Aberrant early-phase ERK inactivation impedes neuronal function in fragile X syndrome

Soong Ho Kim^{*†}, Julie A. Markham^{*}, Ivan Jeanne Weiler^{*}, and William T. Greenough^{*†‡§}

^{*}Beckman Institute, [†]Neuroscience Program, and [‡]Departments of Psychology, Psychiatry, and Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

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Fragile X syndrome (FXS) has so far resisted efforts to define the basic cellular defects caused by the absence of a single protein, fragile X mental retardation protein (FMRP), because the patients have a wide variety of symptoms of varying severity. Immature-appearing dendritic spines on neurons found in FXS patients and *fmr1*-KO mice suggest a role for FMRP in modulating production of synaptic structural proteins. We isolated cortical synaptoneurosomes from WT and KO mice and studied MAPK pathway activation after group I metabotropic glutamate receptor (mGluR) stimulation. Here, we show that ERK in KO synaptoneurosomes is rapidly dephosphorylated upon mGluR1/5 stimulation, whereas it is phosphorylated in WT mice, suggesting that aberrant activation of phosphatases occurs in KO synapses in response to synaptic stimulation. In KO synapses, protein phosphatase 2A (PP2A) is overactivated after mGluR1 stimulation, and tyrosine phosphatase is overactivated after mGluR5 stimulation, causing the rapid deactivation of ERK. ERK activation can be restored in KO by pretreatment with phosphatase blockers; blocking of PP2A by okadaic acid could successfully restore normal ERK activation in KO synaptoneurosomes. We propose that overactivation of phosphatases in synapses may be a key deficit in FXS, which affects synaptic translation, transcription, and synaptic receptor regulation.

dephosphorylation | metabotropic glutamate receptors | phosphorylation | protein translation | synaptoneurosomes

Abnormal numbers and/or shapes of dendritic spines on neurons are associated with fragile X syndrome (FXS) and other neurological and psychiatric diseases such as schizophrenia, mood disorder, Alzheimer's dementia, autism, and mental retardation (1). We previously reported that the brains of FXS patients and the *fmr1*-KO mouse model have immature-appearing, long and thin dendritic spines and higher dendritic spine density (2), suggesting that production of synaptic structural proteins is not appropriately modulated by neuronal activity. Neurotransmitter-triggered synaptic protein synthesis is regulated by a number of signaling pathways. We have chosen to study phosphorylation of the extracellular-signal-regulated kinase ERK because it is a point of convergence of several signaling cascades; thus misregulated ERK activation can be used as a first indicator for deficient translational regulation in synapses. Blocking of overactivated upstream factors to restore normal ERK activation might be a focus for development of treatments and understanding of FXS.

FXS is an inherited, X-linked disorder, caused by hypermethylation of an extreme expansion (200–1,000 repeats) of a (CGG)_n trinucleotide repeat in the 5' UTR of the FMR1 gene, blocking transcription of the gene. The resulting absence of the fragile X mental retardation protein (FMRP) leads to a wide variety of symptoms, including mental retardation, macroorchidism, and behavioral abnormalities. Mental retardation and behavioral abnormalities may be caused principally by disrupted synaptic transmission as a result of a lack of FMRP's role in maintaining synaptic structure and function. Mice lacking FMRP exhibit generalized dysregulation of total protein trans-

lation (3–5), but local (synaptic) translation of specific “cargo” mRNAs is affected as well (6).

We have shown that the rapid initiation of synaptic protein translation, triggered by metabotropic receptor stimulation, is defective in mice lacking a functional *fmr1* gene (7). Synaptic translation of specific mRNAs for AMPA receptor and PSD-95 was also seen to be defective in the absence of FMRP (8). A translational response follows neurotransmitter-initiated phosphorylation and activation of second messengers, prominently ERK 1/2. Here, we investigate changes in the cellular signaling cascade triggered by neurotransmitter receptors. We have examined the early time course of ERK phosphorylation, using cortical synaptoneurosomes from WT and *fmr1*-KO mice. We show here that rapid activation of ERK1/2 subsequent to group I metabotropic glutamate receptor (mGluR) stimulation, as seen in WT mice, is dramatically altered in *fmr1*-KO mice, where there is instead a long-lasting dephosphorylation of ERK after glutamate receptor agonist stimulation. Inhibition of the serine-threonine protein phosphatase 2A (PP2A) by okadaic acid, protein phosphatase 1 (PP1) and PP2A by calyculin A or tyrosine phosphatases by orthovanadate restores agonist-stimulated ERK activation in KO synaptoneurosomes, suggesting that overactivity of phosphatases may be a key deficit in FXS. *Fmr1*-KO mice show, in addition, a residual phosphatase activation in the presence of okadaic acid or orthovanadate.

