

ORIGINAL
ARTICLEc-Jun *N*-terminal kinase regulates mGluR-
dependent expression of post-synaptic FMRP
target proteins

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*Department of Pediatrics and the Waisman Center, University of Wisconsin-Madison, Madison, Wisconsin, USA***Abstract**

Fragile X syndrome (FXS) is caused by the loss of functional fragile X mental retardation protein (FMRP). Loss of FMRP results in an elevated basal protein expression profile of FMRP targeted mRNAs, a loss of local metabotropic glutamate receptor (mGluR)-regulated protein synthesis, exaggerated long-term depression and corresponding learning and behavioral deficits. Evidence shows that blocking mGluR signaling in FXS models ameliorates these deficits. Therefore, understanding the signaling mechanisms downstream of mGluR stimulation may provide additional therapeutic targets for FXS. Kinase cascades are an integral mechanism regulating mGluR-dependent protein translation. The c-Jun *N*-terminal kinase (JNK) pathway has been shown to regulate

mGluR-dependent nuclear transcription; however, the involvement of JNK in local, synaptic signaling has not been explored. Here, we show that JNK is both necessary and sufficient for mGluR-dependent expression of a subset of FMRP target proteins. In addition, JNK activity is basally elevated in *fmr1* knockout mouse synapses, and blocking JNK activity reduces the over-expression of post-synaptic proteins in these mice. Together, these data suggest that JNK may be an important signaling mechanism downstream of mGluR stimulation, regulating FMRP-dependent protein synthesis. Furthermore, local, post-synaptic dysregulation of JNK activity may provide a viable target to ameliorate the deficits involved in FXS.

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Activity-dependent control of synaptic proteins plays an important role in learning and memory. Synaptic protein levels are controlled in part by local turnover, both through local translation and degradation (Huber *et al.* 2000; Bingol and Sheng 2011). Metabotropic glutamate receptor (mGluR) stimulation promotes dendritic translation of a number of post-synaptic proteins including amyloid precursor protein (APP), activity-regulated cytoskeleton-associated protein (Arc/Arg-3.1), and post-synaptic density protein 95 (PSD95), among others (Westmark and Malter 2007; Park *et al.* 2008; Muddashetty *et al.* 2011). This process is perturbed in a number of neurodegenerative diseases, including Alzheimer's disease, as well as inherited, developmental disabilities such as fragile X syndrome (FXS) and trisomy 21 (Oka and Takashima 1999; Albasanz *et al.* 2005; Malter *et al.* 2010). Several signaling mechanisms have been implicated in regulating translation upon mGluR stimulation, including the extracellular signal-related kinase (ERK) and PI3K/Akt/mammalian target of rapamycin (mTOR) pathways (Gallagher *et al.* 2004; Ronesi and Huber 2008a;

Sharma *et al.* 2010). While the c-Jun *N*-terminal kinase (JNK) pathway has been implicated in nuclear transcription following mGluR stimulation (Yang *et al.* 2006), its role in local, dendritic protein expression has not been investigated.

Fragile X syndrome is a prototypical disease with impaired mGluR-dependent translation of dendritic proteins (Waung and Huber 2009). FXS is the most commonly inherited form of mental retardation and a cause of autism affecting

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Abbreviations used: APP, amyloid precursor protein; DHPG, (S)-3,5-Dihydroxyphenylglycine; FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; JNK, c-Jun *N*-terminal kinase; KO, knockout; mGluR, metabotropic glutamate receptor; MPEP, 2-Methyl-6-(phenylethynyl)pyridine; PSD95, post-synaptic density protein 95; SNs, synaptoneuroosomes; SP600125, 1,9-pyrazoloanthrone anthrapyrazolone; WT, wild-type.

approximately one in 4000 males and one in 6000 females (Hagerman 2008). Patients with FXS display impaired cognitive abilities, autistic behaviors, an increased incidence of epilepsy and characteristic facial dysmorphisms (Jin and Warren 2000). The most commonly used animal model of FXS, *fmr1* knockout (KO) mice, display similar 'clinical' phenotypes, including impaired learning and memory and abnormal long-term depression and long-term potentiation, electrophysiological measurements of synaptic plasticity (Huber *et al.* 2002; Lauterborn *et al.* 2007; Ronesi and Huber 2008b; Shang *et al.* 2009). FXS is typically caused by a tri-nucleotide repeat within *FMR1* resulting in gene silencing and a deficiency of fragile X mental retardation protein (FMRP) (Fu *et al.* 1991; Kremer *et al.* 1991; Yu *et al.* 1991), an mGluR responsive mRNA-binding protein (Ashley *et al.* 1993). Normally, FMRP binds to target mRNAs blocking their translation, though the mechanism of this inhibition is incompletely understood (Brown *et al.* 1998; Darnell *et al.* 2011). Upon mGluR stimulation, FMRP-dependent inhibition is relieved allowing for local, nucleus-independent protein translation.

