Augmented noncanonical BMP type II receptor signaling mediates the synaptic abnormality of fragile X syndrome

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Epigenetic silencing of fragile X mental retardation 1 (FMR1) causes fragile X syndrome (FXS), a common inherited form of intellectual disability and autism. FXS correlates with abnormal synapse and dendritic spine development, but the molecular link between the absence of the FMR1 product FMRP, an RNA binding protein, and the neuropathology is unclear. We found that the messenger RNA encoding bone morphogenetic protein type II receptor (BMPR2) is a target of FMRP. Depletion of FMRP increased BMPR2 abundance, especially of the full-length isoform that bound and activated LIM domain kinase 1 (LIMK1), a component of the noncanonical BMP signal transduction pathway that stimulates actin reorganization to promote neurite outgrowth and synapse formation. Heterozygosity for BMPR2 rescued the morphological abnormalities in neurons both in Drosophila and in mouse models of FXS, as did the postnatal pharmacological inhibition of LIMK1 activity. Compared with postmortem prefrontal cortex tissue from healthy subjects, the amount of full-length BMPR2 and of a marker of LIMK1 activity was increased in this brain region from FXS patients. These findings suggest that increased BMPR2 signal transduction is linked to FXS and that the BMPR2-LIMK1 pathway is a putative therapeutic target in patients with FXS and possibly other forms of autism.

INTRODUCTION

Fragile X syndrome (FXS) is the most common heritable form of intellectual disability and autism spectrum disorder (ASD) (1–7). FXS is caused by an expansion of trinucleotide (CGG) repeats in the fragile X mental retardation 1 (FMR1) gene that leads to transcriptional silencing and loss of the FMR1 protein (FMRP) (3–7). FMRP is an RNA binding protein that contains three RNA binding domains: two K homology (KH) domains (KH1 and KH2) and one arginine-glycine-glycine–rich domain (8–11). FMRP binds mRNAs directly, modulating transport and stability and negatively regulating translation (12–15). A missense mutation in the KH2 domain (I304N) that abolishes RNA binding activity has been identified in a patient with FXS (16, 17). This suggests that the pathogenesis of FXS might be caused by the absence of the RNA binding function of FMRP. Both postmortem brain tissues from FXS patients (18, 19) and from young adult mice lacking FMRP (20, 21) exhibit widespread defects in synaptic plasticity and development, including an increased number of long and thin dendritic spines instead of the mature and strong mushroom-shaped spines. Transcripts of 40 genes have been identified as FMRP targets (22–25). Most encode proteins that are implicated in synaptic growth, plasticity, cell adhesion, or cytoskeletal structure and remodeling (15, 26). However, it is not known whether an increase in the abundance of these targets is responsible for the FXS phenotype or whether there are additional FMRP targets that contribute to the disease.

Bone morphogenetic proteins (Bmps) are members of the transforming growth factor–β (TGFβ) superfamily of secreted proteins (27, 28). Their receptors consist of type I and type II subunits, which are both serine/threonine kinases (28). BMPR2 is a type II subunit that, in addition to the kinase domain (KD) in its C-terminal domain (CTD) that is typical of type II subunits, has a unique, evolutionarily conserved ~500-amino acid region. BMP binding leads to activation of the type I receptor and activation of the canonical pathway through phosphorylation of SMAD1, SMAD5, and SMAD8 (28); it also leads to a “noncanonical” signal that is mediated by proteins, such as LIM (Lin-11 Isl-1 Mec-3) domain kinase 1 (LIMK1), that interacts with the CTD (29). Once activated by BMPR2, LIMK1 phosphorylates and inhibits cofilin to promote neurite outgrowth and dendritogenesis (29). In cortical neurons, activation of the noncanonical pathway also involves the p21-activated kinase 1 (PAK1), which facilitates the recruitment of the type 1 BMP receptor to BMPR2 and the subsequent activation of LIMK1 (30).

The BMPR2-CTD-LIMK1 signaling pathway is conserved between human BMPR2 and the Drosophila BMPR2 ortholog wishful thinking (Wit). Although the CTD sequence homology is only ~30%, both CTDs interact with LIMK1, promote cofilin phosphorylation, and induce actin remodeling (29–36). At the Drosophila neuromuscular junction (NMJ), Glass bottom boat, a Drosophila BMP ortholog that is released from the postsynaptic muscle, binds Wit and regulates neuromuscular synapse formation, stability, and function (31, 32, 37, 38). Mutant wit larvae exhibit a reduced number of mature boutons, and this phenotype can be rescued by Wit but not by Wit that lacks the CTD (32), supporting an essential role of the CTD-LIMK1 interaction and noncanonical signaling in synaptic...
stability and growth at the NMJ. BMPR2 mRNA is not among the previously known targets of FMRP. However, because published genome-wide analysis data imply that BMPR2 mRNA could be an FMRP binding partner and because BMP signaling is involved in synaptic formation in Drosophila, we investigated the role of BMPR2 and noncanonical BMP signaling in FXS.

