Fragile X Mental Retardation Protein (FMRP) controls diacylglycerol kinase activity in neurons

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Fragile X syndrome (FXS) is caused by the absence of the Fragile X Mental Retardation Protein (FMRP) in neurons. In the mouse, the lack of FMRP is associated with an excessive translation of hundreds of neuronal proteins, notably including postsynaptic proteins. This local protein synthesis deregulation is proposed to underlie the observed defects of glutamatergic synapse maturation and function and to affect preferentially the hundreds of mRNA species that were reported to bind to FMRP. How FMRP impacts synaptic protein translation and which mRNAs are most important for the pathology remain unclear. Here we show by cross-linking immunoprecipitation in cortical neurons that FMRP is mostly associated with one unique mRNA: diacylglycerol kinase kappa (Dgkk), a master regulator that controls the switch between diacylglycerol and phosphatidic acid signaling pathways. The absence of FMRP in neurons abolishes group 1 metabotropic glutamate receptor-dependent DGK activity combined with a loss of Dgkk expression. The reduction of Dgkk in neurons is sufficient to cause dendritic spine abnormalities, synaptic plasticity alterations, and behavior disorders similar to those observed in the FXS mouse model. Overexpression of Dgkk in neurons is able to rescue the dendritic spine defects of the Fragile X Mental Retardation 1 gene KO neurons. Together, these data suggest that Dgkk deregulation contributes to FXS pathology and support a model where FMRP, by controlling the translation of Dgkk, indirectly controls synaptic proteins translation and membrane properties by impacting lipid signaling in dendritic spine.

Fragile X syndrome (FXS), the most common cause of inherited intellectual disability and autism, is due to the transcriptional inactivation of the Fragile X Mental Retardation 1 gene (FMR1) (1, 2). The FMR1 knockout (KO) mouse (Fmr11/1) replicates phenotypes similar to human symptoms—including autistic-like behaviors, cognitive deficits, and hyperactivity—as well as abnormal dendritic spine morphology (3). FMR1 encodes Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein associated to polyribosomes and involved in the translational control of mRNAs important for synaptic plasticity (4). Consistent with a posttranscriptional function, the absence of FMRP in Fmr11/1 mouse causes abnormal signaling of group 1 metabotropic glutamate receptors (mGLUR1), leading to several forms of abnormal synaptic plasticity that rely on protein translation (5, 6). Protein expression analyses confirmed a role of FMRP in neuronal translational control (7), but the extent of this control remains unclear. Although several studies focusing on individual mRNA targets (e.g., Fmr1, Map1b, Pdd5, App, etc.) suggest specific control by FMRP (2), studies of global translation, including in vivo labeling in the Fmr1+− mouse (8), showed thousands of neuronal proteins deregulated in the absence of FMRP, pointing toward a general translational derepression. Studies seeking to identify the transcripts controlled by FMRP identified candidate mRNAs numbering in the hundreds to thousands (9–13). The lack of obvious overlap between several of these candidate lists calls into question the RNA binding specificity of FMRP (13). How FMRP controls the translation of hundreds of distinct proteins, whether by direct mRNA binding or in an mRNA-independent manner [e.g., through its direct binding with the ribosome (14)], is still unclear. With these hundreds of potential target mRNAs, another key question still awaits an answer: Are there specific mRNAs whose deregulation matters the most for the pathology? Cross-linking immunoprecipitation (CLIP) is a powerful technique to capture the cognate targets of RNA binding proteins (15). Intriguingly, prior CLIPs on FMRP showed rather modest overlaps (13). Whether influenced by the choice of starting material (brain...
extracts vs. nonneuronal proliferating cells) or the experimental construction (endogenous vs. tagged protein; whole cell content vs. subcellular fraction; etc.), these studies focused more on determining the binding motives rather than on identifying all of the mRNA species bound to FMRP. Using a CLIP strategy in mouse cortical neurons that preserves the integrity of mRNAs and comparing the RNAs immunoprecipitated in Fmr1−/− vs. Fmr1+/− extracts, we find that FMRP mostly associates with a single mRNA species. This transcript encodes diacylglycerol kinase kappa (Dgkκ), a member of the master regulator DGK family. Dgkκ acts as a spatiotemporal switch between the diacylglycerol (DAG) and phosphatidic acid (PA) signaling pathways, downstream of mGluRI and upstream of general translation activation control. Our data reveal that the deregulation of mGluRI-dependent DGK activity is a substantial contributor to FXS symptoms observed in the mouse model, one that can explain the paradoxical global protein synthesis deregulation observed in Fmr1−/− neurons.

