Brain-Specific Disruption of the eIF2α Kinase PERK Decreases ATF4 Expression and Impairs Behavioral Flexibility

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SUMMARY

Translational control depends on phosphorylation of eIF2α by PKR-like ER kinase (PERK). To examine the role of PERK in cognitive function, we selectively disrupted PERK expression in the adult mouse forebrain. In the prefrontal cortex (PFC) of PERK-deficient mice, eIF2α phosphorylation and ATF4 expression were diminished and were associated with enhanced behavioral perseveration, decreased prepulse inhibition, reduced fear extinction, and impaired behavioral flexibility. Treatment with the glycine transporter inhibitor SSR504734 normalized eIF2α phosphorylation, ATF4 expression, and behavioral flexibility in PERK-deficient mice. Moreover, the expression levels of PERK and ATF4 were reduced in the frontal cortex of human patients with schizophrenia. Together, our findings reveal that PERK plays a critical role in information processing and cognitive function and that modulation of eIF2α phosphorylation and ATF4 expression may represent an effective strategy for treating behavioral inflexibility associated with several neurological disorders such as schizophrenia.

INTRODUCTION

In mammals, long-lasting memory formation requires new mRNA and protein synthesis (Costa-Mattioli et al., 2009; Kandel, 2001; Kelleher et al., 2004; Richter and Klann, 2009). The translation of mRNAs is highly regulated at the level of initiation by numerous translational control molecules, including the translation initiation factor eIF2 (Sonenberg and Dever, 2004). During this rate-limiting step, eIF2 interacts with initiator tRNA and GTP to facilitate loading of the ternary complex onto the 40S ribosomal subunit, which is essential for new rounds of protein synthesis (Harding et al., 1999). Although phosphorylation of the α subunit of eIF2 results in general inhibition of translation, it paradoxically stimulates the translation of several upstream open reading frame (uORF)-containing mRNAs, including the transcriptional modulator activating transcription factor 4 (ATF4) (Vattem and Wek, 2004). Notably, ATF4 and its homologs play critical roles as repressors of cAMP response element-binding protein (CREB)-mediated synaptic plasticity and memory in diverse phyla (Abel et al., 1998; Bartsch et al., 1995; Chen et al., 2003). Thus, eIF2α phosphorylation controls two distinct processes that are essential for the consolidation of new memories: de novo general protein synthesis, and gene-specific translation of ATF4 mRNA. Furthermore, reduction of eIF2α phosphorylation in mice lacking the eIF2α kinase GCN2 and in heterozygous knockin mice with a mutation on serine 51 of eIF2α results in a lowered threshold for inducing long-lasting late-phase long-term potentiation (L-LTP) and consolidation of long-term memory (Costa-Mattioli et al., 2005, 2007). Moreover, mice harboring a deletion of the double-stranded (ds) RNA-activated protein kinase (PKR) show a similar decrease in threshold for inducing L-LTP and consolidation of long-term memory (Zhu et al., 2011). Conversely, increased eIF2α phosphorylation in transgenic mice overexpressing PKR causes increased expression of ATF4, impaired L-LTP, and memory deficits (Jiang et al., 2010). Collectively, these findings suggest that the proper regulation of eIF2α phosphorylation is required for normal synaptic plasticity and memory.

The PKR-like ER kinase (PERK) was initially characterized as a ubiquitously expressed ER-localized protein kinase that phosphorylates eIF2α to rapidly reduce protein synthesis during ER stress (Harding et al., 2000). Global inactivation of PERK in mice results in multiple developmental defects, including early-onset diabetes, growth retardation, skeletal abnormalities, and pancreatic atrophy (Harding et al., 2001; Zhang et al., 2002). Consistent with this, mutations in the human PERK gene (EIF2AK3) causes Wolcott-Rallison syndrome (WRS), a rare autosomal recessive disorder characterized by permanent neonatal diabetes, multiple epiphysseal dysplasia, liver dysfunction, and pancreas insufficiency (Delépine et al., 2000; Julien and Nicolin, 2010; Rubio-Cabezas et al., 2009). In some cases of WRS, clinical features associated with mental retardation develop in patients (Delépine et al., 2000; Reis et al., 2011; Senée et al., 2004; Thornton et al., 1997). Moreover, cell lines lacking either tuberous sclerosis complex (TSC) 1 or TSC2, the TSC proteins,
Moreover, treatment with the glycine transporter inhibitor 
with PERK-deficient mice revealed multiple phenotypes con-
expression in the prefrontal cortex (PFC). Behavioral studies 
identifying of cellular processes requiring precise translational control, we 
However, the precise role of PERK 
expression, and restored behavioral flexibility in the PERK 

discussion of PERK in hippocampal area CA1. 
Bottom view shows whole brain 
Comparison of PERK levels in cKO mice are expressed at similar 
PERK levels in KO mice are also detected at levels similar to those in WT mice in tissues outside the 
(F) Immunohistochemical detection of phosphorylated eIF2α in layer II/III of the mPFC showing decreased expression in PERK cKO (right panel) compared to WT (left panel). Scale bar, 200 μm. 
(ATF4) Representative western blot showing decreased ATF4 expression in the PFC of PERK 
expression from the western blot 

despite the reduction of PERK in these regions, Nissl-stained sections indicated that mutant mice did not show gross alterations in brain 
the presence of PERK levels to those in WT mice in tissues outside the 
(Figure 1C). Notably, the PFC region harbored the greatest ablation 
(Figure 1D and S1B). To confirm that 

and both mouse and human tumors from TSC model mice and 
patients with TSC, respectively, exhibit activation of the PERK- 
elF2α axis (Ozcan et al., 2008). Together, these analyses suggest 
a crucial role for PERK in normal cellular homeostasis, growth, 
viability, and development. However, the precise role of PERK 
in memory formation and cognition has not been explored. 