RESULTS

Two isoforms of BMPR2 are differentially expressed

In mammals, alternative splicing of BMPR2 produces two mRNA forms: one “full length” (FL) that contains 13 exons and encodes a 1038-amino acid protein, and a shorter one (ΔCTD) that encodes a 530-amino acid protein. ΔCTD lacks exon 12 (ex12), which encodes 80% of the entire CTD (Fig. 1A). Immunoblot analysis of various tissues from adult mice reveals that the two isoforms are differentially expressed (Fig. 1B). However, although the ratio between BMPR2 protein isoforms varied from tissue to tissue, it did not correlate with the amount of the corresponding mRNA isoform (for example, ΔCTD in liver and heart; Fig. 1B). To investigate the cause of this discrepancy, we expressed the two BMPR2 mRNA isoforms from cytomegalovirus promoter complementary DNA (cDNA) constructs in COS-7 cells. Despite similar mRNA amounts, the ΔCTD protein was ~7 times more abundant than the FL protein in immunobLOTS probed with the BMPR2 antibody (Fig. 1C, lanes 2 and 3), or with a FLAG antibody and FLAG-tagged BMPR2 protein constructs (fig. S1, lanes 2 and 3). The different amounts of the two protein isoforms could not be explained by the stability of the correspondent transcripts because the ΔCTD mRNA was slightly less stable than the FL mRNA in an experiment in which de novo mRNA synthesis was blocked by actinomycin D, an inhibitor of RNA polymerase II (Fig. 1D). Furthermore, the stability of FL and ΔCTD proteins was similar over a period of 24 hours when new protein synthesis was inhibited by cycloheximide (Fig. 1E). These results strongly suggest that the BMPR2 isoforms are regulated posttranscriptionally.

BMPR2 translation is inhibited by the mRNA sequence encoding the CTD

To test whether this posttranscriptional regulation is caused by protein size or by the presence of the CTD coding sequence (hereinafter CTDseq), we introduced a nonsense mutation at amino acid 530 in the FL construct (530X; Fig. 2A). The 530X transcript matches the FL transcript in length, but the 530X-encoded protein has the size of the ΔCTD deletion mutant (Fig. 2A). The difference between the transcripts encoding ΔCTD and 530X is the presence of CTDseq (Fig. 2A). Upon transfection in COS-7 cells, we observed that the ΔCTD protein (Fig. 2A) was about three times more abundant than the FL protein (Fig. 2A), which is consistent with the expression data above (Fig. 1C). On the contrary, the expression of 530X and FL (Fig. 2A) was similar. Similar results were obtained by in vitro transcription and translation, suggesting that a cis-acting sequence (or sequences) within the CTDseq affects translational efficiency.

To test the effect of the CTDseq on transcripts originating from the endogenous genomic locus, we used two in vivo systems expressing a ΔCTD protein, one in which the CTDseq is deleted and one in which a frameshift mutation truncates the BMPR2 protein but preserves the CTDseq in the mRNA. We established two independent lines of primary pulmonary artery smooth muscle cells (PASMCs) from a heterozygous knock-in mouse in which ex12 of the BMPR2 gene has been replaced by a cassette encoding the enhanced green fluorescent protein (eGFP); the other allele is intact (Fig. 2B, top). Independent cell lines were established from two wild-type (Fig. 2B, lanes 1 and 3) and two Δex12 mice (Fig. 2B, lanes 2 and 4). In both wild-type PASMCs lines (Fig. 2B, lanes 1 and 3), only the FL protein is detected by immunoblot, indicating that FL is the dominant form of BMPR2 protein in PASMCs (Fig. 2B, right). In the two Δex12 cells lines (Fig. 2B, lanes 2 and 4), the amount of FL protein is reduced to ~50% (Fig. 2B, lanes 2 and 4) compared to wild-type cells.

Fig. 1. Different abundance of BMPR2 isoforms. (A) Schematic of the exon/intron structure (left) and the protein structure (right) of two isoforms of BMPR2: the FL and the ΔCTD-truncated isoform (ΔCTD). ECD, extracellular domain; TM, transmembrane domain; aa, amino acid. (B) Immunoblot analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) for FL and ΔCTD BMPR2 in brain, heart, liver, and kidney isolated from 12-week-old wild-type (WT) mice. Data are means ± SD of five experiments. **P < 0.05 by Student’s t test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (C) qRT-PCR analysis for BMPR2 expression in COS-7 cells transfected with vectors encoding MBP-BMPR2 [in which the BMPR2 ECD was replaced by maltose-binding protein (MBP)], FL or MBP-ΔCTD (ΔCTD) BMPR2, and treatment with actinomycin D (ActD) (5 μg/ml) 24 hours afterward for the period of time indicated. Expression was normalized to GAPDH. Data are means ± SD of five experiments. *P < 0.05 by Student’s t test. (D) Immunoblot analysis for BMPR2 in COS-7 cells transfected with optimized amount of MBP-BMPR2-FLAG (FL) or MBP-ΔCTD-FLAG (ΔCTD) expression vectors (to adjust for similar abundance of expressed protein) and treated with cycloheximide (CHX) (10 μg/ml) 48 hours afterward for the period of time indicated. Protein was normalized to β-actin. Data are means ± SD of four experiments.

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the lack of L504fs (Fig. 2C), which results in a premature termination at Met505. This result demonstrates a more efficient translation of the L504fs/wild-type heterozygous cells, L504fs and wild-type proteins were encoding the CTD.

Fig. 2. Translational regulation of BMPR2 through the mRNA sequence encoding the CTD. (A) Top: Schematic of the expected mRNA and protein structures of C-terminal FLAG-tagged FL, ΔCTD, and 530X BMPR2. CTDseq is the mRNA sequence encoding CTD. Bottom: Immunoblotting and qRT-PCR of FLAG or FL BMPR2 in WT and heterozygous cells. Blots in (B) and (C) are representative of two experiments. *P < 0.05; **P < 0.01 by ANOVA with post hoc Tukey’s test.