Results

FMRP Associates with One Main mRNA Species in Mouse Cortical Neurons, Dgkκ: mRNA. To identify the mRNAs associated with FMRP, we performed a CLIP approach on dissociated cortical neurons. To control for cross-reaction of anti-FMRP antibodies to other RNA-binding proteins (such as the FMRP paralogs FXXRIP or FXR2P), we performed the CLIP both on neurons from wild-type (Fmr1+/+) and Fmr1 KO (Fmr1−/−) mice (Fig. S1 A–C). (All raw data of the figures can be provided upon request.) The H120 polyclonal anti-FMRP antibody was used based on its ability to efficiently immunoprecipitate FMRP (Fig. S1D) together with several mRNAs considered as validated FMRP targets (e.g., Dlg4, Map1b, CamK2a, and Arc), in comparison with mRNAs considered as non-FMRP targets (PO, Ghbp, Actb, and 28S) (Fig. S1E). Because prior CLIP-sequencing studies did not identify the G-quadruplex motif, the highest affinity binding motif known for FMRP (11, 12), we kept the RNAs intact to perform random reverse transcription followed by microarray so that GC-rich or other sequencing-resistant motifs would not impede the mRNA identification. Aside from Fmr1 itself (whose mRNA expression is prevented by the KO design), only two genes showed significantly altered expression when comparing the total RNA levels from Fmr1−/− and Fmr1+/− neurons: ApoC7 and Insm1/S (Dataset S1). Both transcripts showed an approximately 50% reduction in expression in Fmr1−/− mutant neurons, and neither has an obvious link to FXS, indicating that a loss of FMRP has virtually no impact on the whole transcriptome profile of neurons. Among 28,853 interrogated transcripts, 596 are enriched by CLIP in Fmr1−/− compared with Fmr1+/− neurons (as scored by enrichment in Fmr1−/− compared with Fmr1+/− extracts, relative to input amounts; Fig. 1A and B) at a P value ≤ 0.05 compared with 298 transcripts that are depleted (Fig. 1L). This difference increases exponentially with decreasing P value (Fig. 1B), confirming the FMRP specificity of the approach. More than 20% (126 of 596) of the CLIP-identified transcripts have been identified in the previous study by Darnell et al. (11) (Fig. S1F). Of the 596 CLIP-identified transcripts, only 7 mRNAs are enriched by more than twofold. The remaining transcripts have CLIP efficiencies that are thus mostly resulting from nonspecific binding. Dgkκ mRNA strikingly stands out as the sole mRNA with a CLIP efficiency well above any other mRNA. Consistent with this observation, all 27 microarray probe sets covering Dgkκ mRNA show a high CLIP signal, indicating that Dgkκ mRNA is cross-linked to FMRP as an entirely 8.2-kb transcript (Fig. S1G).

The results of the microarrays were confirmed by quantitative RT-PCR (qRT-PCR) for Dgkκ and 42 other RNAs, including the RNAs with next-best high CLIP score (Thn2, Pyp1a3, Ogdh, Apc, etc.) and previously proposed as targets (11, 12) (Dgkκ, CamK2a, Agap2, Shank3, etc.) (Fig. 1C). Comparison of CLIP efficiencies of microarray and by qRT-PCR for these 43 RNAs shows that Dgkκ is by far the mRNA most efficiently and reproducibly communoprecipitated with anti-FMRP H120 in cortical neurons (Fig. 1D). Apart from Dgkκ, 10% of the other tested mRNAs (10 of 42) have CLIP efficiencies that are consistent between the microarray and qRT-PCR data. The variability in CLIP efficiency of the remaining mRNAs is probably a result of their weak interaction or cross-linking efficiency with FMRP. To control that the CLIP was not biased by the antibody used, we repeated it with validated 7G1-1 antibody (9). Again, the comparison of Dgkκ CLIP efficiency with that of 14 different mRNAs, including mRNAs previously established as target (APC, Map1b, Dlg4, etc.) as well as “negative” mRNAs (Acot, 28S, etc.), shows that Dgkκ is the mRNA with the strongest CLIP efficiency for FMRP (Fig. S1H).

To determine whether the FMRP–Dgkκ mRNA interaction revealed by the CLIP approach was due to a cross-linking artifact, we analyzed the interaction between FMRP and Dgkκ mRNA by in vitro binding assays. Human His-tagged FMRP binds to a FMR1 mRNA fragment (N19) with the highest affinity known previously (12, 16) (Fig. 2A). Dgkκ transcript easily displaces FMR1 mRNA fragment N19, contrary to nonspecific RNAs (antisense Dgkκ transcript) or RNAs with lower CLIP efficiency (Dlg4) (Fig. 2A). These data indicate that human FMRP binds to mouse Dgkκ mRNA with the highest affinity currently identified compared with any other transcript.

FMRP Positively Controls Dgkκ Translation. We developed a polyclonal antibody against Dgkκ and validated it using Dgkκ expressing vector in COS cells (Fig. S2A). Remarkably, in dissociated cortical neurons [7 days in vitro (DIV)], the main protein product detected by the antibody is severely reduced in absence of FMRP (Fig. 2B). These data suggest that FMRP is required for the effective translation of Dgkκ mRNA. To confirm an impact of the absence of FMRP on Dgkκ translation in brain, we analyzed the profile of Dgkκ mRNA in mouse brain polysomes using qRT-PCR. In Fmr1−/− brain extracts, Dgkκ mRNA is primarily associated with light and heavy polysomes, consistent with a normal translation rate (Fig. 2C). By contrast, in Fmr1−/− extracts, Dgkκ mRNA is less present in polysomal fractions and more present in monosomal fractions, whereas the distribution of other tested mRNAs Dlg4 and Map1b is not altered. These data are in agreement with an impairment of Dgkκ translation in absence of FMRP.