Given that PERK functions as a critical modulator of a number of 
cellular processes requiring precise translational control, we 
explored the role of PERK in regulating protein synthesis-depen-
dent cognitive function in the mammalian brain. Here, we show that 
a targeted disruption of PERK in the mouse forebrain results in 
significant reduction of phosphorylated eIF2α and ATF4 
expression in the prefrontal cortex (PFC). Behavioral studies with 
PERK-deficient mice revealed multiple phenotypes consistent with impaired cognition and information processing. 
Moreover, treatment with the glycine transporter inhibitor 
SSR504734 normalized eIF2α phosphorylation and ATF4 
expression, and restored behavioral flexibility in the PERK 
mutant mice. Furthermore, the molecular deficits observed in the mutant mice were recapitulated in the frontal cortex of patients with schizophrenia, but not in patients with bipolar disorder. These findings expand the repertoire of PERK as a regulator of translational control to include cognitive processes in the adult brain and highlight the modulation of eIF2α phosphorylation as a key site for influencing translational control underlying the pathophysiology of cognitive deficits commonly associated with neurological disorders.

RESULTS

PERK Regulates eIF2α Phosphorylation and ATF4 Expression in the Brain

To evaluate the precise role of PERK in translation-dependent forms of memory in the mammalian brain, we generated mice containing a forebrain-specific CamkIIα-Cre transgene (Tsien et al., 1996) and conditional alleles of Perk (Zhang et al., 2002) (PerkloxP; Figure 1A). The onset of Cre activity occurs at approximately 2–3 weeks after birth (Tsien et al., 1996), permitting normal brain development in the presence of Perk. The CamkIIα-Cre; PerkloxP/loxP conditional knockout mice (PERK cKO hereafter) contain an ablation of PERK that is predominantly limited to the forebrain. The presence of the conditional PerkloxP allele and Cre transgene was determined using PCR-specific primers (Figure 1A). Western blot assays confirmed efficient Cre-mediated deletion of PerkloxP in several areas of the forebrain isolated from PERK cKO mice, including the hippocampus and the PFC (Figures 1B and 1D; Figure S1A). Despite the reduction of PERK in these regions, Nissl-stained sections indicated that mutant mice did not show gross alterations in brain morphology compared to their wild-type (WT) littermates (Figure 1C). Notably, the PFC region harbored the greatest ablation of PERK, which was correlated with a significant reduction of phosphorylated eIF2α (Figures 1D and S1B). To confirm that the genetic disruption was specifically restricted to the forebrain, we examined PERK levels in the cerebellum, muscle, and

Figure 1. Forebrain-Specific Deletion of Perk Suppresses eIF2α Phosphorylation and ATF4 Expression in the PFC

(A) Schematic for the conditional allele for Perk. In the top view black triangles represent two loxP sites flanking exons 7–9 (E7–E9) of the Perk gene, and gray boxes represent primers (568F and 568R1) designed to detect recombination. Middle view shows that upon recombination with CamkIIα-Cre, Perk is deleted in a forebrain-specific manner. Bottom view is PCR identification of alleles of PerkloxP and CamkIIα-driven Cre.

(B) Representative western blot analysis confirming disruption of PERK in hippocampal area CA1. 
(C) Top view illustrates Nissl-stained coronal sections of PFC. Bottom view shows whole brain 

(D) Representative western blot showing CamkIIα-driven Cre disruption of PERK in other 
regions of the brain from WT and PERK cKO mice. 
(E) Representative western blot showing that PERK levels in KO mice are expressed at similar 
levels to those in WT mice in tissues outside the central nervous system. 
(F) Immunohistochemical detection of phosphorylated eIF2α in layer II/III of the mPFC showing decreased expression in PERK cKO (right panel) compared to WT (left panel). Scale bar, 200 μm. 
(G) Representative western blot showing decreased ATF4 expression in the PFC of PERK 
cKO mice compared to their WT littermates. Quantification of PERK, eIF2α phosphorylation, and ATF4 expression from the western blot analyses is shown in Figure S1.
Peripheral organs and observed that PERK expression was indistinguishable between mutant and WT mice (Figures 1D, 1E, and S1A). Moreover, examination of layer II/III of the medial PFC (mPFC) by immunohistochemistry (IHC) showed nearly a complete absence of phosphorylated eIF2α staining in PERK cKO mice (Figure 1F). Although robust phosphorylation of eIF2α can inhibit global protein synthesis, gene-specific translation of ATF4 is most sensitive to eIF2α phosphorylation (Vattem and Wek, 2004). We observed that a decrease in eIF2α phosphorylation was associated with reduced ATF4 expression (Figures 1G and S1C) in the PFC of PERK cKO mice. However, we found that the decrease in eIF2α phosphorylation had a minimal effect on global translation using a puromycin-based assay adapted from the SUNSET technique that labels newly synthesized proteins (Schmidt et al., 2009) (Figure 2A) and polysome-profiling analysis from sucrose gradients (Figure 2B). Thus, these findings indicate that a forebrain-specific disruption of PERK results in reduced eIF2α phosphorylation and ATF4 expression in the PFC.