In FXS, the loss of FMRP results in increased steady-state levels of FMRP target proteins and a lack of mGluR-dependent protein translation (Waung and Huber 2009). This is similar to a state of constitutive activation of the mGluR receptor (the 'mGluR theory of FXS') (Bear *et al.* 2004). Interestingly, blockade of mGluR receptors ameliorates the enhanced protein synthesis, spine dysmorphology, electrophysiology and some of the behavioral phenotypes in *fmr1* KO mice (Yan *et al.* 2005; Westmark *et al.* 2009; Osterweil *et al.* 2010; Choi *et al.* 2011; Su *et al.* 2011), and multiple mGluR antagonists have entered clinical trials in FXS (Krueger and Bear 2011). This suggests that altered mGluR signaling, independent of (or in addition to) FMRP-mediated events, contributes to the pathobiology of FXS. Indeed, many proteins downstream of mGluR stimulation, including mGluR5 itself, are FMRP mRNA targets and expression and/or activity is elevated in FXS (Darnell *et al.* 2011). A comprehensive review of mGluR5 targets, including those that have been targeted either genetically or pharmacologically, has been recently published (Darnell and Klann 2013). Examples include mGluR5 itself; important mGluR5 interacting proteins including Homer, PIKE, and Shank; various signaling cascades such as the MEK (MAPK/ERK kinase)-ERK and PI3K-Akt-mTORC1 pathways; and proteins involved in the translational machinery (Darnell and Klann 2013). Given the complex function of each of these pathways, pharmacological manipulation could result in numerous undesirable off-target effects. Therefore, further understanding of signaling events downstream of mGluR stimulation that are perturbed in FXS will improve therapeutic targets.

Given that the JNK pathway is activated upon mGluR stimulation (Yang *et al.* 2006), and has been implicated in translation in other models (Patel *et al.* 2012), we sought to determine whether JNK contributes to regulation of local

post-synaptic protein levels. Here, we show that JNK activity rapidly increases after mGluR stimulation in the synapses of wild-type (WT) mice and is necessary and sufficient for local mGluR-dependent changes for a subset of post-synaptic FMRP target proteins. Conversely, *fmr1* KO mice display elevated steady-state JNK activity, increased levels of FMRP target proteins and loss of responsiveness to mGluR stimulation. Furthermore, inhibiting JNK markedly reduces levels of these proteins, even in the absence of FMRP, while direct activation of the JNK pathway increases levels of post-synaptic proteins. These findings support a model in which the JNK pathway is both necessary and sufficient for mGluR-induced increases of multiple post-synaptic proteins. Furthermore, this data suggest that the JNK pathway may provide an additional therapeutic target in FXS.

Materials and methods

Reagents and antibodies

DHPG ((S)-3,5-Dihydroxyphenylglycine), SP600125 (1,9-pyrazoloanthrone anthrapyrazolone), and MPEP (2-Methyl-6-(phenylethynyl)pyridine) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). D-JNKi and HIV-trans-activator of transcription (TAT) control peptide were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Anti-APP antibody was purchased from Millipore (Billerica, MA, USA). Anti-Phospho-JNK1/2/3 (Y185/Y185/Y223), anti-Total Jun and anti-PSD95 antibodies were purchased from Epitomics, Inc. (Burlingame, CA, USA). Anti-Arc and β -Actin antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Neurobasal media and B27 supplement were purchased from Life Technologies (Grand Island, NY, USA).

Animal husbandry

Mice, WT and *fmr1* KO on C57B6 background (a gift from Dr James Malter), were housed in the Waisman Center's Rodent Models Core. In studies comparing WT and *fmr1* KO animals, heterozygous females (\pm) were bred with wild-type males (+/Y) allowing for the use of male littermate controls (+/Y and -/Y). Offspring were genotyped before synaptoneurosome preparation from tail clips using standard protocols. All husbandry and euthanasia was performed in accordance with NIH, ARRIVE guidelines, and University of Wisconsin-Madison Research Animal Resources Center protocol approval.

Primary neuronal culture

Embryonic day 18 pregnant females were killed and the uterine sac transferred to ice cold Hank's balanced salt solution (HBSS). Pup cortices were washed in HBSS, and incubated in HBSS + 0.05% trypsin for 25 min at 37°C. Trypsin was deactivated with complete Eagle's modified essential medium (Eagle's MEM, 10% fetal bovine serum, 10% horse serum, 2 mM L-Glutamine, 0.01% penicillin/streptomycin). Cell suspension was triturated gently and filtered through 70 μ M mesh strainer. Cell density was calculated excluding dead cells using trypan blue staining. 1×10^6 neurons were plated onto poly-D-lysine coated coverslips in neurobasal media with B27 supplements for 12–15 days. Each experimental date was done with individual pup cultures and each treatment performed in duplicate

giving two biological replicates per experimental date. Cultures were treated with DHPG (100 μ M), SP600125 (20 μ M), MPEP (10 μ M) or their respective vehicle controls [phosphate-buffered saline (PBS) or dimethylsulfoxide] for 2, 5, or 10 min for phosphorylation experiments, or 60 min for protein level evaluations. Prior to stimulation, neurons were pre-incubated with SP600125, MPEP, or their respective controls for 10 min at 37°C.

Synaptoneurosome preparation and treatment

Synaptoneurosomes were prepared essentially as described (Westmark and Malter 2007). Briefly, synaptoneurosomes (SNs) were prepared from WT and *fmr1* KO mouse cortical tissue obtained from 14- to 17-day-old pups. For comparisons between WT and *fmr1* KO samples, only male littermates were used. Cortices were homogenized in ice cold gradient medium buffer (0.25 M sucrose, 5 mM Tris [pH 7.5], and 0.1 mM EDTA) using a dounce homogenizer, the homogenate was spun at 1000 g for 10 min at 4°C to pellet cellular debris and nuclei. The supernatant was applied to percoll/sucrose gradients (23%, 15%, 10%, and 3%) and spun at high-speed (32 500 g) for 5 min at 4°C. The 23%/15% interface containing intact SNs was removed and pooled. The salt concentration of the SNs was adjusted with the addition of 10X Stimulation Buffer (100 mM Tris [pH 7.5], 5 mM Na₂HPO₄, 4 mM KH₂PO₄, 40 mM NaHCO₃, 800 mM NaCl), and 1 μ M tetrodotoxin was added to suppress non-specific excitation. Prior to stimulation, SNs were pre-incubated with SP600125 (20 μ M), MPEP (10 μ M), or their respective controls for 10 min at 4°C with rotation. DHPG (100 μ M) was then added to activate Group-1 mGluRs. Samples were mixed at 37°C for the indicated times and then snap frozen until analysis. For short stimulation time points, SNs were quickly equilibrated to 37°C prior to DHPG stimulation.

