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**Increased Expression of the PI3K Enhancer PIKE Mediates Deficits in Synaptic Plasticity and Behavior in Fragile X Syndrome**

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**SUMMARY**

The PI3K enhancer PIKE links PI3K catalytic subunits to group 1 metabotropic glutamate receptors (mGlu1/5) and activates PI3K signaling. The roles of PIKE in synaptic plasticity and the etiology of mental disorders are unknown. Here, we show that increased PIKE expression is a key mediator of impaired mGlu1/5-dependent neuronal plasticity in mouse and fly models of the inherited intellectual disability fragile X syndrome (FXS). Normalizing elevated PIKE protein levels in FXS mice reversed deficits in molecular and cellular plasticity and improved behavior. Notably, PIKE reduction rescued PI3K-dependent and -independent neuronal defects in FXS. We further show that PI3K signaling is increased in a fly model of FXS and that genetic reduction of the *Drosophila* ortholog of PIKE, CenG1A rescued excessive PI3K signaling, mushroom body defects, and impaired short-term memory in these flies. Our results demonstrate a crucial role of increased PIKE expression in exaggerated mGlu1/5 signaling causing neuronal defects in FXS.

**INTRODUCTION**

Dysregulated signaling through phosphoinositide-3 kinase (PI3K) has been recognized as a common pathological mechanism underlying diverse brain disorders, such as epilepsy, schizophrenia, intellectual disability, and autism (Hoeffer and Klann, 2010; Law et al., 2012; Schick et al., 2007; Gross and Bassell 2014). Receptor-mediated PI3K/mTOR signaling plays an essential role in synaptic plasticity and neuronal function (Banko et al., 2006; Hou and Klann, 2004). Analyzing the neuronal functions of proteins that directly mediate receptor-induced activation of PI3K signaling is therefore of particular interest in order to understand molecular defects leading to mental diseases.

The PI3K enhancer PIKE (*Centg1*, a.k.a. *Agap2*) is an important regulator of receptor-mediated PI3K activity. PIKE binds and activates PI3K and Akt and plays roles in many different cellular functions, such as apoptosis, migration, and receptor trafficking (Chan and Ye, 2010). In the brain, PIKE-mediated PI3K activity downstream of group 1 metabotropic glutamate receptors (mGlu1/5) is essential for neuronal survival, leading to reduced neuronal density and decreased dendritic complexity in the neocortex of *Centg1* knockout (*Centg1*KO) mice (Chan et al., 2011b; Rong et al., 2003). PIKE’s role in mGlu1/5-dependent synaptic plasticity and possible implication for the etiology of mental disorders is unknown.

The inherited intellectual disability and autism spectrum disorder fragile X syndrome (FXS) is characterized by increased and stimulus-insensitive signaling through mGlu1/5 (Bear et al., 2004), but the underlying mechanisms are unknown. Recent phase 3 clinical trials in patients with FXS using mGlu5-negative modulators have been unsuccessful to improve the outcome measures in behavior, corroborating the critical need to better understand the mechanisms underlying dysregulated mGlu1/5 signaling in FXS. The detailed analysis of these mechanisms might reveal alternative therapeutic strategies in FXS.

FXS is caused by loss of function of the fragile X mental retardation protein (FMRP), an mRNA-binding protein that binds to numerous mRNA targets and often represses their translation (Bhakar et al., 2012). Previous studies showed that *Centg1* mRNA associates with FMRP (Darnell et al., 2011; Gross et al., 2010; Sharma et al., 2010), leading to increased PIKE protein levels in *Fmr1* knockout (*Fmr1*KO) mice, a mouse model of FXS.
Drosophila invertebrate

Centg1

Genetic Reduction of behavioral impairments that underlie FXS. confirmed FMRP target, as a crucial contributor to mGlu1/5-dependent molecular and cellular plasticity and

Levels and Excessive PI3K Activity in

PIKE in two different animal models of FXS: rescue FXS-associated phenotypes, we genetically reduced 728 Cell Reports gous Fmr1 impaired signaling, synaptic function, and behavior in FXS. Due to loss of FMRP-mediated repression may contribute to
defect in mGlu1/5-mediated signaling and synaptic
dent PI3K activation and given the well-described, but not fully understo ured, PI3K/Ser increases in FXS, we hypothesized that elevated PIKE levels in the mouse brain are increased in the absence of FMRP (Gross et al., 2010; Sharma et al., 2010). To evaluate the functional impact of increased PIKE levels in FXS, we chose a strategy to genetically reduce, but not to delete, PIKE in the FXS mouse model by breeding female Fmr1 heterozygous (Fmr1KO) mice with male mice heterozygous for Centg1 (Chan et al., 2010). Male offspring of the following genotypes were analyzed: Fmr1WT/Centg1HET; and Fmr1KO/Centg1HET (Figure 1A). Western blot analyses validated that Centg1 heterozygosity reduced the expression of PIKE-L protein in the cortex of Fmr1KO and Fmr1WT mice (Figure 1A).