**PERK cKO Mice Show Impaired Information Processing and Behavioral Flexibility**

To examine whether the disruption of PERK resulted in altered cognitive function, we tested male PERK cKO mice in a series of behavioral paradigms. We examined the PERK cKO mice for prepulse inhibition (PPI) of the startle response, a reliable and robust measure of sensorimotor gating and information-processing abilities (Swerdlow et al., 2008). PPI involves a short, low-intensity acoustic stimulus (prepulse) that inhibits the reaction of an organism to a startling stimulus (Swerdlow et al., 2008). The PERK cKO mice showed a deficit in PPI of the auditory startle response compared to their WT littermates (Figure 3A). No difference between genotypes was observed with the presentation of the startling stimulus alone, suggesting a normal baseline startle response in PERK cKO mice (data not shown). PERK cKO mice display significantly enhanced vertical locomotor activity in the open field arena (Figure S2A). However, we observed no difference between genotypes for the distance traveled, time spent in the center zone of the open field, and in the closed arms versus the aversive open arms of the elevated plus maze (Figures S2B–S2D), suggesting normal anxiety levels in PERK cKO mice. These findings indicate that disruption of PERK-directed translational control results in deficits in information processing and enhanced vertical activity.

To further examine the role of PERK in memory and cognitive function, we subjected adult PERK cKO mice to behavioral tasks that require precise cognitive control. First, we found that PERK cKO mice exhibit enhanced preference for the familiar object in the novel object recognition task (Figure 3B). Next, we determined that PERK cKO mice and their WT counterparts were able to use spatial cues to learn the position of a hidden platform (Figure 3C) in the Morris water maze (MWM), a hippocampus-dependent water escape task (Morris, 1984). During probe trials both genotypes showed similar preferences for the target quadrant of the pool that previously contained the platform (Figure S3A), suggesting that PERK cKO mice have normal spatial reference memory. To specifically examine whether PERK is involved in spatial reversal learning in the MWM, we switched the location of the hidden platform to the opposite quadrant and challenged the ability of the mice to learn the new platform position. Notably, on the second day of reversal training, the WT mice exhibited normal spatial reversal learning (Figure 3C; Movie S1), but the PERK cKO mice displayed perseveration for the originally learned platform position (Figure 3C; Movie S2). However, these perseverative behaviors were not associated with repetitive or obsessive-compulsive-like behavior because the PERK cKO mice displayed normal marble burying and grooming behavior (Figures S2E and S2F). PERK cKO mice also spent more time in the previous training quadrant but exhibited normal escape latency during the visible platform task (Figures S3B and S3C). Taken together, these results indicate that a forebrain-specific disruption of PERK causes enhanced perseverative behaviors and impaired spatial reversal learning.

As a further test of our hypothesis that PERK regulates memory and cognitive flexibility, PERK cKO mice were trained in a Y-water maze reversal task. Briefly, mice were trained to locate an escape platform in one arm of a water-based Y maze. After 24 hr, the escape platform was switched to the opposing arm, and the behavioral flexibility of the mice to learn the new escape location was measured. The number of trials to criterion (nine out of ten correct) was achieved at trial 30 by all WT mice (Figure 4A; Movie S3). In contrast, 12 out of 13 mutant mice persistently swam toward the previously trained arm.
arm choice, exhibiting severe behavioral inflexibility (Figure 4A). A retraining paradigm was employed to allow these mice to make three consecutive correct choices (Figure 4A). During retraining a physical wall was used to block the incorrect arm; however, PERK cKO mice continued to swim toward the incorrect path and into the wall (Movie S4). The PERK cKO mice required an additional 28 trials to meet this criterion (Figure S3D). Upon retraining, PERK cKO mice showed comparable rates of reversal learning as their WT counterparts (Figure 4A). Interestingly, PERK cKO mice exhibited normal behavioral flexibility if the escape platform was switched to the adjacent arm immediately after training on the first day (Figure 4B). This finding suggests that the behavioral inflexibility exhibited by PERK cKO mice requires memory consolidation, a protein synthesis-dependent process (McGaugh, 2000). PERK cKO mice exhibited normal motor ability on the rotarod task, suggesting that the disruption of PERK does not affect cerebellar-dependent motor functions (Figure S4). Together, these findings reveal that a postdevelopmental disturbance of PERK is sufficient to trigger impaired behavioral flexibility.

The behavioral results described above suggest that eIF2α phosphorylation is normally altered during reversal learning. To test this hypothesis and to determine whether the removal of PERK has any effect on learning-dependent changes in eIF2α phosphorylation, PERK cKO mice and their WT littermates were subjected to the Y-water maze reversal task and frontal cortices from both genotypes were harvested and assayed for phosphorylated eIF2α 30 min following training for reversal learning. We found that WT mice exhibited decreased eIF2α phosphorylation after reversal learning (Figure 4C). In contrast, although basal phosphorylation of eIF2α was reduced, there was no further reduction in eIF2α phosphorylation in the PERK cKO mice (Figure 4C). Furthermore, we found that mice lacking the eIF2α kinase GCN2 exhibited normal reversal learning (Figure S5), suggesting that behavioral flexibility is regulated specifically by a pool of eIF2α that is normally phosphorylated by PERK. Taken together, these findings suggest that reversal learning normally decreases eIF2α phosphorylation and that PERK phosphorylates a specific pool of eIF2α to regulate behavioral flexibility.

