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Decreased Nociceptive Sensitization in Mice Lacking the Fragile X Mental Retardation Protein: Role of mGluR1/5 and mTOR

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Fragile X mental retardation is caused by silencing of the gene (*FMR1*) that encodes the RNA-binding protein (FMRP) that influences translation in neurons. A prominent feature of the human disorder is self-injurious behavior, suggesting an abnormality in pain processing. Moreover, FMRP regulates group I metabotropic glutamate receptor (mGluR1/5)-dependent plasticity, which is known to contribute to nociceptive sensitization. We demonstrate here, using the *Fmr1* knock-out (KO) mouse, that FMRP plays an important role in pain processing because *Fmr1* KO mice showed (1) decreased (~50%) responses to ongoing nociception (phase 2, formalin test), (2) a 3 week delay in the development of peripheral nerve injury-induced allodynia, and (3) a near absence of wind-up responses in ascending sensory fibers after repetitive C-fiber stimulation. We provide evidence that the behavioral deficits are related to a mGluR1/5- and mammalian target of rapamycin (mTOR)-mediated mechanism because (1) spinal mGluR5 antagonism failed to inhibit the second phase of the formalin test, and we observed a marked reduction in nociceptive response to an intrathecal injection of an mGluR1/5 agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG) in *Fmr1* KO mice; (2) peripheral DHPG injection had no effect in KO mice yet evoked thermal hyperalgesia in wild types; and (3) the mTOR inhibitor rapamycin inhibited formalin- and DHPG-induced nociception in wild-type but not *Fmr1* KO mice. These experiments show that translation regulation via FMRP and mTOR is an important feature of nociceptive plasticity. These observations also support the hypothesis that the persistence of self-injurious behavior observed in fragile X mental retardation patients could be related to deficits in nociceptive sensitization.

Key words: **pain; translation regulation; nociceptor; FMRP; mGluR; neuropathic pain; mTOR**

Introduction

Fragile X mental retardation is the most common form of inherited mental retardation (Youings et al., 2000) and is caused by the expansion of a CGG repeat in the 5′-untranslated region of the fragile X mental retardation 1 (*FMR1*) gene, leading to hypermethylation and transcriptional silencing. The *FMR1* gene encodes the fragile X mental retardation protein (FMRP), an RNA-binding protein that participates in the trafficking of mRNAs to distal sites in neurons (Bagni and Greenough, 2005; Bardoni et al., 2006). FMRP represses translation of mRNAs that it binds (Mazroui et al., 2002; Barbee et al., 2006) but also regulates the activity-dependent translation of these mRNAs, especially in response to group I metabotropic glutamate receptor (mGluR1/5) activation (Todd et al., 2003; Weiler et al., 2004; Antar et al., 2005; Hou et al., 2006). Studies in *Fmr1* knock-out (KO) mice have indicated that FMRP regulates synaptic plasticity through mechanisms that are linked to its role in activity-dependent translational regulation (Antar and Bassell, 2003; Bear et al., 2004; Bardoni et al., 2006). In the hippocampus and cerebellum of *Fmr1* KO mice, long-term depression (LTD) is increased (Huber et al., 2002; Koekkoek et al., 2005). In contrast, in the somatosensory and neocortical areas of the *Fmr1* KO mouse, long-term potentiation (LTP) is absent (Li et al., 2002; Wilson and Cox, 2007).

A prominent behavioral feature of fragile X mental retardation is self-injurious behavior (Symons et al., 2003), suggesting alterations in pain processing. We have previously demonstrated that FMRP is expressed by nociceptors and localizes to axons of these pain-sensing neurons as well as to neurons throughout the superficial dorsal horn (Price et al., 2006). These findings suggested that FMRP could play a role in nociception; however, this possibility has not been studied experimentally. The notion that FMRP might be involved in nociception is further supported by the established role of mGluR1/5 in pain. mGluR1/5 are involved in nociceptive sensitization both in the periphery (Bhave et al., 2001) and the CNS (Fisher and Coderre, 1996; Karim et al., 2001; Adwanikar et al., 2004). mGluR1/5 are required for spinal LTP (Azkue et al., 2003) and are implicated in the increased excitabil-

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ity of dorsal horn neurons evoked by a persistent noxious stimulus (Fisher and Coderre, 1996; Adwanikar et al., 2004). Despite the connection between FMRP and mGluR1/5, the role of FMRP in the function and regulation of nociceptors is unknown. Likewise, the possible pronociceptive role of activity-dependent translation in neurons, which appears to be controlled, at least in part, by the mammalian target of rapamycin (mTOR) (Kelleher et al., 2004), a kinase that regulates cap-dependent translation (Richter and Sonenberg, 2005), is also unknown. We hypothesized that FMRP might be an important regulator of nociceptor sensitization, especially as it relates to mGluR1/5 and mTOR. We have used the *Fmr1* KO mouse to assess this hypothesis and have found a previously unknown role of FMRP in nociception related to mGluR1/5- and mTOR-dependent enhancement of nociceptive excitability.

Materials and Methods

Experimental animals. Male *Fmr1* KO mice and female *Fmr1* $^{+/-}$ mice on a C57BL/6 background were obtained from Neuromice.org (Northwestern University, Chicago IL). All animal care and use procedures were approved by the Animal Care and Use Ethics Committee of McGill University and were in line with National Institutes of Health (NIH) and International Association for the Study of Pain guidelines. All animals were housed on a 12 h light/dark cycle and had *ad libitum* access to food and water. Male *Fmr1* KO mice were bred at McGill University with female *Fmr1* $^{+/-}$ mice to generate experimental animals. Only male KO and wild-type animals were used. Genotyping was performed via ear punches. All experiments were conducted on KO and wild-type littermates between the ages of 8 and 12 weeks of age.

Antibodies and chemicals. The 7G1-1 antibody was obtained from the Iowa Hybridoma Bank (University of Iowa). Isolectin B_4 (IB_4) conjugated to Alexa-Fluor 488 and secondary Alexa-Fluor antibodies were from Invitrogen (Carlsbad, CA), and the calcitonin gene-related peptide (CGRP) antibody was from Sigma (St. Louis, MO). The phospho-ERK (Thr202/Tyr204) antibody was from Cell Signaling Technology (Danvers, MA), and the total ERK antibody was from Millipore (Nepean, Ontario, Canada). The mGluR5 and βIII-tubulin antibodies were from Millipore. (*RS*)-3,5-Dihydroxyphenylglycine (DHPG) was from Tocris Bioscience (Ellisville, MO), rapamycin was from LC Laboratories (Woburn, MA), and 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) was a gift from FRAXA (produced by Technically, Woburn, MA). All other chemicals were obtained from Sigma.