DGK Activity Is Deregulated in the Absence of FMRP. Dgkκ converts DAG into PA (Fig. 2D). We assessed PA and DAG levels in dissociated cortical neurons using a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) lipidomics approach. PA profiling using multiple reaction monitoring (MRM) MS/MS mode revealed three major PA species in Mouse Cortical Neurons, (12, 16) (Fig. 2E). We assessed PA and DAG levels in dissociated cortical neurons using a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) lipidomics approach. PA profiling using multiple reaction monitoring (MRM) MS/MS mode revealed three major PA species (Fig. S2C), namely, 36:1, 38:1, and 38:2. PA level is maintained at low levels in cells by the activity of potent lipid phosphate phosphohydrolase (17), but increases rapidly in neurons on activation of mGluRs and its G protein-coupled phospholipase C, immediately upstream of DGKs (18). Increased 36:1, 38:1, and 38:2 PAs by 40% compared with nonspecific RNAs (antisense Dgkκ transcript) or RNAs with lower CLIP efficiency (Dlg4) (Fig. 2A). These data indicate that human FMRP binds to mouse Dgkκ mRNA with the highest affinity currently identified compared with any other transcript.
with \( \text{Fmr1}^{+/y} \) neurons. This increase affected most major DAG species (Fig. S2E). Upon Quis stimulation, a similar increase of total DAG level in \( \text{Fmr1}^{+/y} \) and \( \text{Fmr1}^{-/-} \) neurons indicated that the absence of FMRP does not impair DAG synthesis. Together, these data indicate that the defect of Quis-dependent PA synthesis and the excess of DAG observed in \( \text{Fmr1}^{-/-} \) neurons is due to a loss of DGK activity. To test whether a loss of DGK activity could be detected in humans, we analyzed postmortem cerebellar extracts of

Fig. 1. FMRP mostly targets \( \text{Dgk} \) mRNA in murine cortical neurons. (A) Volcano plot representation of the FMRP CLIP-microarray results. The x axis is log2 of fold change of average intensity for each individual dataset (AI) from \( \text{Fmr1}^{+/y} \) immunoprecipitated samples relative to \( \text{Fmr1}^{+/y} \) total RNA input compared with \( \text{Fmr1}^{-/-} \) immunoprecipitated samples relative to \( \text{Fmr1}^{-/-} \) total RNA input [i.e., \( \log2(\text{AI}^{\text{Wt CLIP}}/\text{AI}^{\text{Wt Input}})/\log2(\text{AI}^{\text{KO CLIP}}/\text{AI}^{\text{KO Input}}) \)]. The y axis is \(-\log(p\text{ value})\) with \( p\text{ value} \) determined by using the significance analysis of microarrays test (Materials and Methods) with \( n = 5 \) (i.e., one microarray per independent CLIP experiment per biological replicate). The name of a few mRNAs with high \( p\text{ value} \) or previously proposed as targets is given, and the arbitrary 0.05 \( p\text{ value} \) thresholds with corresponding number of genes are shown. (B) Representation of CLIP-identified transcripts number as a function of the \( p\text{ value} \) determined as in A. (C, Upper) qRT-PCR validation of immunoprecipitated mRNAs. The name of a few mRNAs with high \( p\text{ value} \) or previously proposed as targets is given, and the arbitrary 0.05 \( p\text{ value} \) thresholds with corresponding number of genes are shown. (C, Lower) Absence of differential expression of the 43 RNAs between \( \text{Fmr1}^{+/y} \) and \( \text{Fmr1}^{-/-} \) neuron extracts. Fold change of expression was calculated with \( \Delta\Delta\text{CT} \) method with \( \text{Rplp0} \) or \( \text{ActB} \) as normalizer. (D) Scatter plot representation of the fold change of clip efficiency of the 43 RNAs as in C, determined by microarray vs. by qRT-PCR (Left) and of their \( p\text{ value} \) (Right); gray zones indicate >0.05 \( p\text{ value} \).
FXS patients, the cerebellum being a region of the brain affected by FXS (20). Remarkably, an excess of DAG is observed in the FXS compared with the nonaffected samples (Fig. 2H).

Dgk\(\kappa\) Loss of Function Reproduces FXS-Like Phenotype. Dgk\(\kappa\) mRNA is expressed throughout the brain (Fig. S3A), but its involvement in synaptic plasticity was unknown. We focused on the
Fig. 3. Synthetic alterations induced by interference with Dgk expression. (A) Decrease in LTD in slice cultures infected with shRNA-Dgk. (n = 7) vs. shRNA-scramble (n = 6) expressing AAV viruses. *P < 0.05 (two-way ANOVA). (B) Increase in LTD in slice cultures infected with shRNA-Dgk (n = 7) vs. shRNA-scramble (n = 6) expressing AAV viruses. *P < 0.05 (two-way ANOVA).