JNK activity assay

The non-radioactive JNK activity assay was purchased from Cell Signaling Technologies (Danvers, MA, USA) and performed as suggested by the manufacturer. In short, after treatment, SNs were subjected to immunoprecipitation by c-Jun conjugated beads to specifically immunoprecipitate JNK. After washing, the beads were mixed with kinase buffer containing ATP (200 μ M) for 30 min at 30°C and the reaction halted with sodium dodecyl sulfate buffer, boiled and separated for immunoblot analysis. Blots were probed for phosphorylated c-Jun (provided in kit) as a marker for JNK activity, and subsequently re-probed for total Jun to demonstrate equal substrate immunoprecipitate and loading.

Transduction with TAT fusion proteins

Kinase-Dead-MAP kinase kinase 7 (MKK7) and Wild-type-MKK7 were cloned into pHisTAT (provided by James Malter) providing an HIV-1-TAT conjugated MKK7 expression vector and subsequently expressed using BL21 *E. coli* (New England Biolabs; Ipswich, MA, USA) and purified using Ni-NTA agarose (Qiagen; Valencia, CA, USA) following manufacturer's suggested protocols. A protein concentration of each peptide was determined by bicinchoninic acid Assay (Thermo Scientific; Rockford, IL, USA). A final treatment concentration of 50 nM was used based on expression analysis of each peptide. TAT control and TAT-D-JNKi peptides were purchased from Enzo Life Sciences. Final treatment concentration of D-JNKi and control peptide was 5 μ M.

Immunoblotting

Immunoblots were performed as previously described (Wilhelm *et al.* 2012). Signals were detected using the Li-Cor system (Lincoln, NE, USA). Equal loading was confirmed by probing for β -Actin. Band intensity from at least three independent experiments was quantitated using the Li-Cor software, normalized to β -Actin, and presented as percentage of control.

Immunocytochemistry and confocal immunofluorescence

After treatments, neurons were fixed with 100% methanol and permeabilized with 0.5% Triton-X 100 in PBS and then blocked for 1 h at 21°C in 2% bovine serum albumin in PBS plus 0.1% Triton-X 100 (blocking buffer). Neurons were probed with primary antibodies (1 : 250 dilution) in blocking buffer overnight at 4°C. Coverslips were washed with blocking buffer three times and subsequently probed with Alexa Fluor secondary antibody (1 : 500) (Life Technologies) for 1 h, washed and mounted. Images (composites of twelve 0.3 μ M z-stacks) were acquired with a Nikon C1 laser-scanning confocal microscope with EZ-C1 v2.20 software (Melville, NY, USA) at 40X magnification. Immunofluorescent experiments were performed at least three times in triplicate. For each treatment, dendrites were traced using ImageJ beginning a minimum of 10 μ m from the soma and the fluorescence levels of five neurons per treatment per biological replicate were quantitated with ImageJ software using the Analyze Particles function (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2011). Standardized threshold intensity was set and used across all treatments to eliminate quantitation of background signal noise. Output data used was the average integrated density calculated as total pixel integrated intensity divided by area of the dendrite above baseline threshold for each neuron.

Statistics

Average integrated density fluorescence was used for immunofluorescent data. Immunofluorescent data were performed a minimum of three times and quantitative analysis was performed on five neurons per treatment per experiment. Synaptosomal experiments were performed a minimum of three times using pooled WT or *fmr1* KO male pup cortices. Immunoblot analysis was performed on each individual experiment (including JNK activity assay) and data were normalized to loading control (β -Actin or total Jun for JNK activity assays) and expressed as a percentage of control. Both immunofluorescent intensity and immunoblot levels were compared between genotypes and treatments by two-way ANOVA with Bonferroni correction and significance set at $p < 0.05$.

Results

JNK is activated locally in dendrites by mGluR stimulation

Kinase cascades, including the ERK and PI3K/Akt/mTOR pathways, are essential regulators of local protein translation within synaptic spines (Gallagher *et al.* 2004; Ronesi and Huber 2008b; Sharma *et al.* 2010). Previous work has shown that mGluR stimulation regulates JNK-dependent nuclear transcription (Yang *et al.* 2006). However, the role of JNK locally within the dendrites has not been evaluated. We used

immunofluorescence of cultured neurons to determine if JNK is phosphorylated in an mGluR-dependent fashion in dendrites of wild-type (WT) cortical neurons. We used JNK phosphorylation as a surrogate marker for JNK activity in cultured neurons and an immunoprecipitation based JNK activity kit using recombinant c-Jun as a substrate for synaptosomal experiments because we were unable to detect the defining substrate of JNK, c-Jun, in synaptosomal preparations (Figure S1). Cultured neurons were stimulated

with the group-1 mGluR agonist DHPG (100 μ M). Within 2–5 min, there is a significant increase of 45% ($p < 0.05$) in dendritic phosphorylated JNK (P-JNK) after mGluR stimulation (Fig. 1a and c). This induction is transient as P-JNK staining levels are reduced to control by 10 min (Figure S2). The increase in P-JNK is completely prevented by pretreatment with the mGluR5 antagonist MPEP (10 μ M) (Fig. 1a and c) indicating that DHPG-induced activation of JNK is mediated through mGluR5 rather than mGluR1.