Immunohistochemistry. Slide-mounted sections were fixed in ice-cold 3.7% paraformaldehyde in 1× PBS for 1 h and then washed 3 times for 5 min in PBS. Slides were transferred to a solution containing 0.1 M sodium citrate and 0.05% Tween 20 and then microwaved on high power for 3 min in a 900 W microwave oven for antigen retrieval. After a 30 min cooling period, slides were again transferred to 1× PBS, washed 3 times for 5 min, and then permeabilized in 1× PBS, containing 0.05% Triton X-100. Slides were then blocked for at least 1 h in 1× PBS, containing 10% normal goat serum, before the addition of anti-FMRP (7G1-1; 1 μg/ml) antibody overnight at 4°C. The CGRP antibody (1:1000 dilution) and IB_4 (1:1000 dilution) were applied together, and the antigen retrieval step was not used. Immunoreactivity was visualized after subsequent incubation with goat anti-rabbit or goat anti-mouse Alexa-Fluor antibody for 1 h at room temperature. All immunohistochemistry (IHC) images are representative of samples taken from three animals. Confocal IHC images were taken using an Axiovert 100M confocal microscope (Zeiss, Thornwood, NY) at McGill University. Image quantification was done as described previously (Price and Flores, 2007).

Behavior. In all experiments, animals were habituated to a Plexiglas behavior chamber under ambient light for 1 h before the beginning of the experiment. Mechanical thresholds were measured by the up–down method (Chaplan et al., 1994) with calibrated von Frey hairs. Thermal thresholds were measured by the hot plate and radiant heat (Hargreaves et al., 1988) methods. For experiments using the radiant heat method, the radiant heat source intensity was adjusted to 70% of maximum. This is a higher intensity than is normally used in mice. This procedure was needed because of mild hyperactivity in *Fmr1* KO mice (The Dutch-Belgian Fragile X Consortium, 1994). In all formalin experiments, formalin (5% in saline) was injected into the hindpaw in a volume of 20 μl, and nociceptive behaviors (licking, biting, or shaking in the affected paw) were measured for 45 min. The first phase is defined as 0–10 min and the second phase as 15–45 min. In MPEP experiments, MPEP (25 nmol in 5 μl) or dilution vehicle was diluted in 2× artificial CSF (ACSF) to yield 1× ACSF. Mice were anesthetized with isoflurane, and MPEP or vehicle was injected intrathecally (by lumbar puncture) 15 min before formalin injection. Rapamycin was diluted in 100% DMSO. For intrathecal injections with rapamycin, 2 μl injections were made 15 min before formalin injection or DHPG (see below), and vehicle controls were the same volume of DMSO. For intraplantar injections, rapamycin or DMSO (vehicle) was injected 15 min before formalin in a volume of 5 μl. DHPG or vehicle was injected intrathecally (5 μl) in anesthetized mice or into the hindpaw (20 μl). For intrathecal experiments, caudally directed nociceptive behaviors were recorded for 30 min as described. For thermal hyperalgesia experiments, thermal withdrawal latencies were recorded before injection and every 30 min after injection for 3 h by the radiant heat method. Spared nerve injury was performed as described previously (Bourquin et al., 2006), and mechanical thresholds were measured before and after surgery as noted above. In all experiments, the observer was blinded to the genotype of the mice and the drug condition.

Western blotting. Animals were injected with DHPG or vehicle intrathecally, and spinal cords were pressure ejected 15 min after injection. Lumbar spinal cord was dissected and snap frozen in liquid nitrogen. Frozen tissues were homogenized in homogenization buffer (50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA at pH 7.4) containing protease and phosphatase inhibitor mixtures with a motorized Dounce homogenizer, sonicated for 15 min at 4°C, and cleared of cellular debris and nuclei by centrifugation at 13,000 relative centrifugal force for 5 min at 4°C. Ten micrograms of protein per well were loaded and separated by standard 7.5% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and then blocked with 5% dry milk for 3 h at room temperature. Phospho-ERK antiserum (1:2000) was incubated overnight at 4°C and detected the following day with donkey anti-rabbit antibody conjugated to horseradish peroxidase (Dako, Ottawa, Canada). Signal was detected by ECL-plus (GE Healthcare, Piscataway, NJ) on chemiluminescent films (Kodak, Rochester, NY). Blots were then stripped using Restore reagent (Pierce, Rockford, IL) and reblotted with a 1:1000 dilution of anti-total ERK antibody. For total mGluR5 levels, naive mice were used, and spinal proteins were prepared by the same method. The mGluR5 antibody was used at a 1:2000 dilution, and the βIII-tubulin antibody was used at a 1:10,000 dilution. Densitometry was conducted using ImageJ version 1.36b for Macintosh OS X (NIH, Bethesda, MD).

Electrophysiology. Extracellular multiunit and single-unit recordings of spike activity in ascending axons of the contralateral ventral quadrant were performed using a modified version of a previously described preparation (Martinez-Gomez and Lopez-Garcia, 2005). Briefly, 4-week-old mice were anesthetized with urethane (2 g/kg, i.p.), and a rostrocaudal laminectomy was performed to reveal the spinal cord. The whole cord was excised with attached dorsal roots and put in cold (0–4°C), oxygenated (95% O_2/5% CO_2) ACSF and cleared of dura mater. The cord was then placed in a recording chamber, pinned down to a Sylgard base, and continuously superfused with oxygenated ACSF of the following composition (in mM): 117 NaCl, 3.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 11 D-glucose, pH 7.4. Multiunit and single-unit activity in ascending axons of the contralateral ventral quadrant was recorded after stimulation of sacral dorsal roots, amplified, and bandpass filtered using a commercial amplifier (CyberAmp 380; Molecular Devices, Union City, CA). The data were digitized at 10 kHz using a CED analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and analyzed with Spike 2 software (Cambridge Electronic Design). To induce wind-up, a 1 Hz train of 15 stimuli (200 μs duration, 20 times threshold) was applied to the dorsal root. This stimulus strength was sufficient to activate C fibers (Martinez-Gomez and Lopez-Garcia, 2005). Wind-up was defined as a progressive increase in the number of spikes elicited by the