Figure 1. Genetic Reduction of PIKE Expression Decreases Elevated PI3K Activity in the Cortex of Fmr1KO Mice

(A) Centg1 heterozygosity reduces PIKE-L protein levels in both Fmr1WT and Fmr1KO background (two-way ANOVA; significant effect of Centg1 heterozygosity on PIKE-L protein levels [F(1,37) = 29.9; p < 0.001]; no effect of Fmr1KO [F(1,37) = 3.5; p = 0.071]; no interaction [F(1,37) = 0.12; p = 0.727]). Representative western blots are shown at the left. Protein levels were normalized to α-tubulin. Also see Figure S1A for breeding scheme and Figures S1B and S1C for quantification of PIKE mRNA levels in Centg1 heterozygous mice.

(B–D) Increased p110β- and mGlu5-associated PI3K activity is reduced to WT levels in cortical synaptic fractions (p110β) or cortical lysates (mGlu5) from Centg1 heterozygous Fmr1KO mice, whereas IRS2-associated PI3K activity is not affected by Fmr1 or Centg1 genotype. PI3K enzymatic activity of p110β-, mGlu5-, or IRS-2-specific immunoprecipitates was measured by ELISA (B) two-way ANOVA, p(Fmr1) = 0.079, F(1,25) = 3.3; p(Centg1) = 0.053, F(1,25) = 4.0; p(interaction) = 0.011, F(1,25) = 7.3; *p = 0.015, #p = 0.011; (C) two-way ANOVA, p(Fmr1) = 0.08, F(1,25) = 3.3; p(Centg1) = 0.018, F(1,25) = 6.4; p(interaction) = 0.036, F(1,25) = 4.9; *p = 0.042, #p = 0.017; and (D) two-way ANOVA, p(Fmr1) = 0.115, F(1,27) = 2.7, p(Centg1) = 0.228, F(1,27) = 1.5; p(interaction) = 0.947, F(1,27) = 0.01.

Elevated PIP3/PIP2 ratios in

KO mice and centaurin gamma-1A (CenG1A), 727–736, May 5, 2015 The Authors

The expression of PIKE-L protein in the cortex of Fmr1WT heterozygous mouse (Figure 1A). To further confirm that Centg1 heterozygous mice have reduced levels of all PIKE isoforms, we performed qRT-PCR analyses with primers detecting PIKE-A, -S, and -L isoforms. All PIKE mRNA isoforms were significantly reduced in Centg1 heterozygous mouse cortices (Figures S1B and S1C), suggesting that likewise all protein isoforms were reduced.

We have previously shown that activity of the PI3K catalytic subunit p110β is increased in the absence of FMRP (Gross and Bassell, 2012; Gross et al., 2010). Here, we show that Centg1

RESULTS

Genetic Reduction of Centg1 Decreases PIKE Protein Levels and Excessive PI3K Activity in Fmr1KO Mice

PIKE protein levels in the mouse brain are increased in the absence of FMRP (Gross et al., 2010; Sharma et al., 2010). To
heterozygosity reduced p110β-associated PI3K activity in Fmr1KO cortical synaptic fractions to wild-type levels (Figure 1B).

PIKE links PI3K catalytic subunits to mGlu1/5 receptors via the scaffolding protein Homer (Rong et al., 2003). We thus hypothesized that elevated PIKE results in increased mGlu5-associated PI3K activity in Fmr1KO mice. In contrast, PI3K activity associated with PIKE-L-independent receptors, such as insulin receptor substrate 2 (IRS-2) complexes, may be unaffected. We therefore hypothesized that elevated PIKE results in increased mGlu5-associated PI3K activity was significantly increased in Fmr1KO cortex and was reduced by Centg1 heterozygosity (Figure 1C). We did not observe any significant changes in IRS-2-associated PI3K activity in Fmr1KO cortex, and PIKE reduction had no significant effect on IRS-2-associated PI3K activity (Figure 1D).

We have previously reported that the levels of the PI3K product phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) are increased at Fmr1KO synapses (Gross et al., 2010). Here, we show that the ratio of PIP3 and the PI3K substrate phosphatidylinositol-(4,5)-bisphosphate (PIP2) in hippocampal acidophilic lipid fractions is increased in Fmr1KO cortex (Figure 1E). The increased PIP3/PIP2 ratio was normalized to wild-type levels by Centg1 heterozygosity (Figure 1E). Heterozygosity for Fmr1 heterozygosity reduced p110β-associated PI3K activity after treatment with the mGlu1/5 agonist DHPG in Fmr1KO mice. In contrast, PI3K activity associated with PIKE-L-independent receptors, such as insulin receptor substrate 2 (IRS-2) complexes, may be unaffected.