Flexible behavior also is afforded by proper extinction, an active, protein synthesis-dependent learning process driven by the mPFC (Santini et al., 2004), which triggers the formation of a newly updated memory that inhibits the initial memory (Sotres-Bayon et al., 2004). In light of our findings suggesting that PERK regulates behavioral flexibility, we next determined whether fear extinction was compromised by the absence of PERK. Interestingly, we found that PERK cKO mice have

Figure 3. PERK cKO Mice Display Reduced PPI and Enhanced Behavioral Perseveration in Novel Object Recognition and MWM Tasks

(A) PERK cKO mice exhibit impaired PPI of the acoustic startle reflex across varying prepulse intensities: 74, 78, 82, 86, and 90 dB. WT, n = 11; cKO, n = 9 (**p < 0.05, two-way repeated-measure ANOVA followed by Tukey’s post hoc test).

(B) PERK cKO mice display enhanced perseveration for the familiar object in the novel object recognition task. WT, n = 6; cKO, n = 14 (***p < 0.001, two-way repeated-measures ANOVA).

(C) Left view illustrates escape latency across 5 days of MWM reference platform task and shows that PERK cKO mice acquired the spatial hidden platform task similarly to WT controls. Right view shows that PERK cKO mice exhibit higher number of previous-day platform position crossing during day 2 reversal phase of task compared to controls. WT, n = 12; cKO, n = 9 (**p < 0.05, two-way repeated-measures ANOVA, followed by Tukey’s post hoc test).

See also Figure S3 and Movies S1 and S2.
impaired fear extinction memory compared to their WT littermates (Figure 4D). Collectively, these findings suggest that PERK-directed translation regulates extinction learning. Moreover, disruption of PERK leads to profound behavioral symptoms associated with deficits in information processing and cognitive function.
NMDAR Hypofunction Modulates eIF2α Phosphorylation in the PFC

Compelling evidence suggesting a role for N-methyl-D-aspartate receptor (NMDAR) hypofunction underlying the pathophysiology of cognitive impairments stems from findings that NMDAR blockers such as PCP, ketamine, and MK-801 induce impaired working memory, behavioral flexibility, reversal learning, and extinction in mice and humans (Abdul-Monim et al., 2006; Dix et al., 2010; Javitt and Zukin, 1991; Krystal et al., 1994). Because the PERK-deficient mice display multiple cognitive impairments associated with NMDAR hypofunction, we proceeded to record NMDAR-mediated excitatory currents in layer II of the mPFC of PERK cKO mice and their WT littermates. However, we found that the NMDA-to-AMPA ratio was indistinguishable between PERK cKO mice and their WT littermates (Figures 5A and 5B), suggesting that NMDAR function is normal in the PERK cKO mice. We then proceeded to determine whether NMDAR hypofunction could cause abnormal PERK-directed translation by determining whether administration of MK-801 in WT mice could mimic the decreased eIF2α phosphorylation observed in the PERK cKO mice. WT mice received a single dose (acute) or 15 daily injections (chronic) of saline or MK-801 (0.2 mg/kg for acute; 0.1 mg/kg for chronic) via intraperitoneal (i.p.) injections. Frontal cortices from saline- and MK-801-treated WT mice were harvested and assayed for phosphorylated eIF2α. We found that acute MK-801 treatment enhanced eIF2α phosphorylation (Figure 5C), whereas chronic MK-801 treatment decreased eIF2α phosphorylation (Figure 5D), suggesting that the time course of NMDAR hypofunction bidirectionally regulates eIF2α phosphorylation. Consistent with this idea, longer-term increases in eIF2α phosphorylation elicit protein phosphatase 1/GADD34-directed feedback dephosphorylation of this translation factor (Marciniak et al., 2004). These data suggest that NMDAR hypofunction alters eIF2α phosphorylation in the PFC.

SSR504734 Restores Cognitive Function and PERK-Regulated Translation

One therapeutic strategy for the treatment of impaired behavioral flexibility involves the enhancement of NMDAR function by increasing synaptic glycine, an NMDAR coactivator, by blocking glycine reuptake via inhibition of glycine transporter-1 (GlyT1) (Javitt, 2008). Several previous studies have shown that the GlyT1 inhibitor SSR504734 (Depoortere et al., 2005) improves behavioral flexibility, reversal learning, and overall cognitive function (Black et al., 2009; Singer et al., 2009). To evaluate whether SSR504734 could rescue the cognitive impairments exhibited by the PERK cKO mice, the Y-water maze reversal task was revisited to test WT and PERK cKO mice following chronic administration of SSR504734 (20 mg/kg; 21 days) by i.p. injection. Remarkably, following chronic treatment with SSR504734, the PERK cKO mice displayed normal reversal learning when compared to their WT littermates (Figures 6A and 6B; Movies S5 and S6). However, we found that chronic treatment with SSR504734 did not reverse either the enhanced vertical activity (Figure S6A) or the sensorimotor-gating impairment (Figure S6B) displayed by the PERK cKO mice. These data demonstrate that chronic SSR504734 administration can uniquely enhance cognitive ability and restore behavioral flexibility in the PERK cKO mice.

To determine whether chronic SSR504734 treatment also could rescue aberrant PERK-directed translation, frontal cortices from SSR504734-treated WT and PERK mutant mice were analyzed for total PERK, phosphorylated eIF2α, and ATF4 expression levels by western blotting. We found that PERK cKO mice treated with SSR504734 had significantly enhanced levels of phosphorylated eIF2α and increased ATF4 expression compared to untreated PERK mutants (Figures 6D and 6E). There was no significant difference in total PERK levels between the treated and untreated PERK cKO mice (Figure 6C). Collectively, these results indicate that chronic treatment with the GlyT1 inhibitor SSR504734 can rescue disrupted PERK-regulated translation in the PFC, presumably by either activating another eIF2α kinase such as GCN2 and/or PKR or by inhibiting...
the protein phosphatase 1/GADD34 complex that dephosphorylates eIF2α (Ma and Hendershot, 2003).