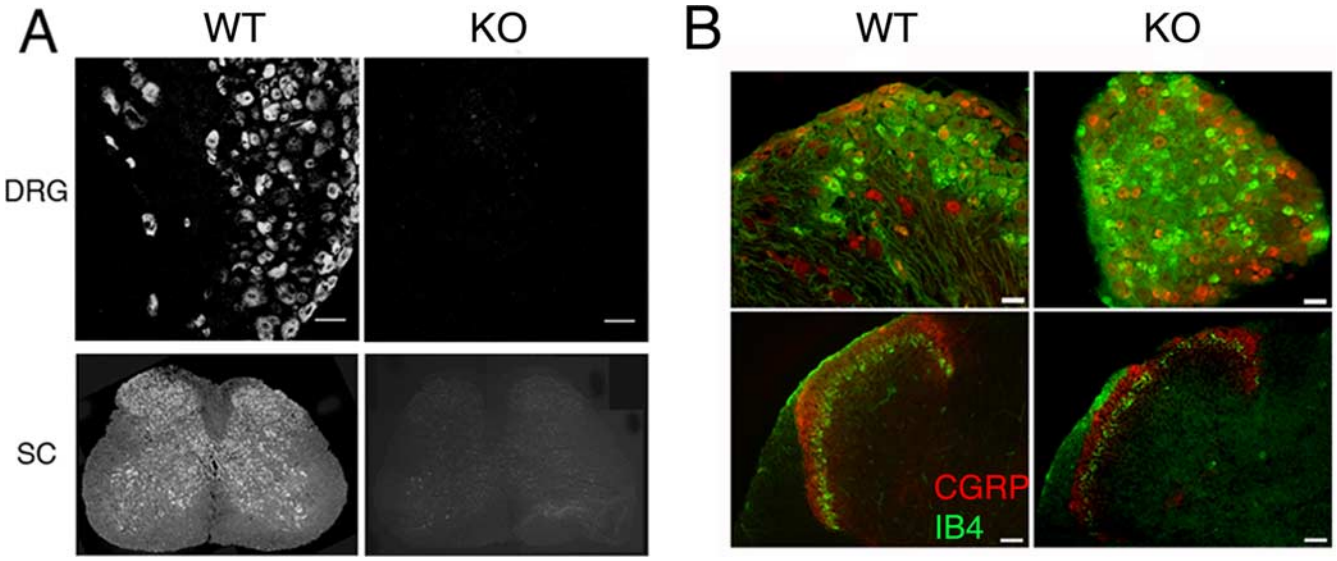

Figure 1. FMRP is expressed in dorsal root ganglion and spinal cord neurons. Distribution of CGRP and IB_4 in Fmr1 KO mice: ***A***, FMRP immunoreactivity was detected in all DRG neurons and throughout the spinal cord (SC) with the anti-FMRP 7G1-1 antibody (left). Immunoreactivity was completely absent in *Fmr1* KO mice (right). Scale bars: DRG panels, 200 μm. ***B***, Immunohistochemistry was performed on wild-type (WT) and KO dorsal root ganglion and spinal dorsal horn for CGRP (red) and IB_4 (green). Top, CGRP and IB_4 labeling were segregated in both wild-type and KO mouse dorsal root ganglion. Scale bars, 200 μm. Bottom, In both wild-type and KO animals, IB_4 (green)-positive afferent terminals localized to the superficial portion of lamina I and lamina II inner. CGRP (red) was found in lamina I and II outer. As in wild-type mice, these subpopulations did not overlap and maintained their laminar distribution in *Fmr1* KO mice. Scale bars, 50 μm.

test stimulation over the 15 stimulations. Wind-down was defined as a progressive decrease of the number of spikes elicited by the test stimulus over the same period.

Data and statistics. All data are presented as mean ± SEM. Single comparisons were made by two-tailed Student's *t* test. Multiple comparisons by genotype were made by two-way ANOVA with Bonferroni post-test. Comparisons of incidence of wind-up in ascending fibers was made by χ^2 test.

Results

FMRP expression and sensory neuron markers in Fmr1 KO and wild-type mice

We first examined the expression of FMRP in the dorsal root ganglion, trigeminal ganglion, and spinal cord of wild-type mice. Using the same antibody (7G1-1) previously used to characterize FMRP expression in rat dorsal root ganglion and spinal cord neurons, we observed FMRP immunoreactivity in all dorsal root ganglion (Fig. 1*A*) and trigeminal ganglion neurons (data not shown) as well as neurons throughout the spinal cord (Fig. 1*A*). Immunoreactivity was completely absent in *Fmr1* KO mice, which confirms the specificity of this antibody (Price et al., 2006). Because fragile X mental retardation is a developmental disorder and FMRP has been suggested to play a role in axonal development (Antar et al., 2006), we assessed the distribution of two common sensory/nociceptor markers in the dorsal root ganglion and dorsal horn in *Fmr1* KO mice. CGRP and the IB_4-binding populations of sensory neurons are segregated in the dorsal root ganglion, and their projections to the dorsal horn have a distinct laminar distribution (Molliver et al., 1997; Zwick et al., 2002). There was no difference in the distribution of CGRP-immunoreactive (wild type, 25.9 ± 1.74%, $n = 3$; *Fmr1* KO, 24.5 ± 1.16%, $n = 3$) or IB_4-binding (wild-type, 24.8 ± 3.72%, $n = 3$; *Fmr1* KO, 23.3 ± 1.15%, $n = 3$) sensory neuronal populations in the dorsal root ganglion, and these populations showed equivalent segregation (colocalization of CGRP and IB_4: wild-type, 2.82 ± 0.30%, $n = 3$; *Fmr1* KO, 2.36 ± 0.13%, $n = 3$) (Fig. 1*B*). Moreover, the laminar distribution of CGRP-immunoreactive and IB_4-binding sensory afferents appeared normal in laminas I and II in both *Fmr1* KO and wild-type mice (Fig. 1*B*). These observations indicate that, at least for these markers, the projection of nociceptors to the dorsal horn develops normally in *Fmr1* KO mice.

Mechanical and thermal thresholds in Fmr1 KO mice

Next we sought to measure basal nociceptive thresholds in *Fmr1* KO mice for mechanical and thermal stimuli. Mechanical thresholds, as determined by von Frey hair stimulation of the hindpaw, did not differ between wild-type (1.04 ± 0.077 g, $n = 13$) and KO (1.22 ± 0.10 g, $n = 14$) mice. Noxious heat sensitivity was assessed in the hot plate and radiant heat tests. In the 50°C hot plate test, latencies to a behavioral response were not different between KO (17.1 ± 0.90 s, $n = 19$) and wild-type (16.4 ± 1.70 s, $n = 9$) mice. In the radiant heat test, withdrawal latencies also did not differ between KO (2.58 ± 0.120 s, $n = 31$) and wild-type (2.27 ± 0.140 s, $n = 23$) mice.