**Genetic Reduction of Centg1 Restores Stimulus-Induced Activation of PI3K and Protein Synthesis in Fmr1KO Mice**

A hallmark of FXS in the mouse model is loss of stimulus-induced signaling and protein synthesis (Gross et al., 2010; Osterweil et al., 2010; Ronesi and Huber, 2008; Sharma et al., 2010; Weiler et al., 2004), which is believed to contribute to impaired synaptic plasticity and neuronal function in FXS. To test whether reducing PIKE protein might restore this molecular plasticity in FXS, we analyzed activity-regulated PI3K function and synaptic protein synthesis in Fmr1KO/Centg1WT mice. Centg1 heterozygosity restored mGlu1/5-mediated activation of p110β-associated PI3K activity after treatment with the mGlu1/5 agonist DHPG in Fmr1KO cortical synaptic fractions (Figure 2A), reduced excess basal protein synthesis rates (Figure 2B), and reinstated mGlu1/5-evoked stimulation of protein synthesis rates (Figure 2C). In wild-type, DHPG-induced stimulation of both PI3K activity and protein synthesis was reduced by Centg1 heterozygosity, corroborating the important function of PIKE in mediating mGlu1/5-dependent downstream signaling.

**Genetic Reduction of Centg1 Rescues Excess Dendritic Spine Density and Exaggerated mGlu5-Mediated LTD in Fmr1KO Mice**

Analyses of Golgi staining in the hippocampal CA1 region of adult mice showed that Centg1 heterozygosity reduced the elevated dendritic spine density typical for the FXS phenotype to wild-type levels (Figures 2D, 2E, S2A, and S2B). Moreover, exaggerated mGlu1/5-dependent long-term depression (LTD) in the hippocampus, a hallmark of impaired mGlu1/5-dependent synaptic plasticity in FXS, was rescued to wild-type levels in Fmr1KO mice heterozygous for Centg1 (Figures 2F and 2G).

**Centg1 Heterozygosity Reduces Neocortical Hyperactivity in Fmr1KO Mice**

FXS mice display enhanced neocortical circuit activity, which can be rescued by mGlu5-negative allosteric modulators or genetic reduction of mGlu5 (Gibson et al., 2008; Gonçalves et al., 2013; Hays et al., 2011). Here, we analyzed whether reduction of PIKE, which is associated with mGlu1/5 via Homer proteins, reduces neocortical activity in Fmr1KO mice. We measured the duration of UP states (a type of persistent activity state), which are prolonged in Fmr1KO thalamocortical slices and may reflect network hyperexcitability in the absence of FMRP (Hays et al., 2011). Duration of UP states in Fmr1KO was significantly reduced to wild-type levels by Centg1 heterozygosity (Figures 3A and 3B). Prolonged UP states do not depend on protein synthesis (Hays et al., 2011), and we thus speculated that PIKE might also be mediating PI3K/mTOR-independent functions downstream of mGlu1/5. In line with this hypothesis, pre-treatment with the broad spectrum PI3K inhibitor Wortmannin did not affect UP state duration in thalamocortical slices from Fmr1WT or Fmr1KO mice (Figures S3A and S3B). Decreased PIKE levels also significantly reduced susceptibility to audiogenic seizures in Fmr1KO mice (Figure 3C).

**Genetic Reduction of Centg1 Reduces Repetitive Behavior in Fmr1KO Mice and Improves Nest Building**

FXS is the most-common monogenic cause of autism. To test the influence of increased PIKE levels on autistic-like behaviors in the FXS mouse model, we analyzed marble burying and nest building. As reported previously, Fmr1KO mice buried more marbles in a given time window than their wild-type littermates, which was normalized to wild-type levels by Centg1 heterozygosity (Figure 3D). Similarly, impaired nest building was improved by Centg1 heterozygosity as measured by the amount of unused nestlet after 24 and 72 hr and by using a nest scoring system as described previously (Deacon, 2006) (Figures 3E, 3F, and S3C–S3E). Interestingly, in contrast to most other phenotypes, in which Centg1 heterozygosity did not have an effect on wild-type, Centg1 heterozygosity also improved nest building in wild-type mice.