**Aberrant PERK-Regulated Translation in Frontal Cortex of Patients with Schizophrenia**

Upon discovering that mice with a targeted disruption of PERK exhibit multiple behavioral features consistent with cognitive impairments associated with several mental illnesses including schizophrenia, we hypothesized that aberrant PERK-directed translation might be involved in the pathophysiology of human schizophrenia. To test this idea, we obtained postmortem human schizophrenic frontal cortex samples and employed western blot analysis to examine the expression of PERK, phosphorylation of eIF2α, and expression of ATF4. All samples from the Stanley Medical Research Institute (SMRI) Array Collection were matched for age, sex, and race. A summary of the relevant patient demographic information is provided in Table S1.

Spearman correlation analysis showed no significant effect of age, brain pH, postmortem interval (PMI), or lifetime antipsychotic usage on the levels of PERK or ATF4 expression (Table S2). Brain pH and PMI were negatively correlated with eIF2α phosphorylation and total eIF2α levels. ANCOVA with pH and PMI as covariates failed to confirm the diagnostic differences initially observed with ANOVA for the eIF2α phosphorylation analysis (data not shown). Thus, we were unable to assess the regulation of eIF2α phosphorylation in postmortem schizophrenic samples. Frontal cortex samples from normal control patients displayed a considerable level of PERK, which was significantly reduced in the schizophrenia samples (Figure 7A). Moreover, ATF4 expression was markedly reduced in the schizophrenia brains compared to controls (Figure 7B). To investigate how broadly PERK-directed translation is involved in mental illnesses, we next examined PERK and ATF4 expression in the frontal cortex of patients with bipolar disorder compared to normal controls. In contrast to schizophrenic brains, PERK and ATF4 levels were unaltered in the frontal cortex of patients with bipolar disorder compared to normal control patients (Figures 7C and 7D). Taken together, these findings suggest that disturbances of PERK-regulated translation of ATF4 in the frontal cortex may specifically contribute to the pathophysiology of schizophrenia.
DISCUSSION

PERK is a key regulator of translation control pathways known to be involved in learning and memory formation. Previous studies have shown that global inactivation of PERK causes severe developmental defects, precluding a comprehensive analysis of its role in cellular and molecular processes underlying memory and cognitive function. In our studies we used the Cre-lox expression system to achieve a temporally and spatially restricted inactivation of Perk in the postnatal forebrain. Our findings reveal a previously unrecognized role of PERK-dependent translational regulation in cognitive function and provide molecular insights into the pathophysiology of cognitive impairment. We found that disruption of the PERK-eIF2α-ATF4 signaling pathway in the mouse PFC recapitulates multiple behavioral phenotypes consistent with impaired cognition and information processing. Furthermore, our studies identify the modulation of eIF2α phosphorylation as a potential molecular target for therapeutic agents designed to prevent cognitive symptoms associated with a wide variety of neurological and neuropsychiatric disorders.

Earlier studies provide strong evidence to suggest that gene-specific translation of ATF4 is critical for the modulation of hippocampus-dependent long-term synaptic potentiation and memory formation. In particular, transgenic mice expressing a dominant-negative inhibitor of C/EBP proteins were reported to have reduced ATF4 expression, which was associated with a facilitation of hippocampus-dependent long-term synaptic plasticity and memory formation (Chen et al., 2003). Moreover, a reduction of ATF4 expression in mice lacking the eIF2α kinase GCN2 and heterozygous knockin mice with a mutation on serine 51 of eIF2α results in a lowered threshold for eliciting long-lasting LTP and memory (Costa-Mattioli et al., 2005, 2007). Taken together, these studies suggest that regulation of ATF4 expression, mediated by GCN2-dependent phosphorylation of eIF2α, is required for activity-dependent, enduring changes in neuronal function. Expanding on these findings, we show that in the PFC of PERK-deficient mice, reduced eIF2α phosphorylation and ATF4 expression are associated with severe behavioral inflexibility. Thus, our data suggest that the regulation of ATF4 mRNA translation, mediated by PERK-directed phosphorylation of eIF2α, is critical for normal cognitive function. Because of its conserved role as a memory repressor in diverse phyla (Bartsch et al., 1995; Chen et al., 2003; Costa-Mattioli et al., 2005; Yin et al., 1994), we speculate that ATF4 normally acts to destabilize the initial memory trace, and consequently, when ATF4 expression is reduced in the PFC, the initial memory trace prevails despite changes in sensory and contextual information.

Our data suggest that reduction of PERK expression and ATF4 translation in the frontal cortex may specifically contribute to the pathophysiology of human schizophrenia (Figure 7). Interestingly, ATF4 has previously been shown to interact with Disrupted-In-Schizophrenia 1 (DISC1) (Chubb et al., 2008; Muir et al., 2008; Sawamura et al., 2008), a genetic risk factor for mental illnesses, including mood disorders and schizophrenia. In addition mutations in selective regions of the DISC1 gene result in a loss of interaction with ATF4 (Morris et al., 2003). Moreover, the ATF4 gene is positioned at chromosome 22q13, a hot spot for several schizophrenia-related susceptibility genes (Lewis et al., 2003; Mowry et al., 2004; Williams et al., 2003). Finally, polymorphisms in the ATF4 locus have been associated with schizophrenia in male patients (Qu et al., 2008). Thus, multiple studies support the notion that ATF4, whose translation is tightly regulated by eIF2α phosphorylation, is correlated with schizophrenia. It should be noted that in addition to the central regulator ATF4, other genes recently have been identified that are preferentially translated by eIF2α phosphorylation, which suggests that additional factors participate downstream of PERK to regulate cognitive function (Dey et al., 2010; Jackson et al., 2010).