Decreased formalin-evoked nocifensive behavior in Fmr1 KO mice

The formalin test of chemically induced pain is a well characterized behavioral model consisting of two consecutive pain behavior phases, of which the second has been suggested to involve sensitization of the spinal nociceptive system (Coderre et al., 1990; Coderre and Melzack, 1992; Ikeda et al., 2006). We hypothesized that spinal sensitization might be blunted in *Fmr1* KO mice and have used the second phase of the formalin test to assess this hypothesis because formalin injection induces LTP in a subset of dorsal horn neurons (Ikeda et al., 2006) and nociceptive behaviors in the second phase of this model are dependent on protein synthesis (Hou et al., 1997; Kim et al., 1998). In the acute (first) phase of the formalin test, the duration of nociceptive behavior was not different between genotypes (wild-type, 271 ± 29.4 s, $n = 7$; KO, 196 ± 10.1 s, $n = 7$). However, the second phase of the test was significantly reduced in *Fmr1* KO mice (345 ± 63.7 s, $n = 7$), by ~50% versus their wild-type littermates (608 ± 70.7 s, $n = 7$) (Fig. 2*A*,*B*).

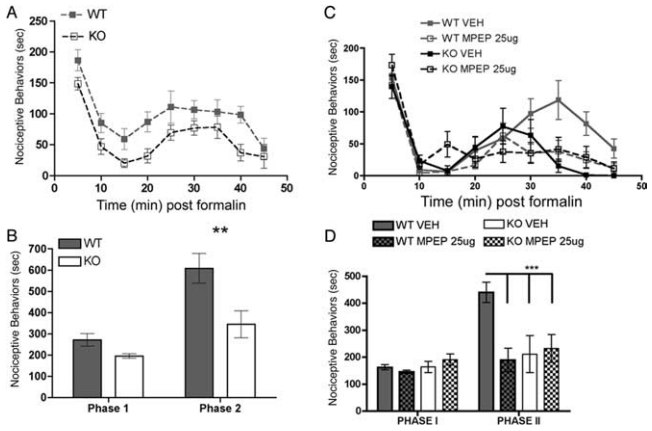

Figure 2. Decreased second-phase formalin responses in Fmr1 KO mice, role of mGluR5. Formalin (5%) was injected directly into the hindpaw of *Fmr1* KO and wild-type (WT) mice, and their nociceptive behaviors were monitored for 45 min. ***A***, Time course of nociceptive behaviors in 5 min blocks. ***B***, Nociceptive behaviors were significantly reduced in the second phase of the formalin test (20–45 min) in *Fmr1* KO mice (**$p < 0.01$). Intrathecal injections of MPEP or vehicle were administered 15 min before intraplantar formalin. ***C***, Time course of nociceptive behaviors in KO and wild-type mice with MPEP or vehicle (VEH) in 5 min blocks. ***D***, MPEP significantly reduced phase 2 responses in wild-type mice but was without effect in *Fmr1* KO mice (***$p < 0.001$).

mGluR1/5-mediated nociceptor plasticity is decreased in Fmr1 KO mice

mGluR1/5-mediated translation is known to be regulated by FMRP (Antar et al., 2004; Aschrafi et al., 2005) and is dysregulated in *Fmr1* KO mice (Hou et al., 2006). Moreover, in the dorsal horn, mGluR1/5 are known to regulate hyperalgesia and LTP (Karim et al., 2001; Azkue et al., 2003) and play an important role in mediating nociceptive behaviors in the second phase of the formalin test (Karim et al., 2001; Zhu et al., 2004). Therefore, we hypothesized that the mGluR5 antagonist MPEP would decrease the second phase of the formalin test in wild-type mice but be ineffective in KO mice. MPEP (25 μg), injected intrathecally 15 min before formalin injection, had no effect on the first phase of the formalin test in wild-type or KO mice (Fig. 2*C*,*D*). In wild-type mice, MPEP significantly decreased the second phase of the formalin test (vehicle, 439 ± 37.5 s, $n = 8$; MPEP, 189 ± 43.3 s, $n = 8$) but did not change it in *Fmr1* KO mice (vehicle, 210 ± 68.5 s, $n = 5$; MPEP, 231 ± 51.9 s, $n = 5$).

The finding that intrathecal MPEP was without effect in *Fmr1* KO mice suggests that mGluR5-mediated mechanisms of nociceptive behaviors in the formalin test are absent in *Fmr1* KO mice. To explore this possibility further, we examined the effect of the mGluR1/5 (mGluR1/5) agonist DHPG on nociceptive behaviors in wild-type and KO mice. When injected intrathecally, DHPG causes a robust nocifensive response that is dependent on mGluR1/5 (Karim et al., 2001). Wild-type and *Fmr1* KO mice received intrathecal injections of DHPG (25 nmol), and their nociceptive behaviors were monitored for 30 min. Nociceptive behaviors were decreased in KO mice versus their wild-type littermates at time blocks of 15–20, 20–25, and 25–30 min (Fig. 3*A*). Over the entire 30 min time period, nociceptive behaviors were decreased by 70% in KO versus wild-type mice. This finding supports the notion that mGluR1/5-mediated spinal nociception is abrogated in *Fmr1* KO mice.

mGluR1/5 not only regulate spinal nociceptive sensitivity but are also involved in peripheral nociceptor sensitization. Peripheral injection of DHPG is known to cause thermal hyperalgesia (Bhave et al., 2001). We have previously shown that FMRP is present in the peripheral axons of nociceptors (Price et al., 2006), indicating that FMRP might play a role in regulating the effects of peripheral mGluR1/5 activation. Hence, we tested the hypothesis that peripherally mediated DHPG-induced thermal hyperalgesia would be reduced or absent in *Fmr1* KO mice. We used a dose of DHPG that has been shown to be peripherally selective and mGluR1/5 dependent in mice (Bhave et al., 2001). Wild-type mice developed thermal hyperalgesia 30 min after DHPG (50 nmol) injection directly into the hindpaw. This thermal hyperalgesia was sustained over the entire 3 h time course of the experiment compared with vehicle-injected wild-type mice (DHPG, $n = 7$; vehicle, $n = 10$) (Fig. 3*B*). However, *Fmr1* KO mice injected with DHPG did not develop thermal hyperalgesia compared with KOs that received vehicle injections (DHPG, $n = 7$; vehicle, $n = 7$) (Fig. 3*B*). Therefore, peripheral, thermal nociceptor sensitization to an mGluR1/5 agonist is absent in *Fmr1* KO mice.