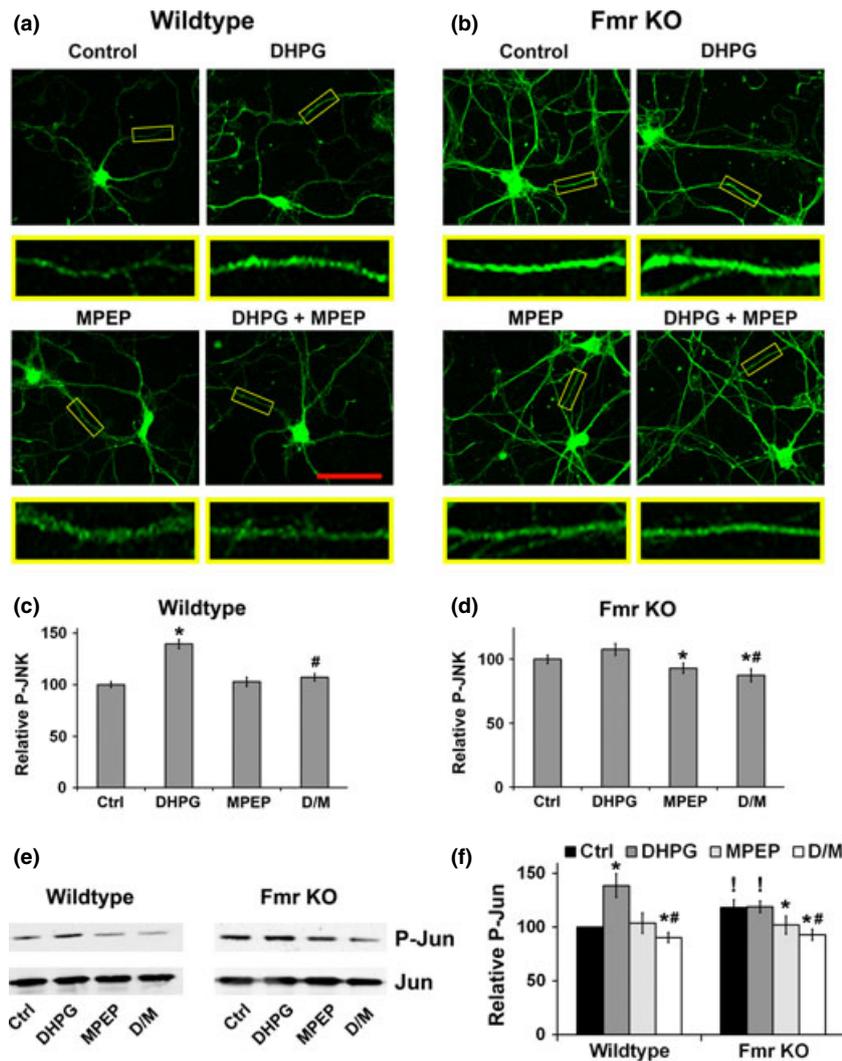


Fig. 1 Local metabotropic glutamate receptor (mGluR)-stimulated c-Jun N-terminal kinase (JNK) activity is constitutively active in *fmr1* knockout synapses. (a and b) Wild-type (WT, a) and *fmr1* knockout [*Fmr* knockout (KO), b] neurons (12–15 DIV) were treated with phosphate-buffered saline (PBS) (Ctrl), DHPG ((S)-3,5-Dihydroxyphenylglycine) (100 μ M), MPEP (2-Methyl-6-(phenylethynyl)pyridine) (10 μ M), or combined DHPG and MPEP (D/M) for 5 min. Neurons were then fixed and stained for phosphorylated JNK (P-JNK). Representative confocal image of each treatment are shown. Scale bar = 50 μ m. (c and d) Wild-type (WT, c) and *fmr1* knockout (Fmr KO, d) five dendrites per experimental replicate were traced using Image J

and the average integrated density per neuron was quantified \pm SEM. (* $p \leq 0.05$ versus Ctrl, # $p \leq 0.05$ vs. DHPG, WT $n = 6$ and *fmr1* KO $n = 8$) (e) Synaptoneurosomes (SNs) from WT and *fmr1* KO littermates (P15) were treated with PBS (Ctrl), DHPG (100 μ M), MPEP (10 μ M), or combined DHPG and MPEP (D/M) for 5 min. SNs then underwent JNK kinase activity assay, separated for immunoblot analysis and probed for phosphorylated c-Jun. Blots were subsequently stripped and re-probed for total Jun to confirm equal substrate loading. (f) Graphical representation of JNK activity shows the mean \pm SEM. (* $p \leq 0.05$ vs. Ctrl, # $p \leq 0.05$ vs. DHPG, ! $p \leq 0.05$ vs. WT, WT $n = 3$ and *fmr1* KO $n = 6$).

The rapid increase in JNK phosphorylation in dendrites suggests that all the components required for mGluR stimulation to activate JNK are present locally within dendrites. However, this data do not exclude the possibility that components in the cell soma or nucleus are required in this process. To confirm that JNK activation is occurring entirely within the dendrites, we examined changes in purified SNs. SNs are purified, resealed vesicles containing both sides of the synaptic cleft and the associated intracellular machinery (Bai and Witzmann 2007). Using a JNK activity assay, we evaluated the changes in JNK activity in WT SNs basally, upon mGluR stimulation, and after pre-treatment with MPEP (Fig. 1e and f). As with intact dendrites (Fig. 1a), we identified a significant increase in JNK activity within 5 min of mGluR stimulation in WT SNs, which was completely abrogated with MPEP pre-treatment (Fig. 1e and f). While a small absolute increase, this degree of JNK activation is consistent with other JNK-dependent signaling events, including pro-apoptotic JNK signaling in the cell soma (Wilhelm *et al.* 2007).