Behavioral studies with the PERK mutant mice revealed multiple phenotypes consistent with cognitive and information-processing deficits, which have been implicated as core features...
of numerous mental illnesses, including schizophrenia, bipolar disorder, attention deficit/hyperactivity disorder (ADHD), and autism spectrum disorder (ASD) (Bora et al., 2009; Goos et al., 2009; Lesh et al., 2011; Solomon et al., 2009). In particular PERK-deficient mice exhibited reduced PPI (Figure 3A), a sensorimotor-gating mechanism that restricts processing of sensory information (Bitsios et al., 2006; Braff et al., 2001). In addition PERK cKO mice showed enhanced preference for the familiar object compared with the novel object in a hippocampus- and entorhinal cortex-dependent novel object recognition task (Figure 3B). One explanation for these results is that PERK is important for frontal and temporal cortex-dependent sensory information processing. Thus, in the absence of PERK, mice are unable to inhibit responses to sensory or cognitive information and have enhanced perseveration. Consistent with this notion, our results from the MWM and Y maze tests indicate that PERK cKO mice have reduced inhibitory control of a previously reinforced response, which causes enhanced perseveration, impaired reversal learning, and behavioral inflexibility (Figures 3C and 4A; Movies S2 and S4). Furthermore, the results from our fear extinction studies highlight an equally important role for PERK in PFC-directed updating of behavior (Figure 4C). Collectively, these results suggest that PERK regulates sensory information processing, thereby inducing deficits in various cognitive paradigms when eliminated.

Previous studies have shown that a reduction of eIF2α phosphorylation in mice lacking the eIF2α kinase GCN2 and in heterozygous knockin mice with a mutation on serine 51 of eIF2α results in a lowered threshold for the consolidation of long-term memory (Costa-Mattioli et al., 2005, 2007). Similar to the PERK cKO mice, GCN2 and eIF2α-S51A mutant mice showed reduced eIF2α phosphorylation and ATF4 expression, although these reductions were global and constitutive rather than forebrain specific and postdevelopmental. However, the behavioral phenotypes of the GCN2 and eIF2α-S51A mutant mice were quite different from the PERK cKO mice. Unlike the PERK cKO mice, we found that GCN2 KO mice showed normal reversal learning in the Y-water maze reversal task (Figure S3). These complementary studies indicate that even in the face of similar biochemical profiles, such as reduced eIF2α phosphorylation and ATF4 expression, additional mechanisms and levels of regulation exist to further modulate pools of eIF2α that are eventually reflected by distinct behavioral phenotypes. It also is important to emphasize that the PERK cKO mice are postdevelopmental knockout mice where the disruption of PERK occurs approximately 2–3 weeks after birth. In contrast, GCN2 KO and eIF2α-S51A mutant mice, as well as PKR (another eIF2α kinase) KO mice, are all global, constitutive knockout mice. Thus, the behavioral phenotypes displayed by the GCN2, eIF2α-S51A, and PKR mutant mouse lines could be due to developmental complications, whereas the PERK cKO mice are not.

One current model for the pathophysiology of PCP-induced psychosis and schizophrenia involves the hypofunction of NMDAR in GABAergic fast-spiking interneurons (Belforte et al., 2010; Lisman et al., 2008; Nakazawa et al., 2012). Loss of NMDAR function in interneurons is thought to result in disinhibition of pyramidal neurons in the cortex and hippocampus, asynchronous pyramidal neuron activation, hyperexcitability of cortical networks, and cognitive impairment. Intriguingly, our findings suggest that whereas selective ablation of PERK in pyramidal neurons does not alter NMDAR function (Figures 5A and 5B), chronic NMDAR hypofunction elicits a decrease of eIF2α phosphorylation in the PFC (Figures 5C and 5D). Based on these results, we speculate that NMDAR hypofunction in interneurons causes cortical excitation, dysregulation of PERK, and decreased eIF2α phosphorylation in pyramidal neurons, resulting in impaired cognition. Consistent with this notion, a recent study showed that deletion of the eIF2α kinase PKR in mice results in reduced GABAergic transmission, increased network excitability, and altered cognition (Zhu et al., 2011). However, whether selective ablation of PERK in pyramidal neurons associates with altered cortical network excitability remains to be determined. Furthermore, it is possible that reduced NMDAR function causes a disruption of PERK-directed translation specifically in GABAergic interneurons to dampen inhibitory control of pyramidal neurons and impair cognitive function. Future studies examining the role of PERK in various neuronal subtypes, in particular GABAergic interneurons, will provide molecular insight into the role of eIF2α in the pathophysiology of cognitive impairment associated with multiple neuropsychiatric disorders.

Interestingly, it has been shown that enhancement of NMDAR function by treatment with the GlyT1 inhibitor SSR504734 improves behavioral flexibility, reversal learning, and overall cognitive function (Black et al., 2009; Depoortère et al., 2005; Singer et al., 2009). Consistent with these studies, our findings indicate that SSR504734 uniquely enhanced behavioral flexibility (Figures 6A and 6B; Movies S5 and S6) without altering enhanced vertical activity and sensorimotor-gating impairments displayed by the PERK cKO mice. Furthermore, chronic SSR504734 treatment was found to restore aberrant eIF2α phosphorylation and ATF4 expression, but not disrupted PERK levels in the PFC of PERK cKO mice (Figures 6C–6E). Thus, our results indicate that chronic inhibition of GlyT1 can normalize disrupted PERK-regulated translation, presumably by either activating other eIF2α kinases such as GCN2 and/or PKR, or by inhibiting the protein phosphatase 1/GADD34 complex that dephosphorylates eIF2α (Ma and Hendershot, 2003). Future studies are required to address whether chronic SSR504734 treatment enhances GCN2 activity, enhances PKR activity, or inhibits protein phosphatase 1/GADD34 complex in the PFC of PERK mutant mice.