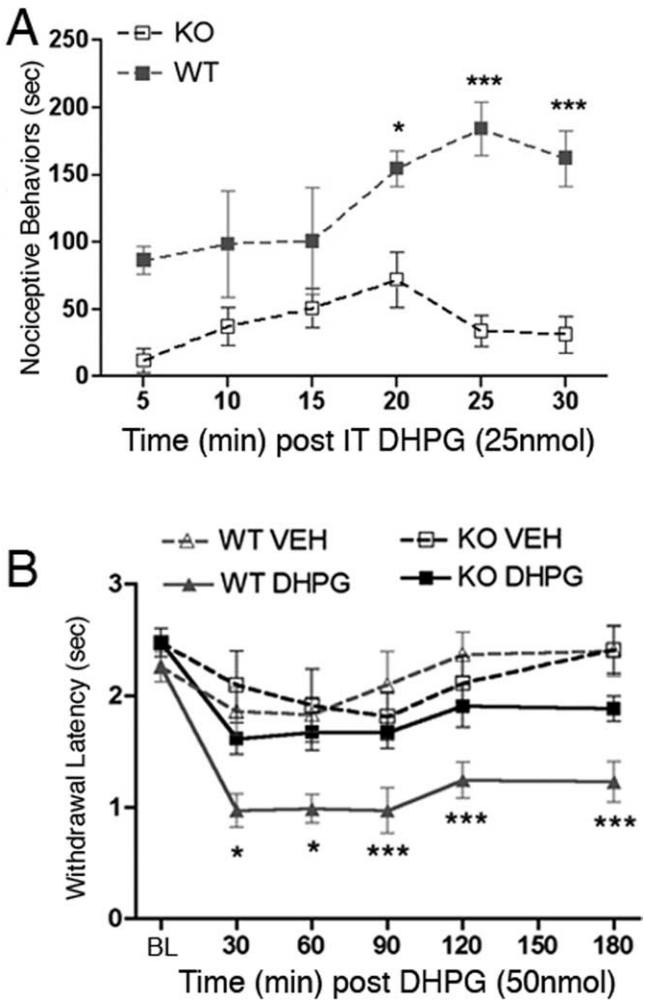

Figure 3. Role of central and peripheral mGluR1/5s in nociceptive sensitization in Fmr1 KO mice. DHPG was injected intrathecally (IT), and nociceptive behaviors were monitored for 30 min. ***A***, Time course of nociceptive behaviors induced by intrathecal DHPG in 5 min blocks (*$p < 0.05$; ***$p < 0.001$). ***B***, Baseline (BL) thermal latencies were measured and then DHPG or vehicle (VEH) was injected directly into the hindpaw. Thermal latencies were measured at the indicated time points for 180 min. Wild-type (WT) mice developed thermal hyperalgesia in response to DHPG (*$p < 0.05$; ***$p < 0.001$). Thermal hyperalgesia was completely absent in *Fmr1* KO mice.

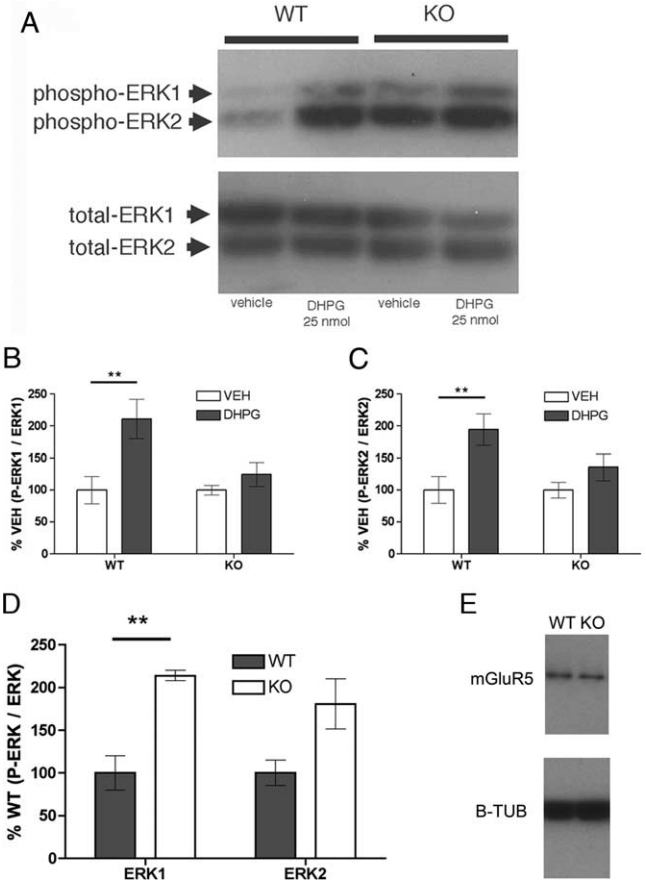

Figure 4. Intrathecal DHPG-induced ERK phosphorylation is impaired in Fmr1 KO mice. DHPG or vehicle was injected intrathecally, and lumbar spinal cords were harvested 15 min later. ***A***, A representative Western blot for phospho-ERK and total ERK in each drug and genotype condition. ***B***, ***C***, Phospho ERK levels are standardized to total ERK and shown as percentage of vehicle (VEH) per genotype. DHPG induced an increase in phospho-ERK1 (***B***) and phospho-ERK2 (***C***) only in wild-type (WT) mice. ***D***, Basal phospho-ERK1 levels were elevated in *Fmr1* KO mice, but a significant increase was not observed for phospho-ERK2. ***E***, Total, spinal mGluR5 protein was also measured and standardized to β-tubulin (B-TUB), and no difference was observed between wild-type and *Fmr1* KO mice. $^{**}p < 0.01$.