BMPR2 mRNA was normalized to GAPDH. Data are means ± SD of three experiments. *P < 0.05 by analysis of variance (ANOVA) with post hoc Tukey’s test. (B) Top: schematic of WT and BMPR2 Δex12 deletion mutant (Δex12) allele. In the Δex12WT mice, the ex12 of one allele of the BMPR2 gene was replaced with eGFP. Right: Immunoblot for BMPR2 abundance in two independent primary vascular smooth muscle cell cultures generated from WT (WT/WT) and heterozygous (Δex12/WT) mice. (C) Top: Schematic of WT and BMPR2 L504fs allele. A frameshift mutation was introduced in at Leu504 (ex11) of BMPR2 gene using TALEN-mediated genome editing technology (L504fs) in rat vascular smooth muscle PAC1 cells. Consequently, L504fs allele produces mutant BMPR2 lacking 504 to 1038 amino acids; however, the transcripts contain CTDseq. Right: Immunoblots for FL, BMPR2 and L504fs in WT and heterozygous cells. Blots in (B) and (C) are representative of two experiments.

FMRP associates with BMPR2 mRNA through CTDseq and controls its translation

FMRP binds a small subset of mRNAs and inhibits their translation (10, 14, 22, 40, 41). On the basis of the results of two independent high-throughput RNA immunoprecipitation (RIP) analyses (11, 22) in which BMPR2 appeared in the list of potentially FMRP-bound mRNAs, we hypothesized that FMRP might be involved in the translational inhibition of FL BMPR2. To first test whether FMRP associates with the CTDseq through putative FMRP binding motifs (FBMs; ACUK, WGGA, or a combination of both) (22), we transfected human embryonic kidney (HEK) 293 cells with the FL or ACTD expression constructs and performed an RIP assay. The FMRP-bound mRNAs were sheared to an average length of ~200 nucleotides to compare the enrichment of different regions of the FL or ACTD mRNAs (Fig. 3A). We investigated eight putative FBMs

Fig. 3. FMRP binds BMPR2-CTDseq and negatively regulates translation. (A) RIP assay for the relative enrichment of FL BMPR2 mRNA regions [schematic (top) marked with FMRP binding regions (black or red) and corresponding primers (orange; see table S2)] in FMRP immunoprecipitates from HEK293 cells transfected with FLAG-tagged FL or ACTD BMPR2 constructs. Data are representative of two experiments. Pulldown with non-specific immunoglobulin G (IgG) served as the control. nc, negative control primer; IP, immunoprecipitation. (B) RIP assays as in (A) and immunoblotting in HEK293 cells transfected with FL or mutant (Δ3240 or A4125) BMPR2. Data are means ± SD of three experiments. *P < 0.05; **P < 0.01 by ANOVA with post hoc Tukey’s test.
Table 1. List of potential FMRP binding sites identified in the BMPR2 transcripts by RIP sequence. On the basis of a previous research (16), potential FMRP binding sites were searched in the BMPR2 transcripts. Its core sequence (WGGA or ACUK) is underlined. Location of the RIP primers and the enrichment of RNA fragments are shown. NA, not applicable.

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Abnormalities at the NMJ in a loss-of-expression FMR1 Drosophila mutant are rescued by reducing Wit

The Drosophila NMJ is a powerful model system for uncovering and characterizing genetic and molecular mechanisms that regulate synaptic formation (36, 42). A loss-of-expression mutation of dFMR1, the Drosophila ortholog of FMR1, causes overgrowth of synaptic boutons in the larval NMJ (43). In contrast, loss-of-expression mutants of the Drosophila ortholog of BMPR2 wit exhibit reduced numbers of synaptic boutons (31, 37, 38). Because both Wit and dFMR1 are expressed in presynaptic motor neurons, we speculated that translational regulation of Wit by dFMR1 might be important for synaptic growth and stability at the NMJ. More precisely, if the overgrowth of boutons in dFMR1 mutants was due to increases of Wit and BMP signaling in presynaptic motor neurons, this phenotype could be rescued by reducing Wit abundance. To test this hypothesis, we crossed heterozygous (wit<sup>12/+</sup>) or homozygous (wit<sup>12B/w1</sup>) wit mutants with heterozygous dFMR1 mutants (dFMR1<sup>D113/+</sup>) and analyzed the number of mature synaptic boutons and branching synapses by immunofluorescence staining of the postsynaptic marker discs large (DLG; Fig. 5A, green) and phalloidin (Fig. 5A, red) on muscle 6/7 in the abdominal segment 3 (A3) of third instar larvae. We also co-stained samples with DLG (Fig. 5B, red) and the presynaptic marker horseradish peroxidase (HRP) (Fig. 5B, green) to distinguish mature synaptic boutons, which are co-stained with both HRP and DLG, from “synaptic footprints,” which are stained only with DLG (32). As previously reported (43), loss of expression of one copy of dFMR1 (dFMR1<sup>D113/+</sup>) resulted in ~75% increase in the number of mature boutons and ~60% increase in branching compared to wild-type animals (Fig. 5C). When one allele of wit was mutated in the heterozygous dFMR1 mutant background (dFMR1<sup>D113/+</sup>; wit<sup>12/+</sup>), the bouton number decreased by ~20% (Fig. 5C). Mutation of both wit alleles in the dFMR1 mutant background (dFMR1<sup>D113/+</sup>; wit<sup>12/w1</sup>) reduced both boutons and branching numbers to indistinguishable quantity from those observed in wit mutants (wit<sup>12/+</sup> or wit<sup>12B/w1</sup>) with wild-type background (Fig. 5C). Thus, the augmented synaptic morphology in the dFMR1 mutant is, at least in part, due to increased Wit abundance and signaling in presynaptic neurons, and both can be decreased by reducing wit gene dosage. Considering that they are orthologs, the epistatic relationship between dFMR1 and wit in the regulation of synaptic development at the NMJ is consistent with the translational regulation of BMPR2 by FMRP.