Discussion

Based on a CLIP analysis of FMRP-bound mRNAs, we provide evidence that Dgks mRNA is a primary target of FMRP in cortical neurons. Surprisingly, although previous analyses have identified hundreds of potential FMRP-binding mRNA candidates, Dgks mRNA was not identified (9–12). Although we have no definitive answer as to why Dgks was not discovered before, several explanations can be proposed. First, it should be noted that Dgks was identified in 2005 (31); thus, it could not have been identified by initial microarray studies. A second factor is the starting biological material. Although we used pure neuron cultures, Ascano et al. (12) performed CLIP in kidney cells where Dgks may have been missed easily because its expression is 100-fold lower in kidney than in neuronal tissues (Fig. S3E). Darnell et al. performed CLIP on brain homogenate where Dgks should have appeared. However, whether Dgks CLIP efficiency is lower in brain homogenates compared with neuron cultures or whether Dgks was lost during the purification step of brain polyribosomes (performed to reduce the high complexity of whole brain extracted material) remains to be determined. Another factor is the bioinformatics treatment of data because Dgks suffers from database annotation problems. Human Dgks is annotated as a noncoding gene in genome build 37 (GRCh37) (this error has been corrected in GRCh38), and mouse Dgks lacks exon 1 (noted as unsequenced). These problems may have screened out Dgks during the bioinformatics analyses. Finally, other factors may have been critical for identification of Dgks. First, as explained in results, we kept mRNAs intact in cases where structured regions are difficult to amplify. Second, we normalized mRNA targets both to the input (to avoid problem of expression level) and to the signal from Fmr1 littersmates (to reduce the noise of unspecific binding). Finally, we quantified results both by microarrays and qPCR.

Our CLIP also identified another 595 mRNAs as significantly associated with FMRP (Fig. 1A). The lower and more variable CLIP efficiency of most of these mRNAs compared with Dgks suggests that they are occasional rather than bona fide interactors.

How FMRP specifically interacts with Dgks mRNA remains to be defined. None of the previously proposed motives bound by FMRP could be evidenced in Dgks (e.g., Dgks does not contain motifs that would readily establish a G-quadruplex structure). However, Dgks mRNA is highly conserved from mouse to human, with 77% nucleotide identity for coding regions and 68% for UTRs. Furthermore, the FMRP binding site is apparently sufficiently conserved to allow cross-species interaction between human FMRP and mouse mRNA (Fig. 2A). It will be important to define whether a specific RNA motif exists in Dgks mRNA specifying FMRP binding. In contrast with most of its previous characterized targets, FMRP positively controls Dgks translation (Fig. 2 B and C). How FMRP can stimulate Dgks translation is the next pending question. The alleviation of a miRNA repression, such as the one identified for FXR1, is a possibility (32). Upon identification of Dgks mRNA as a main interactor of FMRP, we focused our analysis on the biological significance of a Dgks deregulation in FXS Fmr1–/– mouse model. Dgks is a member of the DGK isozymes, master regulators of the balance between DAG and PA signaling (31). Several DGKs have been shown to be involved in structural and synaptic plasticity control (33–36), but no data were available on Dgks neuronal function. We identified well-characterized CA1 region of mouse hippocampus. We performed Dgk silencing in organotypic slices with a validated shRNA (Fig. S3 B–D). We examined the effect of Dgks silencing on long-term potentiation (LTP) and long-term depression (LTD), two forms of synaptic plasticity that are altered in Fmr1–/– mice (5, 21, 22). Theta burst stimulation (TBS)-induced LTP at Schaffer collateral-CA1 region was reduced with shRNA-Dgks (112% ± 3% at 30–40 min, instead of 135% ± 6% with shRNA-scramble) (Fig. 3D), whereas low-frequency stimulation-induced LTD was increased (83% ± 3% at 30–40 min, instead of 95% ± 3% with shRNA-scramble) (Fig. 3B). These data indicate that the reduction of Dgks in CA1 neurons induces synaptic plasticity alterations. Remarkably, these alterations are similar to the one observed in FXS mouse model (6). Approximately 50% of patients with FXS also carry an autism diagnosis, and recent research on hallmark autistic symptoms point toward striatal dysfunction (23, 24). To test whether Dgks loss of function could contribute to FXS phenotype, AAV9-delivered shRNA-Dgks was injected bilaterally into the striatum of wild-type mice. Injected mice were submitted for behavioral testing to assess autism-like behaviors, cognitive impairments, and hyperactivity, the most frequent FXS phenotypes. Four weeks after injection, shRNA expression was broadly spread across the striatal regions (Fig. A4 and Fig. S4 A–C). Consistent with autistic phenotype, animals expressing shRNA-Dgks show deficient social interaction, altered nest-building behavior, and spontaneous motor stereotypes compared with mice expressing shRNA-scramble (Fig. 4 B–D and Fig. S4 D–G), all striatal-dependent symptoms observed in Fmr1–/– mice (25–28). Stereotypic behavior is indicated by an enhanced learning on the accelerating rotarod (24), mainly at late stages of learning (Fig. 4E). In the novel object recognition (NOR) test, shRNA-Dgks-expressing mice showed repetitive patterns of exploration, evocative of cognitive inflexibility, but no detectable memory impairment (Fig. 4F). The absence of memory impairment in these animals, in contrast with Fmr1–/– mice, is a likely consequence of a striatal-centered shRNA expression, sparing cortices and hippocampus. Finally, shRNA-Dgks-expressing animals were hyperactive, crossing quadrants more frequently during NOR testing and traveling longer distances in activity boxes (Fig. 4G). Together, these data indicate that animals with striatal silencing of Dgks mimic autistic hyperactivity and hypersensitivity symptoms and recapitulate the core neurologic phenotypes observed in Fmr1–/– mice.

