-1 Spontaneous seizure occurrence observed in 5XFAD BrPerk KO mice colony.
### Table S-2 PTZ induced seizure behavior at different stages.

<table>
<thead>
<tr>
<th>Five stages of seizure</th>
<th>Seizure phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1: Absence</td>
<td>No movement for at least 2 s, with abdomen in full contact with cage bottom.</td>
</tr>
<tr>
<td>Stage 2: Partial clonus</td>
<td>Brief clonus or control lost affecting part of the body, such as forelimb or hindlimb.</td>
</tr>
<tr>
<td>Stage 3: Myoclonus</td>
<td>Whole body jerk for once; sometimes with Straub tail phenotype.</td>
</tr>
<tr>
<td>Stage 4: Generalized clonus</td>
<td>A series of whole body jerk lasting for couple of seconds</td>
</tr>
<tr>
<td>Stage 5: Generalized clonic-tonic seizure</td>
<td>The maximal seizure type lasting for 10 s-60s. Exaggerated twitches of the limbs usually with hindlimb extension. The animal will roll and stretch with seizure spread, sometimes associated with death.</td>
</tr>
</tbody>
</table>

### Fig. S1 PTZ induced seizure behavior in *BrPerk KO* and wild-type mice.

A. Percentage of male mice showing seizure response at different stages after receiving PTZ i.p. injection at the dosage of 50mg/kg (n=7 for both genotypes).

B. Percentage of female mice showing seizure response at different stages after receiving PTZ i.p. injection at the dosage of 42.5mg/kg (n=9 for both genotypes).
Fig. S2 PTZ induced \( c \) Fos expression in dentate gyrus
A. PTZ treatment strongly induced dentate gyurs \( c \) Fos expression in both wild-type and \( BrPerk \) KO mice.  
B. \( BrPerk \) KO mice exhibited a higher fold of PTZ induced \( c \) Fos expression in dentate gyrus compared to their wild-type littermates.

Male mice were used in the experiment; PTZ dosage: 50mg/kg; \( n=4 \) for each group (per genotype per treatment); **, \( p<0.01 \); *, \( p<0.05 \); student’s t-Test.

Fig. S3 Adult hippocampal neurogenesis is not altered in \( BrPerk \) KO mice
A. Stereological quantification of BrdU positive cells in the dentate gyrus of \( BrPerk \) KO and wild-type mice 1 day and 1 month after BrdU injection. No difference was observed between genotypes. (\( n=4 \) for each genotype in one day group; \( n=5 \) for each genotype in one month group).  
B. Experiment strategy and stereological quantification of BrdU positive cells in the dentate gyurs of ECT treated mice (\( n=2 \) per genotype per treatment).
Appendix B

Functional study of PERK in lipid and glucose regulation in the mice model with high-fat diet treatment

A.

**Fig. S4. Glucose tolerance test in mixed background LiPerk KO mice fed on high-fat diet**

*LiPerk KO* mice and their wild-type littermates in mixed background were fed on high-fat diet (HFD) immediately after weaning (Male group: WT HFD n=5; WT LFD n=4; LiPerk KO HFD n=6; LiPerk KO LFD n=6; Female group: WT HFD n=6; WT LFD n=6; *LiPerk KO* HFD n=6; *LiPerk KO* LFD n=6).

A. Body weight in both genders during the course of HFD treatment. No difference was observed between genotypes.

B. Glucose tolerance test performed in both genders at the end of HFD treatment. No difference was observed between genotypes.
Fig. S5. Gluconeogenesis is not impaired in LiPerk KO mice in mixed background

Gluconeogenesis was measured by pyruvate injection in mice fasted for 24 hours. No difference was observed between genotypes in either gender (Male group: WT n=10; LiPerk KO n=8; Female group: WT n=9; LiPerk KO n=11).

Fig. S6 Councilman body in the liver of LiPerk KO mouse in mixed background

The existence of Councilman body was treatment independent as it was observed in both HFD and LFD treatments. (Liver Councilman body was found in 6 out of 12 LiPerk KO mouse examined, while none of the 7 WT mouse examined exhibited councilman body in their liver)
Fig. S7 TEM imaging of councilman body in the liver of mixed background LiPerk KO mice

Left panel: 1000 magnification

Right panel: 20,000 magnification
A. Body weight in both genders during the course of VHFD treatment. No difference was observed between genotypes.

B. Glucose tolerance test performed in both genders at the end of VHFD treatment. No difference was observed between genotypes.

**Fig. S8 Glucose tolerance test in B6 background LiPerk KO mice fed on very high-fat diet**

LiPerk KO mice and their wild-type littersmates in B6 background were fed on very high-fat diet (VHFD) at the age of 3 month old (Male group: WT VHFD n=6; WT LFD n=6; LiPerk KO VHFD n=6; LiPerk KO LFD n=6; Female group: WT VHFD n=6; WT VLFD n=6; LiPerk KO HFD n=6; LiPerk KO LFD n=6).

A. Body weight in both genders during the course of VHFD treatment. No difference was observed between genotypes.

B. Glucose tolerance test performed in both genders at the end of VHFD treatment. No difference was observed between genotypes.
Fig. S9 Glucose homeostasis in normal fed LiPerk KO mice in B6 background

A. Gluconeogenesis measured by pyruvate injection in mice fasted for 16 hours. The difference in blood glucose level after pyruvate injection was probably due to the blood glucose difference at time 0. The experiment was repeated for 3 times and no consistent results were obtained (n=5 for each genotype).

B. Blood glucose monitoring in LiPerk KO mice fasted for 48 hours (WT n=8; LiPerk KO n=7).

Fig. S10 ER stress markers were not induced in the liver of mice treated with VHFD

WT LFD n=5; WT VHFD n=3; LiPerk KO LFD n=4; LiPerk KO VHFD n=4
Appendix C

Supplementary figures for Chapter 3

Fig. S11 BrPerk KO mice in both genders are impaired in Y-maze spontaneous alternation

A. Male group (WT n=10, BrPerk KO n=15; * p<0.05, n.s. not significant, two-tailed student’s t-Test).

B. Female group (WT n=5, BrPerk KO n=7; ** p<0.01, n.s. not significant, two-tailed student’s t-Test).
Fig. S12 *BrPerk* KO mice in both genders are impaired in the delay match-to place working memory task

A. Male group (WT n=11, BrPerk KO n=11; **p<0.05, n.s. not significant, two-tailed student’s t-Test).

B. Female group (WT n=5, BrPerk KO n=9; *p<0.01, n.s. not significant, two-tailed student’s t-Test).
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Publications
Siying Zhu, Barbara C. McGrath, Yuting Bai, Xin Tang and Douglas R. Cavener. PERK regulates $G_q$ protein-coupled intracellular $Ca^{2+}$ dynamics in primary cortical neurons (under review)
Siying Zhu, Keely Henninger, Barbara C. McGrath and Douglas R. Cavener. PERK regulates working memory and protein synthesis-dependent memory flexibility (under review)

Conference
Poster: PERK EIF2AK3 regulates Alzheimer’s disease progression
Keystone Symposia: New Frontiers in Neurodegenerative Disease Research (J8)
Feb 3-Feb 8 2013, Santa Fe, New Mexico, USA

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