JNK activity is basally elevated and mGluR unresponsive in *fmr1* KO dendrites

Because mGluR signaling appears constitutively activated in FXS (the 'mGluR theory of FXS') (Bear *et al.* 2004), and the basal phosphorylation status of Erk and Akt pathways in *fmr1* KO mice is still inconclusive (Gross *et al.* 2012), we sought to determine whether basal JNK phosphorylation was elevated in the dendrites of *fmr1* KO mice and whether responsiveness to mGluR stimulation is lost. When compared to WT dendrites, P-JNK levels are elevated in the dendrites of *fmr1* KO neurons (Fig. 1b and d). In addition, JNK phosphorylation does not increase upon mGluR stimulation. More importantly, P-JNK levels decrease in the dendrites when pre-treated with the mGluR5 antagonist, MPEP, both in the absence and presence of DHPG.

We then examined JNK activity in SNs prepared from *fmr1* KO mice to determine the local regulation of JNK signaling. As seen in intact dendrites, *fmr1* KO SNs displayed a small, but statistically significant basal elevation of JNK activity that did not increase further upon stimulation

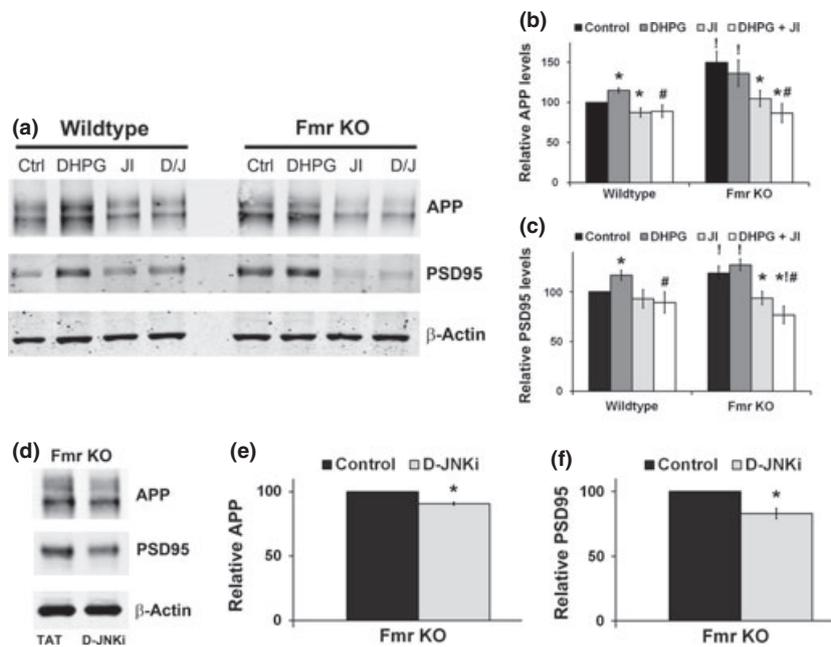


Fig. 2 Inhibiting c-Jun N-terminal kinase (JNK) prevents the metabotropic glutamate receptor (mGluR)-induced increase of multiple fragile X mental retardation protein (FMRP) targets and reduces their excessive expression in *fmr1* knockout synapses. (a) WT and *fmr1* knockout (KO) synaptoneurosomes (SNs) were treated with phosphate-buffered saline (PBS)/dimethylsulfoxide (DMSO) (Ctrl), DHPG ((S)-3,5-Dihydroxyphenylglycine) (100 μ M), SP600125 (1,9-pyrazoloanthrone anthrapyrazolone) (JNK inhibitor, Ji) (20 μ M), or combined DHPG and Ji (D/J) for 1 h. SN protein was separated for immunoblot analysis and probed for two well-defined FMRP targets, amyloid precursor protein (APP) (top row) and post-synaptic density protein 95 (PSD95) (second row). Blots were also re-probed for β -Actin (bottom

row) to confirm equal loading. (b and c) APP (b) and PSD95 (c) expression levels were quantitated relative to β -Actin and expressed as relative to WT control. Mean \pm SEM. (* $p \leq 0.05$ vs. control, # $p \leq 0.05$ vs. DHPG, and $^!p \leq 0.05$ vs. WT, WT APP $n = 9$ and *fmr1* KO $n = 6$, WT PSD95 $n = 5$ and *fmr1* KO $n = 6$). (d) *fmr1* KO SNs were treated with 5 μ M TAT control peptide or TAT-D-JNKi for 1 h. SNs were processed for immunoblot analysis probing for APP (top row), PSD95 (middle row) and β -Actin (bottom row). (e and f) APP and PSD95 expression was quantitated relative to β -Actin and expressed relative to control. Shown is the mean \pm SEM. (* $p \leq 0.05$ vs. control, WT $n = 3$ and *fmr1* KO $n = 6$ for both targets).

with DHPG (Fig. 1e and f). We confirmed that the elevated basal JNK activity was not because of differences in mGluR5 expression by immunoblot analysis for mGluR5 in WT and *fmr1* KO SNs (data not shown) and has been shown by others (Ronesi *et al.* 2012). Interestingly, pre-treatment with MPEP reduced JNK activity in the unstimulated *fmr1* KO SNs. Collectively this data indicate that mGluR stimulation rapidly induces JNK activation locally in WT dendrites and synapses and is mediated through mGluR5. Conversely, basal JNK activity is elevated in *fmr1* KO dendrites and synapses, but is equally inhibited by the mGluR5 antagonist MPEP. This is consistent with a model of constitutively active mGluR-dependent JNK signaling in the *fmr1* KO SNs.