Finally, we examined the impact of Dgks loss of function on dendritic spine morphology and dynamics. Dgks silencing in the CA1 region of mouse hippocampal organotypic slices caused a strong increase of abnormally long and multilayered spines and a marked decrease of the proportion of mature spines (Fig. 5 A–E), whereas spine density remained unchanged (Fig. 5C). Additionally, there was a significant increase of spine turnover, as indicated by the increased rate of spine formation and elimination, associated with spine instability in Dgks silenced neurons (Fig. S5 A–C). These data indicate that Dgks is necessary for spine maturation and maintenance and that its loss leads to structural defects similar to those previously observed in the Fmr1–/– mice (29, 30). To establish a functional link between Dgks and FMRP, we tested whether the overexpression of Dgks within Fmr1–/– neurons could rescue the dendritic spine phenotype. Remarkably, Fmr1–/– neurons transfected with plasmid expressing Dgks had their spine defects corrected (Fig. 5 A–F, Fmr1–/– Dgks), indicating that Dgks overexpression is able to compensate for the lack of FMRP.
that the absence of FMRP leads to the deregulation of mGluRI-dependent DGK activity, causing perturbations in the balance between its substrate, DAG, and its product, PA. These two second-messengers signal via many pathways in a spatiotemporally coordinated manner. DAG signals trafficking, secretion, and cytoskeletal reorganization by binding and activating C1 domain-containing proteins in neuronal and immune tissues. These proteins include PKC isozymes, PKD, chimaerins, Unc13, and RasGRP (37, 38). PA signals cell growth by interacting with many effectors (e.g., mTOR, PAK1, PIP5K, PKCe, sphingosine kinase, Raf1, etc.). Both DAG and PA trigger general protein translation through the allosteric activation of their many effectors; their physiologic concentrations are thus critical for normal protein translational control.

We showed that DAG level is increased in nonstimulated Fmr1−/y neurons, as well as in human FXS cerebellum, suggesting that an excessive DAG signaling occurs in FXS. PKCa, one main DAG effector (39), has been shown to be strongly activated in Fmr1−/y mouse brain (40). One direct consequence of PKC activation in cells is an activation of general translation (41). The perturbation of DAG level could thus explain the excess of translation observed in Fmr1−/y neurons. Furthermore, PKCs have been shown to exert control over sensitization status of mGluRI-LTD by controlling the p38 MAPK pathway (42), and mGluRI-LTP in widespread areas of the brain (39). Thus, PKCs could be an important factor in FXS synaptic plasticity deregulation. We also showed that the mGluRI-dependent PA synthesis is abolished in Fmr1−/y neurons. This finding indicates a transient deficit in PA signaling. Among PA effectors, mTOR is proposed to be abnormally activated in Fmr1−/y mice (43). mTOR signaling regulates translation initiation; however, the deregulation of this pathway does not seem to be the cause of increased protein synthesis because its inhibition does not rescue the excess of translation (44). How the mTOR pathway is precisely deregulated remains unclear. Previous observations indicated that DAG triggers dendritic spine growth and destabilization, whereas PA is spine-stabilizing, indicating that synaptic levels of DAG and PA in neurons are determinants of the dendritic spine growth and stability (45). Thus, an increase of DAG and a lack of PA are expected to produce an increase in spine growth and a defect of spine maturation. This scenario is exactly what we observed in Fmr1−/y neurons. By controlling the switch between DAG and PA signaling, DGK activity is a key regulator of spine maintenance. Remarkably, besides Dgkκ, nine other DGK isozymes exist, but...
only Dgkκ mRNA is targeted by FMRP (Dataset S1). DGK deficiencies cause an imbalance between DAG and PA and affect cellular signaling (45). DGKs are mostly expressed in the brain, where (for those that have been studied) they influence dendritic spine morphology and synaptic plasticity (46, 47). The role of each DGK seems to differ based on its distinct localization or structure, suggesting that each has evolved specialized functions. It will be important to better define the specificity of each of these critical enzymes.

We showed that the reduction of Dgkκ expression in striatal areas of mouse brain reproduced the hyperactive and autistic-like behaviors similar to FXS and that the silencing of Dgkκ reproduces both morphological and functional spine abnormalities of Fmr1−/− mouse model. These results emphasize that Dgkκ is involved in the molecular basis of mGluR1-controlled mammalian synaptic plasticity, the deregulation of which accounts for several of the main neurological phenotypes found in FXS. Remarkably, the overexpression of Dgkκ within Fmr1−/− neurons rescued their spine defects, demonstrating the functional link between FMRP and Dgkκ activity.