**Genetic Reduction of CenG1A, the Drosophila Ortholog of Centg1, Rescues Increased PI3K Signaling, Mushroom Body Defects, and Impaired Short-Term Memory in dFmr1 Mutant Flies**

Previous studies have shown that Drosophila models of FXS, produced by mutations in the Drosophila dFmr1 gene, are a valid tool to analyze molecular, cellular, and behavioral deficits in FXS (McBride et al., 2013). Here, we show that, similarly as in the hippocampus of Fmr1KO mice, PI3K signaling was elevated in flies homozygous for the strong loss-of-function allele Fmr1iso (Zhang et al., 2001). Both PIP3/PIP2 ratios and PI3K downstream signaling, as shown by phosphorylation of S6K and Akt, were increased in dFmr1 mutant fly heads (Figures 4A–4C). To examine whether, similarly as in the mouse model, reducing the gene dosage of CenG1A, the fly ortholog of Centg1, decreases PI3K signaling and reverses neuronal phenotypes observed in dFmr1 mutant flies, we generated flies homozygous for Fmr1iso that were heterozygous for a mutant allele of dFmr1.
Figure 2. Genetic Reduction of Centg1 Rescues Dysregulated mGlu5-Mediated PI3K Activity and Protein Synthesis, Increased Dendritic Spine Density, and Impaired Synaptic Plasticity in Fmr1KO Mice
(A) Genetic reduction of Centg1 restores the mGlu1/5-induced increase in p110 enzymatic activity in cortical synaptic fractions (100 μM DHPG for 10 min; two-way ANOVA, p(Fmr1) = 0.052, F(1,36) = 4.0; p(Centg1) = 0.15, F(1,36) = 2.2; p(interaction) = 0.001, F(1,36) = 12.4; *p = 0.002, #p = 0.006).
(B) Increased basal protein synthesis rates in Fmr1KO cortical synaptic fractions, as measured by incorporation of radiolabeled amino acids, were significantly reduced to wild-type levels by genetic reduction of Centg1 (two-way ANOVA, p(Fmr1) = 0.28, F(1,36) = 1.2; p(Centg1) = 0.043, F(1,36) = 4.4; p(interaction) = 0.011, F(1,36) = 7.1; *p = 0.053, #p = 0.009).
(C) Genetic reduction of Centg1 restores the mGlu1/5-induced increase in protein synthesis rates in Fmr1KO cortical synaptic fractions (50 μM DHPG for 20 min; two-way ANOVA, p(Fmr1) = 0.001, F(1,36) = 18.6; p(Centg1) = 0.011, F(1,36) = 7.1; p(interaction) < 0.001, F(1,36) = 27.1; *p < 0.001, #p < 0.001).

Error bars represent SEM; n represents individual mice from at least five different litters. (D and E) Genetic reduction of Centg1 normalizes dendritic spine density in CA1 apical dendrites to wild-type levels (two-way ANOVA, p(Fmr1) < 0.001, F(1,36) = 32.4; p(Centg1) < 0.001, F(1,36) = 27.0; p(interaction) < 0.001, F(1,36) = 33.1; *p < 0.001, #p < 0.001). Example images are shown in (D); quantification of number of dendritic spines per 10 μm is shown in (E), n indicates number of secondary dendrites analyzed (60–100 μm length each, starting from the primary shaft), three to five mice/genotype, four to eight neurons/mouse, one dendrite/neuron. See Figure S2 for additional analyses. The scale bar represents 3 μm.

CenG1A (CenG1A\textsuperscript{EY0217}). Heterozygosity of CenG1A\textsuperscript{EY0217} significantly reduced PIP3/IP2 ratios and PI3K downstream signaling to or below wild-type levels (Figures 4A–4C). CenG1A\textsuperscript{EY0217} also increased viability of dFmr1 mutant flies (Figure 4D). Offspring from Fmr1\textsuperscript{-/}TM6B X Fmr1\textsuperscript{-/}TM6B crossings or CenG1A \textsuperscript{EY0217}/CyO:Fmr1\textsuperscript{-/}TM6B X Fmr1\textsuperscript{-/}TM6B, respectively, were genotyped and counted at P0 or P1 following eclosion. The values shown are percentages of flies homozygous for the dFmr1-null gene generated by these crossings.

dFmr1 mutant mushroom bodies, which are bilaterally symmetric axonal projections from Kenyon neurons, show a high rate of β-lobe fusion across the midline (Michel et al., 2004). Heterozygosity for the CenG1A\textsuperscript{EY0217} allele rescued this developmental defect in the dFmr1 mutant background but did not affect gross mushroom body morphology in wild-type (Figure 4E).