Results

Group I mGluR Stimulation Triggers Rapid Deactivation of ERK in *fmr1*-KO Cortical Synaptoneurosomes. To study synapse-specific second messenger activation, we used the synaptoneurosomes preparation, based on brief size-selection of synaptic particles so that energy stores are preserved (9). This process makes it possible to take repeated samples from a homogeneous suspension and analyze changes in levels of substrate phosphorylation at successive time points. The two group I mGluRs, mGluR5 and mGluR1, are known to elicit somewhat different cellular responses (10–12). Therefore, we stimulated each receptor independently to test the responses of cortical synaptoneurosomes from WT and *fmr1*-KO mice. Stimulation of mGluR1 [by (S)-3,5-dihydroxyphenylglycine (DHPG) in the presence of the mGluR5 blocker 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP)] in WT led to rapid phosphorylation of ERK, peaking at ≈1 min, and persisting for ≈5 min. In KO synaptoneurosomes, in contrast, there was rapidly decreased phosphorylation of ERK1/2 within the first 1–2 min, which persisted for at least 10 min (Fig. 1A). Activated ERK needs to be deactivated at a certain point after synaptic stimulation, otherwise abnormal accumulation of pERK is toxic to cells (13). It is worth noting that this safety mechanism of shutting off ERK activation (likely

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[§]To whom correspondence should be addressed. E-mail: wgreenou@uiuc.edu.

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Fig. 1. ERK activity after group I mGluR stimulation in WT and *fmr-1* KO cortical synaptoneurosomes. ERK activity was measured by Western blots, using an antibody to ERK1/2 phosphorylated on Thr-202/Tyr-204. (A) mGluR1 stimulation triggered ERK phosphorylation in WT but ERK was rapidly dephosphorylated in KO synaptoneurosomes ($n = 7$ for WT; $n = 4$ for KO). Cortical synaptoneurosomes were preincubated with $10 \mu\text{M}$ MPEP (mGluR5 blocker) for 15 min, then stimulated by $100 \mu\text{M}$ DHPG (group I mGluR agonist) to activate mGluR1s. (B) Stimulation of mGluR5 by specific agonist CHPG ($750 \mu\text{M}$) induced a more transient activation of ERK in WT but a rapid deactivation in KO ($n = 4$ each for WT and KO). Control samples were collected from an unstimulated pool at 0 min (C0') and 10 min (C10'). Total protein level of each lane was measured from an amido black-stained membrane and was used as a loading standard. Data are presented as means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Asterisks between the two curves represent P values for WT versus KO; the rest are for P values of stimulated versus baseline values.

by phosphatases), which was seen after its peak in WT, also functioned in KO (see the parallel decreasing curves after the time points of 2 min in Fig. 1A) even though ERK had already been rapidly dephosphorylated in the initial response to mGluR1 stimulation in KO.

When WT synaptoneurosome were stimulated by mGluR5 [by the specific agonist (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG)] ERK1/2 was rapidly phosphorylated, with somewhat different kinetics than mGluR1, peaking at 1 min, then quickly deactivated. Similarly to mGluR1, we observed dephosphorylation of ERK1/2 in the mGluR5-stimulated preparations from *fmr1*-KO mice (Fig. 1*B*). The safety mechanism to avoid excessive pERK accumulation also operated in both WT and KO.

MEK Is Also Rapidly Deactivated in KO Synapses upon Group I Metabotropic Receptor Stimulation. To understand what causes this activity-dependent rapid dephosphorylation of ERK in KO, we first examined the activity level of its upstream kinase. ERK is activated by a MAPK/ERK kinase (MEK)-catalyzed dual phosphorylation. MEK phosphorylation has been shown to be necessary to the ERK activation observed in hippocampal slices (12, 14). Stimulation of mGluR1 led to a modest and sustained MEK phosphorylation in WT synaptoneurosomes, with slower kinetics than ERK phosphorylation (Fig. 2*A*). Interestingly, although the pERK response rises rapidly, peaking at 1 min, the pMEK response is slower and more prolonged, suggesting a role for an additional enzyme pathway. MEK, again like ERK, is rapidly dephosphorylated in KO samples. Similarly, upon mGluR5 stimulation, MEK is phosphorylated in WT but dephosphorylated in KO samples (Fig. 2*B*). The kinetics of this reaction differ as well: phosphorylation of MEK is stronger and more prolonged than the phosphorylation of ERK. These differences in kinetics suggest involvement of another regulatory factor, such as deactivation of phosphatases.