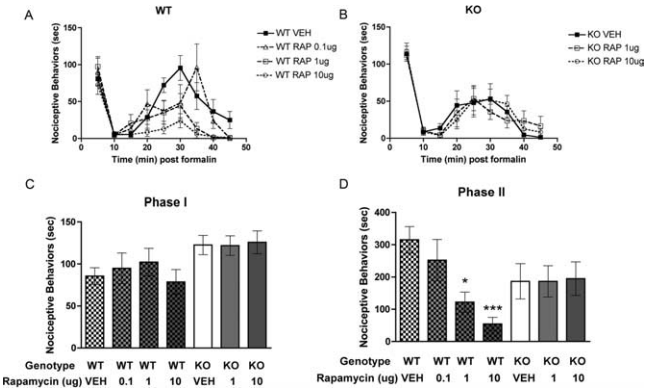

Figure 5. Decreased second-phase formalin responses in Fmr1 KO mice, role of spinal mTOR. Formalin (5%) was injected directly into the hindpaw of *Fmr1* KO and wild-type (WT) mice, and their nociceptive behaviors were monitored for 45 min. Intrathecal injections of rapamycin (RAP) or vehicle (VEH) were administered 15 min before intraplantar formalin. ***A***, ***B***, Time course of nociceptive behaviors in 5 min blocks in wild-type mice with increasing intrathecal doses of rapamycin (***A***) and in *Fmr1* KO mice (***B***). ***C***, Nociceptive behaviors were not altered by rapamycin in the first phase of the formalin test in either genotype. ***D***, Rapamycin significantly reduced phase 2 responses in wild-type mice in a dose-dependent manner but was without effect in *Fmr1* KO mice ($^{*}p < 0.05$, $^{***}p < 0.001$, vehicle vs [rapamycin]).

Misregulation of spinal phospho-ERK in Fmr1 KO mice

Stimulation of spinal mGluR1/5 induces ERK phosphorylation and inhibition of ERK reduces nociceptive behaviors induced by DHPG (Karim et al., 2001). In the hippocampus, DHPG also induces ERK phosphorylation, but this increase in phospho-ERK is absent in *Fmr1* KO mice (Hou et al., 2006). Interestingly, hippocampal phospho-ERK levels are increased in *Fmr1* KO compared with wild-type mice in the absence of mGluR1/5 activation (Hou et al., 2006). We applied DHPG or vehicle intrathecally to wild-type and KO mice and examined ERK phosphorylation status by Western blot 15 min after injection. ERK phosphorylation was standardized to total ERK levels in all conditions. In wild-type mice, DHPG induced a significant, approximately twofold increase in lumbar, spinal phospho-ERK1 and phospho-ERK2 compared with vehicle-injected wild-type mice ($n = 6$/group) (Fig. 4*A–C*). In contrast, DHPG did not induce a significant increase in phospho-ERK1 or phospho-ERK2 levels in *Fmr1* KO mice compared with vehicle ($n = 6$/group) (Fig. 4*A–C*). When vehicle-injected wild-type phospho-ERK levels were compared with vehicle-injected *Fmr1* KO mice, a significant, approximately twofold increase in basal phospho-ERK1 was observed ($n = 6$/group) (Fig. 4*A*,*D*). Therefore, DHPG fails to induce an increase in phospho-ERK in *Fmr1* KO mice, even though basal phospho-ERK1 levels are increased in *Fmr1* KO mice.

Because differences in nociceptive behaviors and signaling between *Fmr1* KO and wild-type mice could be explained by alterations in mGluR5 expression, we examined spinal mGluR5 protein levels in KO and wild-type mice. There was no change in total spinal mGluR5 protein between genotypes in naive animals ($n = 6$/group) (Fig. 4*E*).

Role of mTOR in formalin- and DHPG-induced nociceptive sensitization

Decreased nociceptive sensitization in *Fmr1* KO mice suggests that activity-dependent protein synthesis plays an important role in formalin and DHPG nociception. The kinase mTOR, which phosphorylates inhibitors of elongation initiation factors (namely 4E-BP2 in neurons), allowing for the formation of the eIF–4F complex (Richter and Sonenberg, 2005), plays a key role in stimulating activity-dependent translation and is strongly implicated in synaptic plasticity (Kelleher et al., 2004). Although previous studies have demonstrated a role for protein synthesis in the second phase of the formalin test (Hou et al., 1997; Kim et al., 1998), a role for mTOR in this process has not been explored. Hence, we hypothesized that rapamycin, an inhibitor of mTOR, would inhibit nociceptive behaviors induced by formalin and DHPG in wild-type mice. Intrathecal injection of rapamycin 15 min before formalin injection had no effect on first-phase responses in wild-type or *Fmr1* KO mice (Fig. 5*A–C*). On the other hand, rapamycin dose dependently (0.1–10 μg) decreased second-phase responses in wild-type mice with significant effects observed at 1 and 10 μg (Fig. 5*A*,*D*) (vehicle, 315.0 $\pm$ 41.0 s, $n = 8$; 0.1 μg of rapamycin, 252.0 $\pm$ 64.2 s, $n = 5$; 1 μg of rapamycin, 122.3 $\pm$ 30.8 s, $n = 6$; 10 μg of rapamycin, 54.4 $\pm$ 20.4 s, $n = 8$). In *Fmr1* KO mice, rapamycin did not influence the second phase of the formalin test at any concentration tested (Fig. 5*B*,*D*) (vehicle, 186.4 $\pm$ 54.7 s, $n = 7$; 1 μg of rapamycin, 186. 4 $\pm$ 48.6 s, $n = 7$; 10 μg of rapamycin, 194.9 $\pm$ 52.0 s, $n = 7$).

The absence of peripherally injected DHPG-induced thermal hyperalgesia in *Fmr1* KO mice suggests that protein synthesis in nociceptive axons regulates nociceptor sensitization. Hence, we