JNK is necessary for mGluR-dependent increase in post-synaptic proteins

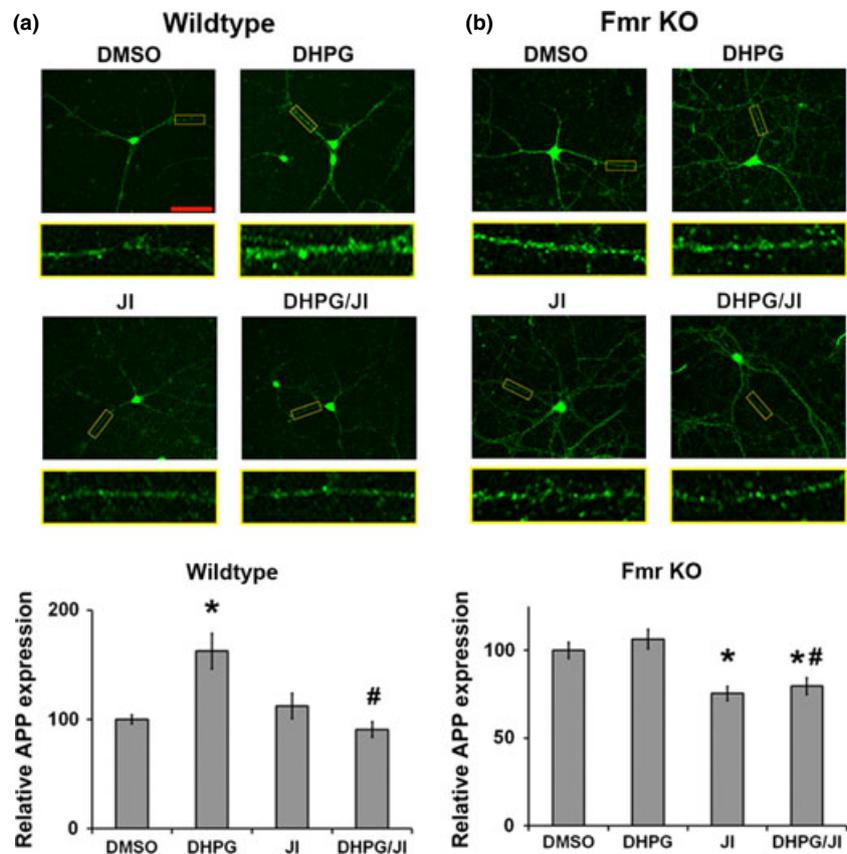
Because mGluR stimulation promotes the local translation of mRNAs that bind to FMRP, we next sought to determine if JNK regulates protein levels of known FMRP targets. We confirmed the role of translation in our synaptosomal system and found cycloheximide does indeed reduce the expression of PSD95 in this system, though not as robustly as SP600125 (see below) (Figure S3). Following pre-treatment with SP600125 (20 μ M) to inhibit JNK, WT and *fmr1* KO SNs were stimulated with DHPG for 1 h and we evaluated the

protein expression levels of two well-defined FMRP targets: APP (Westmark and Malter 2007) and PSD95 (Muddashetty *et al.* 2007, 2011) (Fig. 2a–c). As expected, the expression of both APP and PSD95 increased 1 h after stimulation by DHPG in WT SNs (Fig. 2a, left). In addition, *fmr1* KO SNs demonstrated an elevated basal level of both proteins and no significant change in expression upon mGluR stimulation, consistent with enhanced basal translation in the KO (Fig. 2a, right). JNK inhibition reduced the mGluR-dependent increase in expression in wild-type SNs. Interestingly, in the KO SNs, JNK inhibitor reduced protein levels in both stimulated and unstimulated SNs.

To ensure specificity of our findings, we determined the effects of a second, peptide-based inhibitor, D-JNKi on levels of FMRP targets in the *fmr1* KO SNs. D-JNKi is a cell-permeable, highly specific inhibitor of JNK (Bonny *et al.* 2001; Borsello *et al.* 2003). Importantly, D-JNKi does not inhibit the ERK or AKT pathways (Borsello *et al.* 2003), which have been previously implicated in mGluR-dependent translation of FMRP targets (Gallagher *et al.* 2004; Ronesi and Huber 2008a; Sharma *et al.* 2010). As shown in Fig. 2d–f, D-JNKi (5 μ M) also significantly reduces levels of both APP and PSD95 in *fmr1* KO SNs.

Next, we determined the requirement for JNK activity in FMRP target expression in intact neurons. We treated WT

Fig. 3 c-Jun N-terminal kinase (JNK) activity is necessary for metabotropic glutamate receptor (mGluR)-dependent amyloid precursor protein (APP) expression in wild-type dendrites and reduces excessive APP in *fmr1* knockout neurons. Wild-type (a) and *fmr1* knockout (KO) (b) neurons were treated with phosphate-buffered saline (PBS)/dimethylsulfoxide (DMSO) (Ctrl), DHPG ((S)-3,5-Dihydroxyphenylglycine) (100 μ M), SP600125 (1,9-pyrazoloanthrone anthrapyrazolone) (JNK inhibitor, JI) (20 μ M), or combined DHPG and JI (D/J) as indicated. After 1 h of treatment, neurons were fixed and stained for APP. Representative confocal images are shown. Scale bar = 50 μ m. Quantification of APP intensity within dendrites was performed using ImageJ. Quantification was performed on five dendrites per treatment and displayed as the mean integrated density \pm SEM. (* p < 0.05 vs. control, #vs. DHPG, n = 4).



cortical neurons with DHPG in the absence or presence of SP600125 and evaluated staining of APP within dendrites by immunofluorescence. At 1 h after DHPG stimulation, dendritic APP signal was significantly increased in WT neurons (Fig. 3a). This increase was completely prevented by SP600125.