CTD-LIMK1-dependent signal promotes augmented dendritogenesis in FMR1-null neurons

In the Drosophila NMJ, presynaptic expression of a Wit mutant lacking the CTD fails to rescue synaptic stability in wit-null mutants (32). This failed rescue has been attributed to the failure of the Wit CTD mutant to activate dLIMK1 (the Drosophila ortholog of LIMK1), which is essential for promoting the stability of neuromuscular synapses (32). To compare the effect of FL and CTD in BMP-mediated axon remodeling and filopodia formation, we expressed each isoform in the neural crest–derived neuroblastoma cell line N1E-115 (N1E) and subjected them to BMP7 (also known as Osteogenic protein-1) stimulation followed by phalloidin staining (Fig. 6A). BMP7 was used in this experiment because it is expressed in the developing nervous system and is a potent inducer of dendritic morphogenesis in N1E cells (29) and rat sympathetic neurons (44). Phalloidin staining in control cells showed that BMP7 induced the formation of filopodia, which are fundamental for neurite outgrowth (Fig. 6A). In comparison, FL-expressing cells showed an increased intensity of both basal and BMP7-induced phalloidin staining and filopodia formation (Fig. 6A, top). We observed proper plasma membrane localization (45) and similar amounts of CTD and FL proteins (Fig. 6A, bottom left), which correlated to similar induction of the ID1 gene (Fig. 6A, bottom right), a readout of the

Fig. 4. FMR1 is a negative regulator of FL BMPR2 protein expression. (A) Immunoblotting and qRT-PCR for BMPR2 in COS-7 cells transfected with siRNAs against FMR1 (si-FMR1) or a control sequence (si-Ctr). Data are means ± SD of three experiments. *P < 0.01 by Student’s t test. (B) Immunoblotting and qRT-PCR for BMPR2 in stable HEK293 cell lines with doxycycline (Dox)–inducible expression of FLAG-tagged FMRP or GFP (control). Protein and mRNA abundance was normalized to GAPDH. Data are means ± SEM of three experiments. *P < 0.05 by ANOVA with post hoc Tukey’s test. C) Immunoblotting and qRT-PCR for BMPR2 in brain lysates from P7 FMR1<sup>−/−</sup> or WT mice. Data are means ± SEM of seven individual mice. *P < 0.05 by Student’s t test. Asterisk (*) indicates a nonspecific band in the FMRP immunoblot panel. In (A) to (C), protein and mRNA abundance was normalized to GAPDH.

fibroblasts (MEFs) derived from FMR1-null mice exhibited ~2-fold increase in BMPR2 (FL) protein compared to MEFs derived from wild-type littermates (fig. S3A). Upon BMP treatment, the phosphorylation of SMAD1/5, a hallmark of the “canonical” pathway downstream of BMPR2, was also increased (fig. S3A), whereas TGFβ signaling was not affected, suggesting that the effect of the loss of FMRP is BMP-specific (fig. S3B). Next, we collected brain samples from FMR1-null or control (wild-type) mice at postnatal day 7 (P7) and subjected them to immunoblot and qRT-PCR analysis of BMPR2 abundance and expression, respectively. At protein but not at mRNA level, a greater amount of FL isoform was detected in the brain of FMR1-null mice in comparison with wild-type mice (Fig. 4C). Unlike FL, the amount of CTD isoform was similar in both FMR1-null and wild-type mice (Fig. 4C). These results confirm an increase of FL isoform in the FMRP-depleted mouse brain and, thus, suggest that the increase in the CTD-mediated signal might contribute to the development of the neuronal phenotypes associated with FXS.
Reduction of the BMPR2-LIMK1 pathway rescues abnormalities of neuronal development in FMR1-null mice

To examine whether the aberrant dendritogenesis in FMR1-null neurons (53, 54) can be rescued by inhibition of LIMK1, we isolated cortical neurons from FMR1-null or wild-type mice at P0, followed by BMP7 and LIMK-i or LDN treatment for 24 hours (Fig. 7A). BMP7 treatment significantly increased the dendrite number in FMR1-null neurons, whereas it had only a modest effect on wild-type neurons (Fig. 7A). Treatment with LIMK-i, but not LDN, abolished the BMP7-dependent increase in dendritogenesis in FMR1-null neurons (Fig. 7A). The density of spines on the dendrites of cortical and hippocampal neurons is abnormally high in FMR1-null mice (46–49) and FXS patients (55). Staining with phalloidin revealed a ~40% increase in spine density in FMR1-null cortical neurons compared to that in wild-type neurons after 16 days in culture (Fig. 7B), resembling the morphology described in vivo (46–49). LIMK-i treatment reversed the abnormal dendritic spine morphology of FMR1-null neurons to the wild-type phenotype (Fig. 7B), suggesting that LIMK-i treatment could ameliorate the abnormally high turnover of dendritic protrusions observed in FMR1-null neurons (54). These results suggest that FMRP-deficient neurons develop irregular dendrites and spines at least in part as a result of increased FL BMPR2–LIMK1 signaling activity.