PP2A Is Overactivated in KO Synapses After mGluR1 but Not mGluR5 Stimulation. The level of ERK activation is controlled not only by its upstream kinase MEK but also by phosphatases such as PP2A. PP2A is a serine–threonine phosphatase, long identified as the major Ser/Thr phosphatase involved in ERK inactivation (14).

By staining samples with antibody to phosphorylated (deactivated) PP2A, we were able to demonstrate that stimulation of mGluR1s led to transiently deactivated PP2A in WT, but not KO, synaptoneurosomes (Fig. 2C). This transient deactivation corresponds kinetically to the increase in ERK phosphorylation seen in WT samples. Sustained basal activity level of PP2A in KO samples together with rapidly deactivated MEK can account for the rapid deactivation of ERK upon mGluR1 stimulation. There was no significant difference in PP2A activation of WT and KO after mGluR5 stimulation (Fig. 2D), which suggests that the deactivation of ERK and MEK in KO upon mGluR5 stimulation was caused by overactivation of a different phosphatase than PP2A.

Thus, the aberrant initial responses to mGluR stimulation in KO synaptoneurosomes, rapidly dephosphorylated ERK and MEK, are caused mainly by inappropriate activation of phosphatases, which does not happen in WT. The data so far strongly indicate that mGluR stimulation triggers overactivation of phosphatases in KO synapses, which leads to the deficient early-phase ERK activation, and thus a lack of activity-dependent modulation of synaptic protein synthesis. To test this hypothesis, we applied phosphatase blockers to KO synaptoneurosomes to see whether normal ERK activation in response to mGluR stimulation could be restored.

Inhibition of PP2A Restores Normal ERK Activation in KO Synapses. Okadaic acid and calyculin A are both blockers of PP2A. Fig. 3 shows that in the presence of okadaic acid, both WT and KO synaptoneurosomes respond to metabotropic receptor agonist stimulation with a strong phosphorylation of ERK lasting ≈ 5 min. Calyculin A blocks PP1 and PP2A; when it is used on KO synaptoneurosomes, the deblocked pERK response is even stronger (Fig. 3*B*; $P < 0.05$ at $t_{\min} = 1$, KO + okadaic acid versus KO + calyculin) than when blocking only PP2A. These results suggest that there is a reservoir of ERK-phosphorylating activity that emerges when phosphatase activity is blocked; the dephosphorylation of ERK observed in KO synaptoneurosomes is abolished under these conditions. Interestingly, the release from negative phosphatase feedback seems to be complete in both WT and KO when stimulated by mGluR1 (Fig. 3*A*; WT +

Fig. 4. ERK activity after group I mGluR activation in WT and KO cortical synaptoneurosomes with tyrosine phosphatase blocker. (A) mGluR1 stimulation in the presence of orthovanadate led to a sustained phosphorylation level of ERK in KO, which was significantly higher than in WT at 10 min after stimulation ($n = 4$ each for KO + orthovanadate and WT + orthovanadate). KO with orthovanadate did not achieve the level of ERK activation in WT + orthovanadate. Asterisks between the two curves represent P values for either WT + vanadate versus KO + vanadate or KO + vanadate versus WT. (B) Phosphorylation of ERK in KO synaptoneurosomes rose dramatically in response to mGluR5 stimulation together with orthovanadate, to a peak level that was not statistically different from WT + orthovanadate ($n = 3$ for KO + orthovanadate; $n = 4$ for WT + orthovanadate). Asterisks represent P values for either WT versus KO + orthovanadate or WT + orthovanadate versus KO + orthovanadate. Control samples were collected from an unstimulated pool at 0 min (C0') and 10 min (C10'). Total protein level of each lane was measured from an amido black-stained membrane and was used as a loading standard. Data are presented as means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Although there is interest in the mGluR theory to describe the FXS (30), and evidence for relief of audiogenic seizures by using MPEP (31), an mGluR5 blocker, there are as yet no direct measurements (e.g., ref. 32) demonstrating excessive activity of mGluR5 in fragile X mammalian systems. A more complex effect, caused by dysregulated levels of a series of FMRP cargo enzymes, may cause an imbalance in second messenger systems, an explanation amenable to experimental approaches. Because the resting cellular level of pERK is higher in hippocampus (33) and cortical synaptoneurosomes (data not shown) of *fmr-1* KO mice, the key to signaling efficacy likely lies in the rate of change

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