Finally, we examined APP levels in cultured *fmr1* KO neurons by IF. Because these cultures were prepared and treated at separate times from WT cultures, we cannot directly compare levels between WT and KO. However, as expected based on previously published work (Westmark and Malter 2007) and our SN data in Fig. 2, APP levels are unresponsive to mGluR stimulation in *fmr1* KO neurons (Fig. 3b). Importantly, dendritic APP levels were reduced below basal levels by SP600125, in both DHPG-stimulated or unstimulated neurons. These data confirm that JNK is required for the protein expression of FMRP target mRNAs.

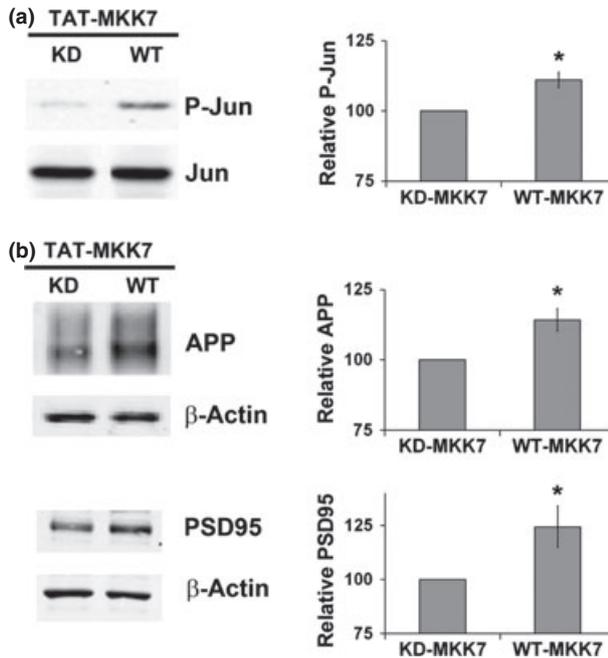


Fig. 4 c-Jun N-terminal kinase (JNK) activity is *sufficient* to increase expression of fragile X mental retardation protein (FMRP) target proteins. Wild-type synaptoneuroosomes (SNs) were treated with kinase dead (KD) or wild-type (WT) MKK7 (50 nM) for 5 min (a) or 1 h (b). (a) After JNK kinase assay reactions, SNs were processed for immunoblot analysis and probed for phosphorylated c-Jun. Blots were subsequently stripped and re-probed for total Jun to confirm equal substrate loading and expressed as relative to total Jun. (Mean \pm SEM, $*p \leq 0.05$ vs. KD, $n = 4$) (b) SN reactions processed for immunoblot analysis and probed for the FMRP targets amyloid precursor protein (APP) (top row) and PSD95 (bottom row). Blots were also probed for β -Actin (bottom row) to confirm equal loading. Protein expression levels were quantitated relative to β -Actin and expressed as relative to control. (Mean \pm SEM, $*p \leq 0.05$ vs. KD, APP $n = 3$, PSD95 $n = 6$).

JNK activation is sufficient for elevating FMRP target protein expression

Evidence above indicates that JNK activity is required for appropriate FMRP target protein expression levels. We next aimed to determine if driving JNK activity is sufficient to increase FMRP target protein expression. To drive JNK activity for these studies we used cell permeable TAT-MKK7 recombinant protein and a KD mutant of MKK7 as control (50 nM). MKK7 is the immediate upstream MAPKK that specifically phosphorylates and activates JNK (Tournier *et al.* 1997). Conjugation to HIV-TAT provides rapid and efficient membrane permeability (Gump and Dowdy 2007). We found that treatment with TAT-MKK7 rapidly drives JNK activity in WT SNs within 5 min (Fig. 4a), though not quite as efficiently as stimulating mGluR5. Despite this lower level of JNK activation, TAT-MKK7 increased APP and PSD95 levels in WT SNs within 1 h of treatment (Fig. 4b). These data indicate that not only is JNK necessary for APP and PSD95 post-synaptic expression, but JNK is sufficient to drive their expression.

Discussion

Local regulation of dendritic protein expression, through both local translation and degradation, plays an important role in learning and memory. This activity-dependent process is perturbed in many disorders, including FXS, in which the absence of FMRP leads to constitutive over-expression of multiple post-synaptic proteins. Here, we show that the JNK pathway is both *necessary and sufficient* for increased expression of several well-characterized targets of FMRP. These findings have significant implications for not only FXS, but for other disorders of learning and memory, such as Alzheimer's disease, that exhibit exaggerated signaling through the JNK pathway (Morishima *et al.* 2001; Thakur *et al.* 2007; Ploia *et al.* 2011; Sclip *et al.* 2011).