Thus, we postulated that reducing the abundance of FL BMPR2 or the activation of LIMK1 in vivo might prevent some aspects of the FMR1-null phenotype. We first tested whether a reduction of the BMPR2 gene dosage could rescue the neuronal morphology in FMR1-null mice. We crossed FMR1 homozygous-null and BMPR2 heterozygous mice to generate FMR1+/−; BMPR2+/− mice. BMPR2 homozygous-null mice die during early embryogenesis and could not be tested. As previously reported (46–49), granule neurons in the dentate gyrus (DG) of FMR1-null mice exhibit an increased dendritic spine density (Fig. 7C, left) with a higher percentage of long thin (>2 μm) immature spines compared to control (wild-type) mice (Fig. 7C, right). The fraction of long immature spines of DG neurons observed in the FMR1+/−; BMPR2+/− mice was similar to that seen in wild-type littermates or BMPR2 heterozygous mice (Fig. 7C). These results demonstrate that genetic perturbations of the BMPR2 signaling pathway are sufficient to modify the abnormal dendrite morphology in an FXS mouse model and in the fly, implying that the pathway is evolutionally conserved.
Next, we examined whether postnatal administration of the LIMK1 inhibitor could rescue the abnormal morphology of hippocampal neurons in the FMR1-null mouse. LIMK-i or vehicle (dimethyl sulfoxide) was injected into the cranium of P1 and P4 mice, followed at P7 by Golgi staining of the central nervous system (CNS) and biochemical analysis. Brain samples from FMR1-null mice treated with LIMK-i showed markedly reduced abundance of phosphorylated cofilin (fig. S5), indicating an effective inhibition of the catalytic activity of LIMK1. As previously reported (46–49), compared to those in wild-type mice, granule neurons in the DG of FMR1-null mice exhibited increased dendritic spine density (Fig. 7D) and an increased proportion of long, thin (>2 μm), and immature spines (Fig. 7E). In FMR1-null mice treated with LIMK-i, both spine density (Fig. 7D) and the proportion of immature spines (Fig. 7E) were reversed to those observed in mock-treated wild-type mice (46–49). These results demonstrate that genetic and pharmacological perturbations of the BMPR2-LIMK1-cofilin pathway are sufficient to modify the abnormal dendrite morphology of an FXS mouse model.

**Induction of the BMPR2-LIMK1-cofilin pathway is seen in brain tissue from FXS patients**

Finally, to test the hypothesis that the activation of the BMPR2-LIMK1-cofilin pathway found in the mouse FXS model also occurs in human FXS patients, we performed immunohistochemical (IHC) analysis to compare the amount of BMPR2 in the prefrontal cortices of FXS patients and of gender- and age-matched control individuals. We observed ~2-fold more FL BMPR2 in FXS compared to control cortices in the upper to middle layers (Fig. 8A). Consistent with the result of IHC, an immunoblot analysis confirmed that loss of FMRP in FXS patients’ cortices correlated with a ~3-fold increase of FL BMPR2 protein (Fig. 8B) and 16-fold increase of phosphorylated cofilin in FXS cortices compared to control cortices (Fig. 8B). These results demonstrate that loss of FMRP leads to an increase of FL BMPR2, which in turn augments the phosphorylation of cofilin, presumably through activation of LIMK1, in the brain tissue of FXS patients. Thus, they support our model of a critical contribution of the BMPR2-LIMK1 signaling axis to the pathology of human FXS and offer a novel opportunity to a therapy for FXS and possibly other ASD conditions.

**DISCUSSION**

The BMP signaling pathway controls morphogenesis of nearly every tissue and organ by coordinating basic properties of the cell, such as differentiation, proliferation, motility, morphology, and death, either during development and in the adult (27, 28). Here, we demonstrate that the BMPR2 mRNA is a target of translational regulation by FMRP and provide evidence supporting a link between augmented BMP signaling and neurological disorder in humans. The epistatic relationship between FMR1 and BMPR2 and the physiological significance of the FMRP-mediated down-regulation of BMP2 during neuronal development have been conserved during evolution from Drosophila to mammals. In particular, the noncanonical signaling pathway downstream of BMPR2, which includes LIMK1, appears to play an essential role in the development of the neuropathology of patients with FXS and in the mouse model of FXS. Tempering this pathway, either by reducing the BMP2 gene dosage or applying a small-molecule inhibitor of LIMK1, rescues the neuromorphological abnormality in the FXS mice model, indicating that the BMPR2-LIMK1 axis is an intervention site for expression of the central nervous system (CNS) and biochemical analysis. Brain samples from FMR1-null mice treated with LIMK-i showed markedly reduced abundance of phosphorylated cofilin (fig. S5), indicating an effective inhibition of the catalytic activity of LIMK1. As previously reported (46–49), compared to those in wild-type mice, granule neurons in the DG of FMR1-null mice exhibited increased dendritic spine density (Fig. 7D) and an increased proportion of long, thin (>2 μm), and immature spines (Fig. 7E). In FMR1-null mice treated with LIMK-i, both spine density (Fig. 7D) and the proportion of immature spines (Fig. 7E) were reversed to those observed in mock-treated wild-type mice (46–49). These results demonstrate that genetic and pharmacological perturbations of the BMPR2-LIMK1-cofilin pathway are sufficient to modify the abnormal dendrite morphology of an FXS mouse model.