The identification of the master-regulator Dgkκ as a main mRNA target of FMRP enables us to propose a novel molecular mechanism driving neuronal defects in the FXS mouse model. Instead of repressing general synaptic translation (the current proposed model), our data support a different model where FMRP is mostly dedicated to the positive control of Dgkκ. The defective regulation of Dgkκ in the absence of FMRP explains the dendritic spines and synaptic plasticity alterations observed in FXS. The increased level of DAG seen in Fmr1−/− unstimulated neurons would be a main triggering factor of the general local increase of synaptic protein synthesis seen in FXS neurons (e.g., by activating PKCs or other effectors) and the induction of abnormal spine growth. The lack of PA synthesis after mGluR activation, conversely, could be responsible for the defect in spine maturation and associated perturbations of synaptic plasticity via the failure to activate its numerous binding effectors (e.g., mTOR) and by impacting actin polymerization. Currently proposed therapeutic approaches mostly target the excess of protein synthesis observed in FXS neurons. Our present data suggest that this strategy may be insufficient: The defect of spine maturation that results from defective PA signaling and its associated lipid membrane alterations should be considered as well. DGK activity may represent a promising and novel therapeutic target.

Materials and Methods

Ethics Statement. Animal work involved in this study was conducted according to relevant national Comité National de Réflexion Ethique en Expérimentation Animale and international guidelines (86/609/CEE).

**Primary Cortical Neuron Cultures.** Cortices from C57BL/6j Fmr1−/+ or Fmr1−/− mouse embryos (E17.5) were dissected in 1× PBS, 2.56 mg/mL b-glucose, 3 mg/mL BSA, and 1.16 mM MgSO4; incubated for 20 min with 0.25 mg/mL trypsin and 0.08 mg/mL DNase I; and mechanically dissociated after supplementation of medium with 0.5 mg/mL trypsin soybean inhibitor, 0.08 mg of DNase I and 1.5 mM MgSO4. The cells were plated on poly-L-lysine hydrobromide-coated six-well culture plates for 8 d in Neurobasal Medium (GIBCO) supplemented with B27, penicillin/streptomycin, and 0.5 μM l-glutamine.

**CLIP on Primary Neurons.** CLIP strategy was adapted from Ule et al. (15) with modifications for neuron cultures. Neurons from Fmr1+/− or Fmr1−/− mice grown in six-well plates for 8 DIV were gently washed with cold PBS and exposed to 254-nm UV (400 μJ/cm2), in Stratalinker 2400 on ice. Neurons from three littermate embryos with the same genotype were pooled (constituting one biological sample) and lysed with 1 mL of lysis buffer [50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, 0.1% SDS, 1% Nonidet-P-40, 0.5% sodium deoxycholate, 30 U of anti-Nase (Ambion), and 10 U of DNase Turbo (invitrogen)] for 5 min at 37 °C. Lysates were spun down at 18,000 × g at 4 °C, and supernatants were precleared by incubation with 50 μL of free Dynabeads protein G (Dynal) and then on 50 μL of Dynabeads protein G coupled to rabbit anti-mouse IgGs (5 μg) for 1 h each. The lysates were then incubated overnight with agitation on 50 μL of Dynabeads protein G coupled to 5 μg of anti-FMRP antibody (H-120; Santa Cruz). After immunoprecipitation, the supernatants were saved for RNA extraction (Fmr1+/− and Fmr1−/− inputs), and the beads were washed three times with 1 mL of high-salt washing buffer (50 mM Tris-HCl, pH 7.4, 1 M KCl, 1 mM EDTA, 1% Nonidet-P-40, 0.5% sodium deoxycholate, and 0.1% SDS; 4 °C). RNAs from Fmr1+/− and Fmr1−/− neurons were recovered by treatment of the beads with 0.4 mg of protease K in buffer (100 mM Tris-HCl, pH 7.4, 4 °C).