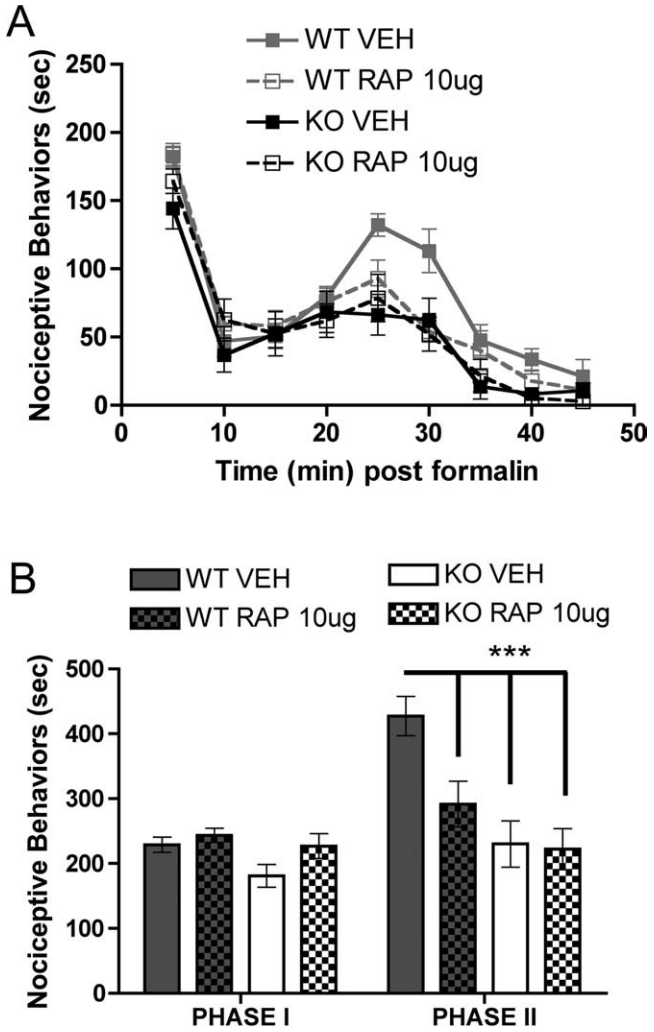

Figure 6. Role of peripheral mTOR in second-phase formalin responses. Rapamycin (RAP, 10 μg) or vehicle (VEH) was injected into the hindpaw 15 min before 5% formalin. ***A***, Time course of nociceptive behaviors in 5 min blocks in wild-type (WT) and *Fmr1* KO mice. ***B***, Rapamycin had no effect on phase 1 responses in either genotype but significantly decreased phase 2 responses only in wild-type mice (***$p < 0.001$).

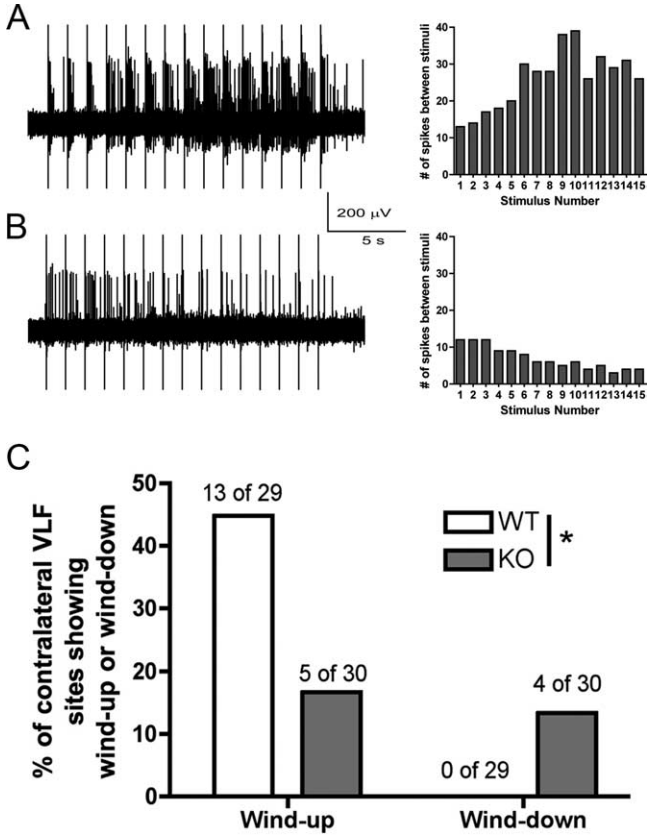

Figure 7. Reduced wind-up and increased wind-down in Fmr1 KO mice. Recordings were made in the contralateral ascending VLF fibers in an *in vitro* whole spinal cord preparation. ***A***, Left, Representative trace of wind-up in a wild-type mouse preparation. ***B***, Left, Representative trace of wind-down in an *Fmr1* KO mouse preparation in response to 15 C-fiber stimuli of the dorsal root at 1 Hz. ***A***, ***B***, Right, The wind-up (***A***) or wind-down (***B***) response as the total number of spikes evoked by each stimulus over the stimulation period. ***C***, The incidence of wind-up was decreased and the incidence of wind-down was increased in *Fmr1* KO mice compared with wild-type (*$p < 0.05$).

tested the ability of an intraplantar injection of rapamycin to inhibit a subsequent nociceptive response to formalin injection into the same hindpaw. In wild-type mice, rapamycin (10 μg) significantly inhibited the second phase of the formalin test without affecting the first phase (Fig. 6*A*,*B*) (vehicle, 427.2 ± 30.3 s, $n = 9$; rapamycin, 291.5 ± 35.2 s, $n = 8$). Rapamycin injection had no effect on formalin responses in *Fmr1* KO mice (Fig. 6*A*,*B*) (vehicle, 230 ± 35.8 s, $n = 6$; rapamycin, 222.2 ± 31.7 s, $n = 6$).

Finally, we tested the role of mTOR in intrathecally injected DHPG-stimulated nociceptive behaviors. We used a dose of rapamycin that was effective in reducing second-phase formalin responses (10 μg). Wild-type mice that received an intrathecal injection of rapamycin showed a significant decrease in nociceptive behaviors over the 30 min observation period compared with mice that received a vehicle injection (vehicle, 1294 ± 54.2 s, $n = 5$; rapamycin, 814 ± 135.9 s, $n = 6$). We did not test the effect of rapamycin in this model in *Fmr1* KO mice because their DHPG-induced behaviors were dramatically decreased compared with wild-type and because rapamycin was not effective in KO mice in the formalin test. Together, these findings demonstrate that mTOR-dependent protein synthesis plays an important role at the spinal and peripheral levels in formalin-induced nociceptive behaviors in wild-type but not *Fmr1* KO mice and that mTOR regulates DHPG-induced nociception at the spinal level.

Reduced wind-up in Fmr1 KO mice

To gain an independent assessment of decreased nociceptive sensitization in *Fmr1* KO mice, we used an *in vitro* spinal cord preparation to examine the incidence of wind-up in ascending nociceptive fibers of the contralateral ventrolateral funiculus (VLF). Wind-up is a form of short-term synaptic plasticity that can be evoked in ascending VLF fibers by repetitive stimulation of the dorsal root at C-fiber strength (Martinez-Gomez and Lopez-Garcia, 2005). Of 29 units recorded from 16 wild-type mice, 13 units (44.8%) gave wind-up responses, and there was no wind-down response in any unit recorded. A representative trace of wind-up in wild-type mice is shown in Figure 8*A*. In eight *Fmr1* KO mice, a total of 30 units were recorded. C-fiber stimulation evoked contralateral VLF unit activity in *Fmr1* KO mice, further confirming the integrity of the basal nociceptive system in these mice. On the other hand, of the 30 units recorded, only five gave a wind-up response (16.7%), and four units (13.3%) displayed a wind-down response (Fig. 7*B*,*C*). The distribution of wind-up and wind-down responses in ascending, contralateral VLF nociceptive fibers was significantly different between wild-type and *Fmr1* KO mice (Fig. 7*C*) ($\chi^2 = 8.217$, 2; $p = 0.016$), indicating