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Inhibition of BMPR2-LIMK1 pathway rescues abnormal neuronal development in the mouse model of FXS. (A) Confocal microscopy detecting the number of dendrites (arrowheads) in neurons isolated from the cortex of FMR1−/− or littermate WT P0 mice, cultured for 3 days, and then transfected with a GFP expression plasmid and treated 24 hours later with LIMK-i (3 μM) or LDN (100 nM) for 12 hours, followed by BMP7 (10 ng/ml) for 24 hours. Cells were stained and imaged at day 6 in vitro. Data are means + SEM from more than seven DG neurons from three mice. *P < 0.01 by ANOVA with post hoc Tukey’s test. Scale bar, 30 μm.

8. FMR1 mRNA and FMRP correlate with a more aggressive form of breast cancer (59). This correlation was attributed to FMRP binding to E-cadherin and vimentin mRNAs and regulating epithelial-to-mesenchymal transition, a hallmark of cancer invasion and metastasis (57, 59–62). Conversely, loss of BMP signaling in mammary carcinomas can accelerate metastasis, indicating that BMP plays a tumor suppressor role (63). Therefore, it is conceivable that increased FMR1 transcripts might permit more invasive breast cancer by reducing the tumor suppressive development of an FXS and ASD therapy. A previous study, which finds that BMPR2 deletion in the mouse hippocampus and forebrain produce reduced anxiety-related behaviors and increased exploration, supports a role of BMPR2 signaling in modulating cognitive and behavioral phenotypes (56).
activity of the BMP signal. Another disease potentially associated with the FMRP-BMPR2 axis is pulmonary artery hypertension (PAH). More than 80% of patients with heritable PAH and ~20% with sporadic PAH carry a loss-of-expression or loss-of-function mutation in the FMR1 gene (64), implying a role in FXS (65). It has been reported that PAK1 binds the KH2 domain of FMRP and phosphorylates it, allowing it to be active in dendritic spine morphology and behavioral abnormalities in FXS (66). Furthermore, expression of a dominant-negative PAK1 mutant in the forebrain (67) or pharmacological inhibition of PAK1 rescues irregular dendritic spine morphology and behavioral abnormalities in FMRP-null mice (68), supporting the inhibition of PAK1 as a potential therapy for PAH among a small subset of BMPR2 carriers. The kinase PAK1, which phosphorylates and activates LIMK1, also appears to play a role in FXS (65). It has been reported that PAK1 binds the KH2 domain of FMRP and phosphorylates it, allowing it to be active in FXS (66). Furthermore, expression of a dominant-negative PAK1 mutant in the forebrain (67) or pharmacological inhibition of PAK1 rescues irregular dendritic spine morphology and behavioral abnormalities in FXS (68), supporting the inhibition of PAK1 as a potential therapy for FXS and ASDs. However, there is yet no evidence of abnormal activity and/or amount of PAK1 in human patients with FXS or other neurological diseases. PAK1 is ubiquitously expressed, activated by various extracellular stimuli, and upstream of various essential biological processes, including cell proliferation, motility, and apoptosis, in addition to the control of the actin cytoskeleton (69). Thus, compared to LIMK1, PAK1 might be a less ideal therapeutic target for FXS because of the expected secondary effects of its inhibition. In contrast, LIMK1, which is expressed mainly in the CNS (70), appears to phosphorylate only cofilin, which directly controls actin polymerization (71). LIMK2, which we used in this study, was originally identified as an anticancer drug that inhibits LIMK1 and LIMK2 activity (median inhibitory concentration equals 7 and 8 nM for LIMK1 and LIMK2, respectively) and blocks tumor cell invasion (72). Under the condition used in mouse primary neuron culture, we detected no cytotoxicity; however, development of LIMK1-specific inhibitor might be more desirable for FXS therapy because of the ubiquitous presence of LIMK2.

MATERIALS AND METHODS

Animals

B6.129P2-FMR1tm1Cgr/J, FVB.129P2-Pde6b−/− Tyr−/− FMR1tm1Cgr/J, B6.129P2-Pde6b−/− Tyr−/−/Anj (The Jackson Laboratory), B6.129S4(Cg)-BMPR2tm1.1Enl/Mmnc (MMRC), and Tg(ACTB-cre)2Mrt mice were maintained according to the University of California, San Francisco (UCSF) Laboratory Animal Resource Center guidelines. To obtain FMR1 knockout littersmates, we used wild-type and knockout (FMR1−/−) male mice derived from FMR1 heterozygous female and hemizygous male mating. To obtain FMR1−/−; BMPR2−/−; BMPR2−/− female and FMR1−/−; BMPR2−/− male mice were mated. Genotyping was performed following the The Jackson Laboratory protocol.

Drosophila lines and genetic crosses

Flies were raised at 25°C. Control wild-type flies used in this study were the w1118 strain. Mutant alleles witA12+, witB11+, and dFMR1D113M were obtained from the Bloomington Stock Center. These lines were crossed to generate witA12−/+ [witA12−/+], dFMR1D113M/+ [dFMR1D113M/+], and witA12−/+ [witA12−/+]. dFMR1D113M/+ [witA12−/+], and witA12−/+ [witA12−/−], dFMR1D113M/+ [witA12−/−] and dFMR1D113M/+ [witA12−/−].