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**Fig. 5.** Dendritic spine alterations and rescue with Dgkκ expression modulation. (A) Illustration of the changes in spine morphology in Fmr1−/− (Left) or Fmr1+/− (Right) CA1 pyramidal neurons transfected with pAAV-EGFP-shRNA-scramble (Upper Left), pAAV-EGFP-shRNA-Dgkκ (Lower Left), pAAV-EGFP (Upper Right), and pAAV-Dgkκ (Lower Right). Note the presence of multihedged spines (stars) as well as very long thin spines (arrows). (Scale bar: 2 μm.) (B) Spine changes (new spines, +, and lost spines, −) occurring in shRNA-scramble-expressing pyramidal neurons. (Scale bar: 2 μm.) (C) Absence of changes in spine density under the four conditions. ns, not significant. (D) Increase in multihedged spines and decrease in mature spines (stubby-mushroom) in shRNA-Dgkκ (sh-Dgkκ) (n = 10) vs. shRNA-scramble (sh-scramble) transfected cells (n = 8) and in Fmr1−/− (n = 9) vs. Fmr1−/−+Dgkκ transfected cells (n = 8). *P < 0.05; **P < 0.01; ***P < 0.001 (two-way ANOVA with Bonferroni posttest). (E) Distribution of spine length in shRNA-Dgkκ (n = 342 spines), shRNA-scramble (n = 297 spines), Fmr1−/− (n = 359 spines), and Fmr1−/−+Dgkκ (n = 377 spines) transfected cells (P = 0.13, Kolgomorov–Smirnov test). (F) Decrease in spine stability over time. *P < 0.05 (two-way ANOVA with Bonferroni posttest).
50 mM NaCl, and 10 mM EDTA) for 20 min at 37 °C. The H120 polyonal anti-FMRP antibody was selected among several anti-FMRP antibodies (i.e., Dilg, Map1b, CamK2a, and Arca) compared with mRNAs considered as non-FMRP targets (PO, Glrb, Actb, and 28S) in Fmr1 Tr/M− compared with Fmr1 Tr/M+ neuron extracts as quantified by qRTPCR. The RNA was resupended in water, and its quality was checked on a 10th of the peak area, determined by using

Brain Polyribosome Profiling. Brain from 10- to 15-day-old C57BL/6J Fmr1 +/+ or Fmr1 Tr/M− mouse embryos were dissected and homogenized with 10 strokes of Dounce homogenizer in 0.7 mL of ice-cold lysis buffer (50 mM Tris HCl, pH 7.4, 100 mM KCl, 5 mM MgCl2, 1 mM DTE, 40 μM RNasin, and 1% Nonidet P-40). Extracts were cleared by 18,000 × g centrifugation for 15 min, and supernatants were loaded on linear sucrose gradient 15-45% (w/v/wt) in buffer (50 mM Tris HCl, pH 7.4, 100 mM KCl, and 5 mM MgCl2) in SW41 tubes. Gradients were centrifuged at 36,000 rpm for 2 h at 4 °C in a Beckman SW41 rotor. Gradients were collected from 12 fractions of 1 mL by using peristaltic pump coupled to AKTA (GE) detector and collector apparatus. Fractions were precipitated with 2 volumes of cold ethanol at −20 °C. Pellets obtained after centrifugation at 20,000 × g for 20 min were washed with 80% cold ethanol and briefly dried and resuspended in 20 μL of water. Total RNA was extracted from each fraction by acidic phenol/chloroform (1:1 v/v) followed by chloroform extraction and an ethanol precipitation. RNA was resupended in 20 μL of water, and its quality was checked on a 10th of the material by electrophoresis in 1% agarose gel with ethidium bromide. cDNA synthesis and qRTPCR were performed as described above.

Neuron Treatments. mGluR1 agonists DHPG resuspended in DMSO (Tocris) or Qis resuspended in water (Tocris) were applied on neurons at concentrations of 100 and 5 μM, respectively, for 10 min at 37 °C. DGK inhibitors R59022 (DGK Inhibitor I; Calbiochem) and R59949 (DGK Inhibitor II; Calbiochem) were applied at concentrations of 3 and 0.2 μM for 15 min at 37 °C prior to an eventual mGluR treatment.

PA Profiling by LC-MS/MS. Neurons of 8 DIV cultures (treated or not) from a single six-well plate well were washed twice with cold PBS 1X and lysed in cell extraction buffer (FNN0011; Invitrogen) supplemented with protease inhibitors (complete protease inhibitor mixture; Roche) and PMSF (1 mM) for 30 min, on ice, with vortexing at 10-min intervals. Afterward, neuron extracts were centrifuged for 18,000 × g for 10 min, and proteins were quantified by the Bradford method to normalize samples. Total lipids were extracted by the method of Bligh and Dyer (50). Typically, extracts were mixed with chloroform:methanol (4:1), vortexed for 10 s, and left under nitrogen for 1 h at 4 °C. After 5-min centrifugation at 18,000 × g, organic phase was recovered and used for MS analyses. The organic phase (150 μL) was analyzed by UPLC/MS/MS (Acquity UPLC System; Waters Corp.) coupled to a Quattro Premier XE triple Quadrapole MS System; Waters Micromass).

Column was a Waters Acquity UPLC BEH Amide column (21 mm × 100 mm, 1.7-μm particle size) together with an Acquity UPLC BEH Amide pre-column (2.1 mm × 5 mm, 1.7-μm particle size). Temperature of column oven was set to 60 °C. Refractive index detectors were set to 15°C and 40 °C. Four MS transitions were monitored for each PA standard (DAG 17:0/17:0) from Nu-Check Prep was added. LC-MS/MS (MRM) analyses were performed with mass spectrometer model QTRAP 5500 (ABSciex) coupled to a LC system (Ultimate 3000; Dionex). Analyses were performed with MassLynx software (Version 4.1) running under Windows XP professional on a Pentium PC.