While mGluR5 stimulation had previously been shown to activate nuclear JNK signaling (Yang *et al.* 2006), our work represents the first evidence that JNK plays a *local* role in dendrites. This does not discount the importance of nuclear signaling in specific effects of mGluR5 stimulation. However, local regulation of post-synaptic protein levels in dendrites is known to be important in modulating synaptic strength. Because JNK is activated and regulates protein levels in synaptoneuroosomes, our data confirm that nuclear events are not required in this process. Furthermore, the blockade of JNK activity by MPEP in both wild-type and *fmr1* KO SNs and dendrites confirms that JNK is downstream of mGluR5 stimulation. Importantly, our work does not rule out a similar role for the JNK pathway downstream of the other type 1 mGluR receptor, mGluR1. However, we chose to focus on mGluR5-mediated signaling given its importance in FXS.

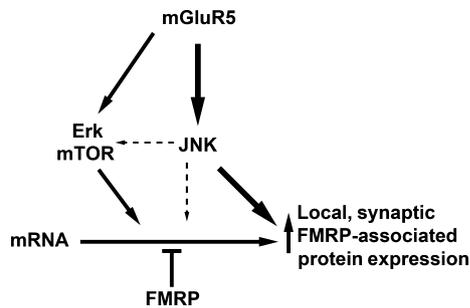


Fig. 5 Model of c-Jun *N*-terminal kinase (JNK)'s involvement in local, post-synaptic protein expression. A schematic displaying what is known from previously published works (medium arrows), what we show herein (heavy arrows) and what remains unknown (hashed arrows) for JNK's role in expression of post-synaptic proteins.

To confirm the specificity of our results we used two complementary approaches. First, two JNK inhibitors having different mechanisms of action (SP600125 and D-JNKi) reduced the levels of post-synaptic proteins. In addition, we show that directly stimulating the JNK pathway with an active, upstream kinase increases levels of post-synaptic proteins further supporting JNK's importance in this process. Together with the activation of dendritic JNK in both neurons and synaptoneuroosomes, this fits with our proposed model of JNK's involvement in mGluR-dependent regulation of post-synaptic protein levels (Fig. 5).

The data presented here identify a new role for the JNK pathway in regulating mGluR-dependent changes in FMRP target proteins. This, in turn, raises the possibility that JNK may provide an additional therapeutic target in patients with FXS. We have shown that JNK is constitutively active in the synaptoneuroosomes and dendrites of neurons lacking FMRP. Importantly, the specific mGluR5 inhibitor MPEP, which provides behavioral benefits in *fmr1* KO mice, reduces the constitutive activation of JNK in these neurons (Fig. 1c). This suggests that elevated JNK signaling could directly contribute to the learning and memory and/or behavioral abnormalities in FXS. In this report, we specifically chose to examine how the JNK pathway regulates post-synaptic levels of known FMRP targets because of the constitutive activation of JNK in the *fmr1* KO dendrites. Furthermore, FMRP is proposed to interact with the RNAs for a large number of proteins, making it too difficult to choose protein targets that are definitely not regulated by FMRP. However, even if JNK regulates only FMRP-regulated proteins, this has important implications in our understanding of the mechanisms of learning and memory, and has specific therapeutic implications for FXS. Importantly, abnormalities of mGluR5- and FMRP-dependent signaling have been identified in a number of neurodevelopmental (i.e., Trisomy 21), neurodegenerative (i.e., Alzheimer's disease), and neuropsychiatric disorders (i.e., schizophrenia and major depression). This raises the

possibility that the JNK pathway is similarly dysregulated in these disorders.

JNK inhibitors have begun to enter the clinical arena, enhancing the clinical implications of this work. An orally active JNK inhibitor, CC-930 has entered clinical trials for pulmonary fibrosis and fibrosis in Discoid Lupus Erythematosus (Celgene Corporation 2010; and Celgene Corporation 2011). In addition, D-JNKi, which has been renamed XG-102 and AM-111, has completed a Phase 1 safety trial and a Phase 2 study in hearing loss (Auris Medical AG 2008 and Xigen 2012). Targeting JNK poses several specific problems including the complexity of the signaling complex resulting from three genes, ten splice variants and several scaffold and substrate proteins (Sabapathy 2012). In addition, these different splice variants and scaffolds can be involved in opposing cellular processes (Sabapathy 2012). Thus, ongoing efforts to further understand the JNK signaling components involved in mGluR5-dependent protein translation will be important, as will the ongoing efforts to find increasingly specific inhibitors of specific JNK molecular complexes. This will be particularly important in diseases that require long-term inhibition of JNK such as fragile X syndrome.

Our model for JNK's role in regulating post-synaptic protein levels is shown in Fig. 5. JNK is activated by mGluR stimulation and is therefore more active in disorders with constitutive mGluR signaling such as FXS. Directly stimulating the JNK pathway increases the level of multiple proteins regulated by FMRP, raising the possibility that JNK may work in conjunction with, or in parallel to, the ERK or Akt pathways (dashed arrow, Fig. 5). Importantly, while FMRP itself is regulated by phosphorylation (Narayanan *et al.* 2007, 2008), our data suggest JNK is not responsible for directly phosphorylating FMRP. FMRP inhibits translation when phosphorylated, which would be opposite the effect one would expect based on our data. However, JNK could be regulating a phosphatase or another kinase that does regulate FMRP activity. In either case, JNK seems to have effects independent of FMRP, as JNK inhibition can reduce protein levels in the *fmr1* KO setting.

Collectively, the data presented here indicate that JNK is *necessary and sufficient* for the mGluR5-dependent increase in post-synaptic proteins. Importantly, JNK is constitutively active in FXS dendrites, and one of the most promising therapeutic approaches for this disease, inhibiting mGluR5, reduces JNK activity.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. c-Jun is undetectable in synaptosomal preparations.

Figure S2. mGluR-dependent JNK phosphorylation is transient.

Figure S3. Cycloheximide blocks new protein translation in synaptosomes.

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