Cell culture and transfection

COS-7, HEK293, N1E, and PAC1 cells were purchased from American Type Culture Correction and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). HEK293 cells stably expressing FLAG-HA-FMR1 isoform 1 and eGFP were reported previously (22). Primary MEFs were prepared from embryonic day 14.5 FMR1−/− and control wild-type mouse embryos and cultured in DMEM containing 10% FBS. PASMCS from heterozygous ∆ex12 and control wild-type mice were obtained from K. D. Bloch and cultured as reported previously (39). Postnatal (P0) cortical neurons were prepared from littermate FMR1−/− and wild-type mice and cultured in Neurobasal Medium (Life Technologies). Cells were cultured at 37°C in the presence of 5% CO2. Plasmid and siRNA transfections were performed using FuGENE 6 (Promega) or Lipofectamine 2000 (Life Technologies) and Lipofectamine RNAiMAX (Life Technologies) reagents, respectively.

TALEN-mediated targeted gene mutagenesis in PAC1 cell line

TALEN arms were designed using TAL Effector Nucleotide Targeter 2.0 (Cornell University). PAC1 cells were transfected with two targeting TALEN effectors [TAL_179Q (NN HD NI HD NI NN NG NN NG HD NN NI NN NI NN NI) and TAL_188R (NG NG HD NG HD NG HD NG HD NI NI NG NI HD NI)] and with pcDNA3.1-cEGFP in a ratio of 5:5:1. Forty-eight hours after transfection, eGFP-positive cells were sorted by flow cytometry, and single cells were reseeded into 96-well plates. After single-cell cloning, the mutations present in each clone were examined by Sanger sequencing.

Fig. 8. Induction of the BMPR2-LIMK1-cofilin pathway is seen in brain tissue from FXS patients. (A) Representative images of BMPR2 staining on the middle layer prefrontal cortex from FXS patients and control subjects (CTR) (top). Insets are magnified ×2. Average intensity of BMPR2 signals of lower, middle, or upper layer of prefrontal cortex of three FXS and three controls was quantitated (bottom). Data are means ± SD of three samples. **P < 0.01 by Student’s t test. P-cofilin, phosphorylated cofilin; T-cofilin, total cofilin.
Plasmid constructs
The N-terminal FLAG-tagged FL BMPR2, BMPR2 mutants, and negative control were subcloned into pcDEF3 for overexpression experiments. The BMPR2 mutants were constructed by a PCR-based approach. The BMPR2 530X was constructed by inserting a FLAG coding sequence and stop codon between amino acids 529 and 530. The MBP-BMPR2-FLAG and MBP-ACTD-FLAG were constructed by replacing the extracellular domain of BMPR2 with MBP. pcDNA3.1-eGFP was constructed by inserting the eGFP sequence into the pcDNA3.1 vector.

siRNAs and adenovirus
The siRNAs targeting human, monkey, and rat FMR1 (5’-GGAGUAGAATAGGGUGUGAGUdTdT-3’ and 5’-GCUAGGGCUUUCUGGAAldTdT-3’) and mouse LIMK1 (5’-UAGUACUGGUGUAGAGGAGACdTdT-3’) were previously reported (73–75) and purchased from Dharmacon. The control siRNA was purchased from QIAGEN. Adenovirus vectors expressing FLAG-HA-FMRP isomir 1 and eGFP were prepared using AdEasy Adenoviral Vector System (Agilent Technologies). The adenovirus vectors that express FL and ACTD BMPR2 were previously reported (45).

qRT-PCR analysis
Total RNAs were isolated using TRIzol (Life Technologies), and real-time PCR was performed as described previously (76) using the oligodeoxynucleotide primers listed in table S1. The primers for human or mouse ID1 and GAPDH were reported previously (77).

Immunoblot analysis
Cells and mouse tissues were lysed in lysis buffer [tris-buffered saline (TBS) containing 1% NP-40, 1 μM NaF, and 1 mM EDTA]. The frontal cortices of FXS patients and controls were lysed in RIPA (radioimmunoprecipitation assay) buffer. Lysed samples were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Millipore), immunoblotted with antibodies, and visualized using a LI-COR Imaging System. The antibodies used were directed against FLAG epitope tag (M2, Sigma), FMRP (ab17722, Abcam), BMPR2 KD (19087-1-AP, Proteintech Group), total SMAD1/5/8 (BMP 00479, Bio Matrix Research), phospho-SMAD1/5/8 (#9511, Cell Signaling), coflin (#5175, Cell Signaling), phospho-cofilin (SC-12912-R, Santa Cruz), β-actin (A5441, Sigma), and GAPDH (MAB374, Millipore).

Phalloidin staining
Eight-well chamber slides (Nunc Lab-Tek) were coated with poly-o-lysine (0.1 mg/ml) overnight, followed by laminin (0.2 μg/ml) for 1 hour at 37°C. N1E cells (50,000) were seeded onto each chamber, transfected or infected with siRNAs or adenovirus vectors, and treated with BMP7 with or without inhibitors [LIMKI-3 (Millipore) and LDN] as indicated in the figure legends. The cells were fixed, permeabilized, stained with Alexa Fluor 568–conjugated phalloidin (Life Technologies), as described in the manufacturer’s protocols, and observed under a point scanning confocal microscope (Leica). The phalloidin signal on filopodia was traced and quantified using the ImageJ program by a blinded investigator.