Next, we tested whether CenG1A heterozygosity improved the impairment in short-term memory in dFmr1 mutant flies. When exposed to an unreceptive (previously mated) female, male wild-type flies learn to reduce mating behavior and retain this memory over 2 or 3 hr. As shown previously (McBride et al., 2006), dFmr1 mutant flies had impaired short-term
The goal of this study was to test the hypothesis that increased expression of the PI3K enhancer PIKE, which is translationally regulated by FMRP and an important regulator of mGlu1/5-dependent PI3K activity, contributes to dysregulated mGlu1/5-dependent synaptic plasticity and neuronal function underlying the pathogenesis of FXS. To this end, we used genetic approaches in a mouse and a fly model to reduce PIKE levels in FXS. Previous studies have genetically reduced or deleted components upstream and downstream of the PI3K-signaling complex, but these were not direct mRNA targets of FMRP, nor were they shown to be translated in excess in FXS (Bhattacharya et al., 2012; Dölen et al., 2007, Ronesi et al., 2012). Moreover, in most cases, the approach taken thus far has been to delete a gene of interest, hence an advantage of the genetic reduction approach taken here is to compensate for loss of translational repression of FMRP-associated mRNAs in FXS. Here, we show that this strategy rescued FXS-associated phenotypes on several functional levels, from signaling mechanisms to synaptic plasticity and memory, including PI3K- and protein synthesis-dependent and -independent neuronal functions. We further corroborate the importance of PIKE dysregulation for the etiology of FXS by showing that PI3K signaling is increased in a fly model of FXS and that reducing the Drosophila ortholog of PIKE, CenG1A in FXS flies rescues molecular, morphological, and cognitive defects. Taken together, our study suggests that gain of function of a critical molecular linker of the mGlu5-PI3K-signaling complex contributes to FXS-associated defects in neuronal and behavioral function.

Receptor-Specific PI3K Signaling Defects in FXS

Numerous studies have reported exaggerated signaling of mGlu1/5 receptors in FXS animal models (Dölen et al., 2007; Huber et al., 2002; Muddashetty et al., 2007; Osterweil et al., 2010; Ronesi et al., 2012), and mGlu5 negative allosteric modulators have been shown to rescue many FXS-associated phenotypes (McBride et al., 2005; Michalon et al., 2012; Yan et al., 2005). Several recent studies have begun to analyze the molecular mechanisms of dysregulated mGlu1/5 signaling in FXS by genetically reducing or deleting mGlu1/5-signaling complex components or downstream regulators of protein synthesis (Bhattacharya et al., 2012; Dölen et al., 2007; Ronesi et al., 2012), but the detailed underlying molecular mechanisms are still unknown. In this study, we show that the PI3K enzymatic activity of the mGlu5 protein complex is increased in Fmr1KO cortex.

F(3,35) = 6.6; p(Centg1) = 0.039, F(1,35) = 4.6; p(interaction) = 0.699, F(1,35) = 0.15. Representative pictures of nests and analyses after 72 hr are shown in Figures S3C–S3E. Error bars represent SEM. n indicates number of slices for (B) and individual mice from at least five different litters for (C)–(F).

Figure 3. Genetic Reduction of Centg1 Reduces Neocortical Hyperactivity and Repetitive Behaviors and Improves Nest Building in Fmr1KO Mice

(A and B) Genetic reduction of Centg1 decreases duration of UP states in acute thalamocortical slices from Fmr1KO mice. Example traces for each genotype are shown in (A) and quantification in (B) (two-way ANOVA, p(Fmr1) < 0.001, F(1,148) = 15.4; p(Centg1) = 0.002, F(1,148) = 9.8; p(interaction) = 0.131, F(1,148) = 2.3; data square root transformed twice to achieve normal distribution). Also see Figures S3A and S3B, showing that UP states in Fmr1 KO mice (Fisher’s exact tests; *p < 0.001; #p = 0.008; p(Fmr1/KO/Centg1KO/Centg1WT) = 0.213).

(C) Genetic reduction of Centg1 reduces increased susceptibility to audiogenic seizures in Fmr1WT mice (Fisher’s exact tests; *p < 0.001; #p = 0.008; p(Fmr1WT/Centg1WT/Fmr1KO/Centg1WT) = 0.213).

(D) Genetic reduction of Centg1 rescues increased marble burying in Fmr1KO mice. Shown are number of marbles buried more than 50% after 15 min (two-way ANOVA, p(Fmr1) = 0.037, F(1,33) = 4.7; p(Centg1) = 0.106, F(1,33) = 2.8; p(interaction) = 0.039, F(1,33) = 4.6; *p = 0.021).