Under normal physiological conditions it has been reported that systemic administration of SSR504734 improves behavioral flexibility and cognitive function in WT mice (Singer et al., 2009). In contrast we found that although chronic SSR504734 treatment had no effect on the performance of the WT mice, it restored the behavioral flexibility of the PERK cKO mice (Figure 6A). Moreover, we found that SSR504734 could not only rescue the behavioral deficits but also could restore the molecular anomalies exhibited by the PERK cKO mice (Figures 6D and 6E). Thus, our findings provide direct evidence that SSR504734 can modulate eIF2α phosphorylation and ATF4 expression that are tightly correlated with reversal of the behavioral inflexibility displayed by the PERK cKO mice. Future studies are required to determine whether chronic SSR504734...
treatment can restore the dysregulated eIF2α-ATF4 axis in WT mice treated chronically with agents such as MK-801 and PCP that induce NMDAR hypofunction and are used to model schizophrenia.

A rapidly expanding list of neurological disorders and neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, fragile X syndrome, TSC, and ASD has been linked to dysregulated protein synthesis (Auluck et al., 2010; Hoeffer and Klann, 2010; Kelleher and Bear, 2008; Ozcan et al., 2008; Palop and Mucke, 2010; Santini and Klann, 2011). Consistent with these findings, our results suggest that postdevelopmental disruption of PERK-regulated translational control is sufficient to trigger cognitive control impairments consistent with several disorders, including schizophrenia. In conclusion these findings emphasize the critical importance of PERK in normal cognitive processes. Further studies elucidating the specific role of PERK-regulated translation in the brain may provide new avenues to tackle such widespread and often debilitating neurological disorders.

EXPERIMENTAL PROCEDURES

Transgenic Mice
Floxed Perk (Perk<sup>loxP/loxP</sup>) mice were generated as described previously by Zhang et al. (2002). Mice expressing the Cre recombinase transgene (T-29-1), referred to as CamkII-Cre, were kindly provided by Dr. S. Tonegawa (Tsien et al., 1996). PERK cKO and WT littermate control mice were on a C57/C14 background.

Genotyping
Genotyping of mice was performed using standard procedures. Please see Extended Experimental Procedures for details.

Western Blotting
Western blots were performed using standard procedures as described previously by Banko et al. (2005). Please see Extended Experimental Procedures for details.

IHC and Nissl Staining
IHC and Nissl staining were performed using standard procedures. Please see Extended Experimental Procedures for details.

SUnSET Technique
Proteins were labeled using an adaptation of the SUnSET method by Schmidt et al. (2009). Briefly, 400 μm coronal prefrontal slices from WT and PERK cKO mice were prepared using a vibratome, slices were allowed to recover in artificial cerebrospinal fluid (ACSF) at 32°C for 1 hr, and subsequently treated with puromycin (P8833 [Sigma-Aldrich, St. Louis] 5 μg/ml) for 45 min. Reactions were stopped by flash freezing the slices on dry ice. Proteins were prepared, blotted, and quantified as described above (see Western Blotting), and 50 μg of puromycin-labeled protein was resolved on 4%-12% gradient gels (Invitrogen) and visualized using an antibody specific to puromycin (12D10; see Western Blotting). Protein synthesis levels were determined by taking the total lane signal from 15 to 250 kDa and subtracting the signal from the control lane that was not labeled by puromycin. Comparisons of protein synthesis levels between both genotypes were made by normalizing to the average WT signal obtained from different experimental replicates.

Polysome Analysis
Three pairs of 3-week-old PERK cKO mice and their WT littermates were terminally anesthetized by isoformurane, and polysome gradients were prepared. Briefly, postmitochondrial supernatants of PFC, dissected free from white matter, were prepared and separated by sucrose density gradients (20%–60%). Gradients were centrifuged at 4°C for 2 hr at 40,000 rpm in an SW41 rotor. Then, 890 μl fractions (16 per gradient) were collected with continuous monitoring of UV absorbance at 254 nm (UA-6 absorbance detector; ISCO).

PPI
Sensorimotor gating was measured by testing the startle response and the PPI of the startle response. Mice were placed individually in a Plexiglas cylinder connected to a startle detector. Upon habituating to the background noise (70 dB), each mouse was presented with a semirandom series of prepulses of varying intensities (74, 78, 82, 86, and 90 dB) paired with an acoustic startle stimulus (120 dB). The percent (%) PPI was calculated as follows: (1 – (startle response to prepulse+pulse)/startle response to pulse alone) × 100.

Novel Object Recognition
Novel object recognition experiments were performed as previously described by Hoeffer et al. (2008). The novel object recognition task is based on the natural tendency of mice to explore a novel object rather than a familiar object. The amount of time spent exploring the novel object was divided by the total time spent exploring both objects to generate a preference index that was multiplied by 100 to calculate percent object preference. The Noldus EthoVision software and video-tracking system were used to monitor and record object interaction time.