Immunofluorescence microscopy
Primary cortical neurons were isolated from littermate FMRI−/− and wild-type P0 mice, and 80,000 cells were plated on 12-mm poly-o-lysine–coated coverslips (Corning). The cells were transfected with pcDNA3.1-eGFP plasmid using Lipofectamine 2000 (Life Technologies) after 3 days in vitro. Twenty-four hours after transfection, the cells were treated with LIMK-i for 12 hours, followed by BMP7 (10 ng/ml) treatments for 24 hours. The cells were fixed with 4% PFA/4% sucrose in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked with 3% bovine serum albumin/0.02% Triton X-100 in PBS, and stained with GFP antibody (AB13970, Abcam) overnight at 4°C, followed by incubation with an Alexa Fluor 488–conjugated secondary antibody. Confocal images were acquired using a point scanning confocal microscope (Leica). Images were from a projection of z sections.

RIP assay
RIP assay was performed as reported previously (11). Briefly, HEK293 cells were transfected with vectors expressing wild-type or mutant BMPR2. Forty-eight hours after transfection, the cells were washed with PBS containing cycloheximide (100 μg/ml), and the cell dishes were placed on an ice pack and ultraviolet (UV)–cross-linked in ice-cold PBS containing cycloheximide (100 μg/ml) for three times at 400 ml/cm2 (254-nm UV light) using an UVC 500 cross-linker (Hoefer). The cells were collected, lysed in SDS-immunoprecipitation buffer, sonicated, and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was heated to 90°C for 10 min with shaking and diluted nine times by volume with CSK buffer. The lysate was treated with ribonuclease T1 (5 U/ml) (Thermo Scientific) and deoxyribonuclease I (DNase I; 0.8 U/ml) (Roche) for 5 min at 37°C, and SUPERase In (100 U/ml) (Life Technologies) was added to stop the reaction. After preclear with Protein A Dynabeads (Life Technologies), FMRP and RNA complexes were immunoprecipitated using Protein A Dynabeads bound to either FMRP antibody (ab17722, Abcam) or control Ig overnight at 4°C. After immunoprecipitation, the beads were washed with high-stringency buffer, high-salt buffer, low-salt buffer (twice), and NT2 buffer, followed by two washes with PK buffer. The beads were resuspended in PK buffer containing proteinase K (0.8 mg/ml) (Roche), incubated for 20 min at 37°C with shaking, mixed with an equal volume of PK-7 M urea buffer, and incubated for 20 min at 37°C with shaking. The RNAs were purified by acid phenol/chloroform extraction, followed by ethanol precipitation. The RNA solution was treated with DNase I (1U/ml) for 30 min at 37°C, purified by acid phenol/chloroform extraction and ethanol precipitation, and used for reverse transcription using SuperScript III Reverse Transcriptase (Life Technologies), followed by real-time PCR. The oligodeoxynucleotide primers used for qRT-PCR are listed in table S2.

Larva immunofluorescence and image quantitation
Wandering late third instar larvae were dissected for analysis of NMJ phenotype. After dissection, the tissues were fixed with 8% formaldehyde in PBS for 10 min, washed with PBT (PBS containing 0.1% Triton X-100), and blocked with 5% normal donkey serum in PBT for 1 hour at room temperature. The larvae were incubated with DLG (4F3, Developmental Studies Hybridoma Bank) antibody overnight at 4°C, followed by incubation with Alexa Fluor 488– or Alexa Fluor 555–conjugated secondary antibodies against mouse Ig, Alexa Fluor 488–conjugated HRP antibody (123-545-021, Jackson ImmunoResearch), and Alexa Fluor 568–conjugated (A12380, Life Technologies) or Alexa Fluor 647–conjugated (#8940, Cell Signaling) phalloidin. Confocal images were acquired using a point scanning confocal microscope (Leica). Images used for quantification of NMJ bouton number and branch number were derived from a projection of z sections. For quantification of the branch number, branches with at least three boutons were counted blindly.

Golgi staining
The mice received LIMKI-3 (20 μg per pup) by intracerebroventricular injection at P1 and P4. At P7, the mice were anesthetized, and the brains were quickly removed and rinsed in distilled water to be used for Golgi’s staining. Golgi’s staining was performed as described in the manual (FD Rapid GolgiStain Kit, FD NeuroTechnologies). Shortly, the brains were incubated in solution A + B for 3 weeks, followed by 3-day incubation in solution C. We
stained 100-µm brain sections and performed spine density and morphological analyses under a microscope at >100 magnification. Quantification of dendritic spine number in 30 µm (~30 µm from cell body) was performed blindly.

FXS patient brain immunohistochemistry

FXS patient and control frontal cortices were fixed by formalin, and the samples in paraffin were sectioned at 7 µm. Antigen retrieval was performed with 1x diva solution (heated-enzyme retrieval buffer, Biocare Medical) at 110°C for 8 min, followed by 3% H2O2 treatment for 7 min. The samples were incubated with blocking buffer (TBS buffer containing 10% donkey serum and 0.0025% Triton X-100) for 1 hour, primary antibodies (in TBS buffer containing 10% donkey serum and 0.0025% Tween 20) for 15 hours at 4°C, biotin-conjugated secondary antibodies (Jackson Immunoresearch; in TBS buffer containing 10% donkey serum and 0.0025% Tween 20) for 1 hour at room temperature, and DAB Substrate (Vector Laboratories Inc.) for 5 min, followed by counterstaining with Harris hematoxylin (Thermo Scientific), dehydration with ethanol, clearing with xylene, and mounting with Permount (Fisher). An antibody with poly(A)* mRNA in actively transcribing polyribosomes. *Human Mol. Cell. 6, 1456–1472 (1997).


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Augmented noncanonical BMP type II receptor signaling mediates the synaptic abnormality of fragile X syndrome
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