(E and F) Impaired nesting behavior is improved in Fmr1WT and Fmr1KO mice by genetic reduction of Centg1. Shown are the nest score (E) and the average amount of unused nestlet after 24 hr (F). (E) Two-way ANOVA, p(Fmr1) = 0.003, F(1,35) = 16.1; p(Centg1) = 0.003, F(1,35) = 10.0; p(interaction) = 0.418, F(1,35) = 0.7; (F), two-way ANOVA, p(Fmr1) = 0.015, memory of courtship suppression, and CenG1A EY01217 heterozygous flies had impaired short-term memory as well, suggesting that this type of memory in flies is dosage sensitive to PIKE.

DISCUSSION

The goal of this study was to test the hypothesis that increased expression of the PI3K enhancer PIKE, which is translationally regulated by FMRP and an important regulator of mGlu1/5-dependent PI3K activity, contributes to dysregulated mGlu1/5-dependent synaptic plasticity and neuronal function underlying the pathogenesis of FXS. To this end, we used genetic approaches in a mouse and a fly model to reduce PIKE levels in FXS. Previous studies have genetically reduced or deleted components upstream and downstream of the PI3K-signaling complex, but these were not direct mRNA targets of FMRP, nor were they shown to be translated in excess in FXS (Bhattacharya et al., 2012; Dölen et al., 2007, Ronesi et al., 2012). Moreover, in most cases, the approach taken thus far has been to delete a gene of interest, hence an advantage of the genetic reduction approach taken here is to compensate for loss of translational repression of FMRP-associated mRNAs in FXS. Here, we show that this strategy rescued FXS-associated phenotypes on several functional levels, from signaling mechanisms to synaptic plasticity and memory, including PI3K- and protein synthesis-dependent and -independent neuronal functions. We further corroborate the importance of PIKE dysregulation for the etiology of FXS by showing that PI3K signaling is increased in a fly model of FXS and that reducing the Drosophila ortholog of PIKE, CenG1A in FXS flies rescues molecular, morphological, and cognitive defects. Taken together, our study suggests that gain of function of a critical molecular linker of the mGlu5-PI3K-signaling complex contributes to FXS-associated defects in neuronal and behavioral function.
and rescued by CenG1 heterozygosity (Figure 1C), which provides direct evidence for a link between impaired mGlu1/5 signaling, excessive PI3K activity, and loss of translational control of FMRP target mRNAs in FXS. In contrast, IRS-2-associated PI3K activity is not significantly altered in Fmr1 KO mice (Figure 1D). This supports a direct role of the mGlu1/5-PI3K-signaling complex in mediating neuronal defects in FXS (Ronesi et al., 2012) and gives rise to the hypothesis that FMRP is not a regulator of general PI3K signaling but controls a specific subset or network of the PI3K-signaling complex in neurons via regulation of proteins that tether PI3K activity to certain receptors (Figure 5).

Reducing PIKE Rescues Impairments in Stimulus-Induced Signaling and Protein Synthesis, Dendritic Spine Morphology, and Synaptic Plasticity in Fmr1KO Mice

The capability of a neuron to adjust intracellular signaling and synthesize new proteins in response to external stimuli is essential for enduring forms of synaptic plasticity underlying behavior and cognition (Sutton and Schuman, 2006). In FMRP-deficient neurons, basal activity of the PI3K subunit p110β and general protein synthesis are increased and stimulus insensitive (Gross et al., 2010; Osterweil et al., 2010; Weiler et al., 2004). Here, we show that genetic reduction of CenG1 restored...
mGlu1/5-induced increases in protein synthesis and p110β activity in Fmr1KO mice (Figures 2A–2C). Our observations suggest that PIKE is a key mediator of FMRP’s function to control activity-mediated neuronal signaling and protein synthesis and thus synaptic plasticity. Further corroborating these results, Centg1 heterozygosity rescued exaggerated mGluR-LTD in Fmr1KO hippocampus (Figures 2F and 2G) and reduced the increased dendritic spine density in hippocampal CA1 neurons from Fmr1KO mice (Figures 2D and 2E).

We noticed that, for the most tested molecular, cellular, or behavioral phenotypes, Centg1 heterozygosity had no significant effects in wild-type mice, although PIKE protein levels were reduced. We speculate that, in wild-type, reduced levels of PIKE are compensated by other known modes of PI3K activation, for example, through PIKE-L association with Homer and regulation of homotypic G proteins (Guillermet-Guibert et al., 2008). However, neurons seem vulnerable to elevated and unregulated PIKE in the absence of FMRP, suggesting that increased PIKE plays an important and specific role in FXS-associated phenotypes.