DAG Measurements by LC-MS/MS. Neuron lysates from one six-well plate well (prepared as for PA) and ≥ 80 mg of postmortem human brain samples (males, aged between 57 and 78, with one FXS aged 25) ground in a liquid nitrogen cooled mortar were extracted with 2 mL of chloroform/methanol 2/1 (v/vol), 1 mL of water, and 10 μL of synthetic internal standard (DAG 15:0/15:0) from Sigma Aldrich, sonicated for 2 min, vortexed, and centrifuged. The lower organic phase was combined and evaporated to dryness under nitrogen. Lipid extracts were resuspended in 50 μL of eluent A and a synthetic internal lipid standard (DAG 17:0/17:0) from Nu-Chek Prep was added. LC-MS/MS (MRM mode) analyses were performed with mass spectrometer model QTRAP 5500 (ABSciex) coupled to a LC system (Ultimate 3000; Dionex). Analyses were achieved in positive mode with a capillary voltage of 3.4 kV, RF lens at 0 V, resolution (LM1, HM1, LM2, and HM2) 15, and ion energy 1 and 2.05. Source and desolavation temperatures were 135 °C and 400 °C. Flow was 0.5 mL/min for nebulization and desolvation. Precisio of the argon collision gas was 3.0 × 10−3 mbar. Full scan, selected ion recording, and daughter scan mode were used for qualitative analyses. Quantitative PA analyses were made based on MS/MS MRM as described (51). Briefly, MRM transitions for each individual PA were determined based on PA standards obtained from Avanti Polar Lipids. The PAs were identified as deprotonated parent ions [M−H]+, and cone energy was optimized for each PA and set to 44 V. MRM transitions and specific retention times were used to selectively monitor PA. Data acquisition and analysis were performed with the MassLynx software (Version 4.1) running under Windows XP professional on a Pentium PC.
by placing a small piece of porous membrane on top of the CA1 area (White FHLC Membrane, Filter grade 0.45 μm, EMD Millipore) injected with 0.3 μL (5 × 10^12 viral genomes per mL) of either AAV9-shRNA-Dgk-κ or AAV-EGFP-shRNA-scrumble viruses. The injection of the porous membrane was carried out by using a picospritzer (Tootey). The porous membrane was removed after 1 h and the slices maintained under culture conditions. The efficacy of infection was verified in all slices before the electrophysiological recordings by checking the level and area of expression of GFP. Injected slice cultures were tested electrophysiologically at DIV 7–8 for LTD and DIV 14–15 for LTP and placed in an interface-type of recording chamber and continuously perfused with a medium containing (in mM): NaCl 124, KCl 1.6, CaCl2 2.5, MgCl2 1.5, NaHCO3 24, KH2PO4 1.2, glucose 10, and ascorbic acid 2; saturated with 95% O2 and 5% CO2 (pH 7.4; temperature 31 °C). EPSPs were evoked by stimulation of a group of CA3 cells by using a stimulating electrode made of twisted nichrome wires, and responses were recorded in the CA1 stratum region by using a glass patch pipette. LTD was induced by a low-frequency stimulation protocol (0 μA, 0.1 ms pulses at 5 Hz) as in ref. S3. EPSPs were recorded by using IGOR software, and variations in response size were assessed by measuring EPSP slopes and expressed as percent of baseline values.

StereoTactic Surgery and AAV Injections. Mice (C57BL/6) were deeply anesthetized with ketamine/xylazine (Virbac/Bayer, 100 mg/kg, 10 mg/kg, intraperitoneal) dissolved in sterile isotonic saline. One microgram of AAV9-EGFP-scrumble viruses in dry ice-ethanol and 37 °C baths, further treated with 100 U/mL Benzonase (Novagen) for 30 min at 37 °C, and clarified by centrifugation. Viral vectors were purified by iodixanol (Optiprep; Axis Shield) gradient ultracentrifugation followed by dialysis and concentration against PBS containing 0.5 mM MgCl2 (Novagen) for 30 min at 37 °C, and centrifuged by brief. Dendritic segments of CA1 transfected neurons (30 μm in length) were imaged from 12 to 15 DIV at 0, 5, 24, 48, and 72 h by using an Olympus Fluoview 300 system, and analysis of the Z-stacked images obtained was performed by using Osiris software.

Stereotoxic Surgery and AAV Injections. Mice (C57BL/6) were deeply anesthetized with ketamine/xylazine (Virbac/Bayer, 100 mg/kg, 10 mg/kg, intraperitoneal) dissolved in sterile isotonic saline. One microgram of AAV9-EGFP-scrumble viruses in dry ice-ethanol and 37 °C baths, further treated with 100 U/mL Benzonase (Novagen) for 30 min at 37 °C, and clarified by centrifugation. Viral vectors were purified by iodixanol (Optiprep; Axis Shield) gradient ultracentrifugation followed by dialysis and concentration against PBS containing 0.5 mM MgCl2 (Novagen) for 30 min at 37 °C, and centrifuged by briefly. Dendritic segments of CA1 transfected neurons (30 μm in length) were imaged from 12 to 15 DIV at 0, 5, 24, 48, and 72 h by using an Olympus Fluoview 300 system, and analysis of the Z-stacked images obtained was performed by using Osiris software.

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