MWM Reversal Learning Task
MWM experiments were performed as previously described by Banko et al. (2005), and the reversal learning protocol was adapted from previous studies by Hoeffer et al. (2008). Escape latency, number of previous platform position crossing, time spent in each quadrant, and trajectories of the mice were recorded with a computerized video-tracking system (Noldus EthoVision). Briefly, the paradigm consisted of a 9 day training period broken into several phases. On days 1–5 (reference), mice were trained to locate a submerged hidden platform. Following completion of the last training trial on day 5, a single-probe trial was given by removing the hidden platform from the pool. On days 6 and 7 (reversal), the originally learned platform location was moved to the opposite quadrant. On days 8 and 9 (visible), mice were tested using a visible cue. During reference, reversal, and visible phases, mice were given four trials per day (60 s maximum, intertrial interval [ITI] 15 min).

Y-Water Maze Arm Reversal Task
Arm reversal in the Y-water maze task was carried out as described previously by Hoeffer et al. (2008). A retraining paradigm was incorporated to allow the 12 out of 13 PERK cKO mice that perseverated to make 3 consecutive correct choices (Figure 4A). This entailed using a Plexiglas wall to section off the incorrect arm. The paradigm consisted of a 9 day training period broken into several phases. On days 1–5 (reference), mice were trained to locate a submerged hidden platform. Following completion of the last training trial on day 5, a single-probe trial was given by removing the hidden platform from the pool. On days 6 and 7 (reversal), the originally learned platform location was moved to the opposite quadrant. On days 8 and 9 (visible), mice were tested using a visible cue. During reference, reversal, and visible phases, mice were given four trials per day (60 s maximum, intertrial interval [ITI] 15 min).

Fear Extinction
Prior to extinction training, mice were fear conditioned by training them to associate two CS-US pairings separated by 1 min (foot-shock intensity [US]; 0.65 mA, 2 s duration; tone [CS]; 85 dB white noise, 30 s duration). The following day, mice were placed in an environmentally altered training chamber and received 2 extinction training sessions consisting of 15 consecutive CS presentations with an average ITI between CSs of 120 s and a 24 hr interval between each session. Twenty-four hours after the last extinction training session (day 3), mice were returned to testing chamber and presented with an LTM test (two tone alone trials). Freezing was recorded continuously during the 15 extinction training trials and 2 trial LTM test sessions. Freezing was scored blind with respect to the mouse genotype.
Intracellular Electrophysiology

Whole-cell recordings from mPFC pyramidal cells from layer II were conducted using standard procedures. Please see Extended Experimental Procedures for details.

Human Samples

Protein samples extracted from the dorsolateral PFC (DL-PFC; Brodmann Area 46) of 35 individuals in each of the three diagnostic groups (schizophrenia, bipolar disorder, and unaffected controls) were obtained from the SMRI Array Collection. These specimens were collected, with informed consent from next of kin, by participating medical examiners between January 1995 and June 2002. All groups were matched for age, sex, and race. A summary of the relevant patient demographic information is provided in Table S1. The specimens were collected, processed, and stored in a standardized way. Proteins were extracted using a protease inhibitor-Tris-glycerol extraction buffer (AEBSF 0.048%, apronitin 0.011%, leupeptin 0.002%, pepstatin A 0.001%, glycerol 50%, Tris Ultra Pure 1.2%) (0.1 g brain tissue: 1.25 ml buffer). Analysis of 20 μg protein samples from schizophrenia (n = 35), bipolar (n = 35), and unaffected controls (n = 35) was conducted; the samples were coded such that the investigator was blind to diagnostic status. Upon decoding, no signal was detected in two samples from the schizophrenia cohort for the ATF4 analysis and eliminated from further analysis.

Pharmaceutical Reagents

For protein synthesis studies using the SUnSET technique (Figure 2A), coronal prefrontal slices from WT and PERK cKO mice were incubated with puromycin (P8833, 5 μg/ml) for 45 min. For NMDA hyposensitivity studies (Figures 5C and 5D), CS7/Bi6 WT mice were given either a single (acute) or 15 daily (chronic) i.p. injections of vehicle (0.9% saline) or MK-801 dissolved in vehicle (Sigma-Aldrich; 0.2 mg/kg for acute studies and 0.1 mg/kg for chronic studies). SSR504734, a GlyT1 inhibitor compound, was provided by Sanofi-Aventis (Paris) and dissolved in water with a few drops of Tween 80 as previously described by Depoortere et al. (2005). For behavioral and molecular rescue studies (Figure 6), injections of SSR504734 (20 mg/kg) were given i.p. to PERK cKO and WT littermates for 21 days prior to behavioral testing and protein expression analysis.

Statistical Analysis

All data are presented as the mean ± SEM. Statistical analyses between two groups were performed using a two-tailed Student’s t test. Comparisons involving drug treatment, time courses, or protein expression among multiple groups or genotypes were performed with an one- or two-way ANOVA. For the human studies, potential association of continuous variables such as age, brain pH, PMI, and lifetime antipsychotic usage was determined using a regression method with Grubbs’ criterion. Extreme outliers were detected by applying Grubbs’ test and eliminated from further analysis by applying Grubbs’ criterion. Extreme outliers were detected by applying Grubbs’ test and eliminated from further analysis by applying Grubbs’ criterion. Extreme outliers were detected by applying Grubbs’ test and eliminated from further analysis by applying Grubbs’ criterion. Extreme outliers were detected by applying Grubbs’ test and eliminated from further analysis by applying Grubbs’ criterion. Extreme outliers were detected by applying Grubbs’ test and eliminated from further analysis by applying Grubbs’ criterion.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, two tables, and six movies and can be found with this article online at doi:10.1016/j.cerep.2012.04.010.

REFERENCES