**A Role for Increased Expression of PIKE in PI3K-Independent Neocortical Hyperactivity and in Impaired Behavior in Fmr1KO Mice**

Loss of FMRP in patients and animal models leads to increased susceptibility to epileptic seizures, increased neuronal network activity, and general neuronal hyperexcitability (Gonçalves et al., 2013; Hays et al., 2011). Here, we show that genetic reduction of PIKE reduces the prolonged duration of bursts of spontaneous neocortical activity (UP states; Figures 3A and 3B) that are observed in Fmr1KO mice (Hays et al., 2011). Notably, whereas UP states are sensitive to mGlu5 inhibition, they do not depend on new protein synthesis (Hays et al., 2011) or on PI3K signaling (Figures S3A and S3B), suggesting additional roles of PIKE in mGlu1/5 function apart from PI3K/mTOR-mediated protein synthesis, for example, through PIKE-L association with Homer and regulation of homotypic G proteins (Guillermet-Guibert et al., 2008). Moreover, PIKE isoforms were shown to have other roles apart from PI3K activation, which could also contribute to the observed rescue of UP states, such as direct binding of PIKE-L to GluA2 and GRIP2 (Chan et al., 2011a).

Centg1 heterozygosity also significantly reduces the susceptibility to audiogenic seizures in Fmr1KO mice (Figure 3C). Absence of PI3K in Centg1 heterozygous mice leads to higher susceptibility to kainic-acid-induced excitotoxicity and seizures (Chan et al., 2012). Our results suggest that Centg1 heterozygosity reduces neuronal excitability also in wild-type, although none of the effects were significant. Reduced PIKE levels may thus protect neurons from hyperactivity in these experimental settings.

Genetic reduction of Centg1 rescued impaired nesting behavior and obsessive marble burying in Fmr1KO mice (Figures 3D–3F), suggesting a role of increased PIKE in autistic-like behavior. Increased PI3K/mTOR signaling has been implicated in autism, but the Centg1 gene has not been previously associated with autism susceptibility.

**Improvement of Neuronal Function by Genetic Reduction of PIKE in FXS Is Conserved across Species**

Here, we show that increased PI3K signaling occurs in a Drosophila model of FXS and can also be genetically targeted to correct phenotypes. Genetic reduction of the Drosophila Centg1 ortholog CenG1A reduced excess PI3K signaling and rescued lethality, axonal overgrowth, as well as impaired synaptic plasticity and activity-regulated protein synthesis (Ben-Asher et al., 2008; Hong et al., 2011a; Hays et al., 2011; Ronesi et al., 2012). Moreover, PIKE isoforms were shown to have other roles apart from PI3K activation, which could also contribute to the observed rescue of UP states, such as direct binding of PIKE-L to GluA2 and GRIP2 (Chan et al., 2011a).

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short-term memory caused by the absence of dFMR1 (Figure 4). These findings in the fly model are similar to observations in Fmr1KO mice and thus suggest a conserved function of FMRP to regulate PIKE and PI3K signaling. However, as of now, it is not known whether CenG1A mRNA is a target of dFMR1 in flies, and more work is needed to identify dFMR1 mRNA targets in flies that are translationally dysregulated in dFmr1 mutant flies.

Whereas PIP3/PIP2 ratios in CenG1A heterozygous flies were reduced in both wild-type and dFmr1 mutant background, downstream signaling and gross mushroom body morphology were not affected in wild-type. However, CenG1A heterozygous flies were impaired in courtship memory, suggesting a PIKE dosage-sensitive regulation of this type of cognition. Given these results and the importance of PI3K signaling in axonal growth and guidance in the Drosophila visual system (Song et al., 2003), we speculate that CenG1a heterozygosity could have caused some more-subtle defects in mushroom body morphology in wild-type flies that were not detected by our assessment of FXS-typical loss of β-lobes. Our results showing a dosage sensitivity of memory function to PIKE and PI3K activity are in line with earlier reports showing that learning in flies is dosage sensitive to the cAMP-signaling pathway: both the dnome mutant, which is impaired in cAMP phosphodiesterase, as well as the rutabaga mutant, which has a gene defect in Ca2+/calmodulin-dependent adenylyl cyclase, are impaired in associative learning (Tully and Quinn, 1985). These observations suggest that, similarly as in the case of PIKE and PI3K activity, either too much or too little cAMP signaling can have adverse effects on cognitive function.

Our study reveals that genetically reducing PIKE, a confirmed FMRP target, can rescue diverse FXS-associated phenotypes at the molecular, physiological, and behavioral levels in animal models. These findings provide insight into the mechanisms of dysregulated mGlu1/5 signaling in the absence of FMRP by showing that reducing the increased expression of PIKE, a critical mediator of mGlu1/5-dependent downstream signaling, rescues FXS-associated neuronal impairments on multiple levels. Notably, this strategy rescues protein-synthesis-dependent (LTD) and -independent neuronal defects (UP states) in FXS, suggesting that increased PIKE is not just a mediator of dysregulated protein synthesis but plays additional roles in mTOR- and protein-synthesis-independent dysfunctions of the mGlu1/5 complex in the absence of FMRP (Figure 5). So far, it is unknown whether the observed rescue is mediated mainly by reduction of the long isoform of PIKE that tethers mGlu1/5 to downstream PI3K signaling or whether the overall reduction of all PIKE isoforms underlies the improvement of FXS-associated phenotypes. In the future, to further test the mGluR theory of FXS, it will be interesting to explore whether targeting specifically the long isoform of PIKE, e.g., through blocking interactions of PIKE with mGlu1/5 receptor complexes, may also rescue phenotypes in the FXS mouse model.

EXPERIMENTAL PROCEDURES

Mice and Flies
Mice were generated by crossing female Fmr1NET mice (The Jackson Laboratory) with male Centg1 heterozygous mice (Chan et al., 2010) and were geno-
and recorded as a function of 10-μm segments on the dendrites using Fiji Imaging software. Dendrites were between 60 and 120 μm in length. We analyzed three to five brains per genotype, four to eight neurons per brain, and one dendrite per neuron.

**Audiogenic Seizures**

Mice were tested in a plastic chamber covered with a Styrofoam lid containing a 120-dB personal security alarm as described in Ronesi et al. (2012). The alarm was presented to the mice for 5 min. Mice were observed or videotaped during the entire procedure, full tonic-clonic seizure (sometimes followed by death caused by respiratory arrest) was counted as positive, and wild running behavior was not counted as seizure.

**UP States**

UP states were performed as described previously (Hays et al., 2011). For details, see the Supplemental Experimental Procedures.

**Hippocampal mGluR-LTD**

DHPG-induced mGluR-LTD on brain slices was performed as described previously (Bhattacharya et al., 2012). For details, see the Supplemental Experimental Procedures.

**Nest-Building Behavior**

Nest building was assessed as described in Deacon (2006). Briefly, male mice at postnatal day 30 were placed in a fresh cage with standard bedding supplemented with 3 g of fresh nestlet between 4 and 6 p.m. at the start of the experiment. 72 hr later, nests were assessed using the score proposed by Deacon (2006), unused nestlet was weighed, and all nestlet material was replaced by 3 g of fresh nestlet. After 24 hr, nests were assessed as described above.

**Marble Burying**

Marble burying was assessed as described previously (Bhattacharya et al., 2012), with the following modifications. Twenty dark blue small glass beads were placed in a 5 × 4 grid on fresh 8-cm deep bedding. Mice were left in the cage for 15 min. Marbles covered 50% or more were scored as “buried.” Mice were always tested between 12 and 3 p.m. Mice were tested in nesting behavior prior to the marble-burying assay.

**Analysis of Drosophila Mushroom Body Morphology**

Flies were collected at days 0–2 post-eclosion. For western blot analyses and PIP3/PIP2 quantifications, fly heads were dissected on dry ice and stored at −80°C until further processing. For mushroom body analyses, fly brains were dissected and immediately fixed in 4% PFA, processed for anti-Fasciclin II staining (Wu and Luo, 2006), and mounted on microscope slides using VectaMount AQ aqueous mounting medium (Vector Laboratories). Sections were imaged using a Zeiss LSM 710 confocal or a Leica SP8 Multi Photon microscope and processed using ImageJ (NIH). Brains were analyzed for β-lobes fusion across the midline as described previously (Michel et al., 2004).

**Analysis of Drosophila Courtship Behavior**

Courtship short-term memory was assessed as described previously (McBride et al., 2005). Briefly, virgin male flies were collected within 4 hr of eclosion, aged for 5–7 days, and transferred to fresh food in individual small food tubes the night before testing. Virgin females were collected within 2 hr of eclosion and kept in groups of 10–15. Mated females were observed to mate with a male the night before training. The courtship index (CI) was calculated as the percentage of time spent courting during the observation time, which was either 10 min or until successful copulation (Siegel and Hall, 1979). Memory index was calculated as the relative difference between the mean CI of trained and naive flies ((CI)naive-CI(trained))/CI(naive); Kelemen et al., 2012.

**Western Blot Quantification**

For western blot analyses, equal amounts of protein were loaded and western blots were quantified densitometrically using ImageJ (NIH) and normalized to α-tubulin.

**Data Acquisition and Statistical Analyses**

Experimenter's were blind to the genotype at the time of the experiments. All statistics were performed with SigmaStat v.3.1, GraphPad Prism6, or SPSS using the appropriate tests as indicated in each figure. For details, see the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cerep.2015.03.060.

**AUTHOR CONTRIBUTIONS**


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