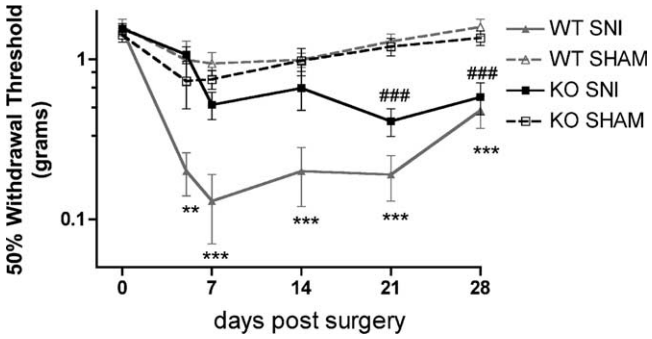

Figure 8. Delayed development of neuropathic allodynia in Fmr1 KO mice. Baseline mechanical thresholds were measured before sham or spared nerve injury (SNI) surgery. Mechanical thresholds were measured at the indicated time points after surgery. Wild-type (WT) spared nerve injury mice developed allodynia versus sham by 5 d after surgery (**$p < 0.01$; ***$p <$ 0.001). *Fmr1* KO spared nerve injury mice did not develop allodynia until 21 d after surgery versus sham ($^{###}p < 0.001$).

that wind-up in response to C-fiber stimulation is severely reduced in *Fmr1* KO mice, whereas wind-down (which was not observed in wild-type mice) is almost as prevalent in these mice.

Delayed development of nerve injury-induced allodynia in Fmr1 KO mice

Axonally synthesized proteins appear to play an important role in nerve regeneration and repair after injury (Willis and Twiss, 2006), and local, axonal translation is required for injury-induced hyperexcitability of *Aplysia* axons (Weragoda et al., 2004). We predicted that, if FMRP regulates the translation of proteins that mediate nerve injury-induced hyperexcitability in axons, the development of neuropathic allodynia would be delayed (because these proteins would have to be transported from the soma, rather than synthesized locally) or even absent in *Fmr1* KO mice. We used the spared nerve injury model (Bourquin et al., 2006) to test this hypothesis. As previously reported (Bourquin et al., 2006), wild-type mice developed mechanical allodynia versus sham wild-type mice at 5 d after surgery, and the allodynia was maintained for the full 28 d of testing ($n = 7$–10/group) (Fig. 8). In contrast, *Fmr1* KO mice did not develop mechanical allodynia until 3 weeks after surgery ($n = 8$–10/group) (Fig. 8). These findings suggest that FMRP-mediated translational regulation in injured axons is involved in the development of nerve injury-induced mechanical allodynia.

Discussion

The experiments described here demonstrate that *Fmr1* KO mice have normal acute nociceptive responses (Zhao et al., 2005) and that the gross development of the dorsal root ganglion (DRG)–spinal nociceptive system is intact. On the other hand, we have observed a reduced mGluR1/5-mediated nociceptive sensitization at the level of the spinal cord and periphery in *Fmr1* KO mice. Our findings also indicate that mTOR, a kinase that regulates cap-dependent translation (Richter and Sonenberg, 2005), plays an important role in nociceptive sensitization on the spinal and peripheral levels in wild-type mice and that this mechanism is lacking in *Fmr1* KO mice. Moreover, Fmr1 KO mice show a drastic reduction in C-fiber stimulation-evoked wind-up in ascending nociceptive pathways and a 3-week delay in the development of allodynia in response to peripheral nerve injury. Hence, our experiments demonstrate that Fmr1 KO mice show decreased nociceptive sensitization in multiple experimental paradigms and that translation regulation via FMRP and mTOR are likely to play a critical role in sensitization of the nociceptive pathway.

New protein synthesis is required for several forms of synaptic plasticity (Steward, 2002; Schuman et al., 2006). Increased excitability in the spinal cord after a persistent noxious stimulus is associated with spinal LTP (Ji et al., 2003; Ikeda et al., 2006; Sandkuhler, 2007), and spinal late-phase LTP requires protein synthesis (Hu et al., 2003). Formalin injection induces LTP in a subset of dorsal horn neurons (Ikeda et al., 2006). Moreover, nociceptive behaviors in the second phase of the formalin test are inhibited by systemic (Hou et al., 1997) or intrathecal (Kim et al., 1998) application of protein synthesis inhibitors. Our findings suggest that FMRP and mTOR are important regulators of translation related to spinal sensitization in the formalin model. Second-phase formalin responses were strongly reduced in *Fmr1* KO mice, and the mTOR inhibitor rapamycin dose dependently blocked second-phase responses in wild-type mice via intrathecal delivery. Rapamycin was without effect in *Fmr1* KO mice in the formalin model, consistent with recent findings demonstrating that activity-regulated protein synthesis is dysregulated in *Fmr1* KO mice (Hou et al., 2006; Nosyreva and Huber, 2006; Muddashetty et al., 2007). The lack of effect of intrathecal MPEP in the second phase of the formalin test coupled with the ~70% decrease in DHPG-mediated nociceptive behaviors in *Fmr1* KO mice indicates that these events are linked to mGluR1/5. Moreover, DHPG-induced nociceptive behaviors were attenuated by intrathecal rapamycin in wild-type mice, indicating that mTOR-dependent translation mediates part of this effect downstream of mGluR1/5. Intrathecal DHPG also failed to increase ERK phosphorylation in the spinal cord of *Fmr1* KO mice, whereas basal phospho-ERK levels were increased in these mice. This finding is consistent with previous experiments in hippocampal slices from *Fmr1* KO mice (Hou et al., 2006) and suggests that the temporal regulation of ERK activity in response to mGluR1/5-stimulated pathways is absent in *Fmr1* KO mice. This apparent failure in mGluR1/5-mediated ERK regulation could explain the paradoxical finding of decreased nociceptive sensitization in the presence of increased ERK phosphorylation. Moreover, in the context of mGluR-dependent synaptic plasticity, ERK and mTOR signaling appear to be parallel pathways that eventually converge on eIF4E to regulate cap-dependent protein synthesis (Banko et al., 2006). Hence, understanding aberrant ERK and mTOR signaling and their effects on cap-dependent translation in *Fmr1* KO mice could reveal mechanisms of disrupted activity-dependent translation that have been observed in this model of fragile X mental retardation (Hou et al., 2006; Nosyreva and Huber, 2006; Muddashetty et al., 2007) and have relevance to nociceptive sensitization.

FMRP plays a well established role in mGluR1/5-mediated translational regulation (Todd et al., 2003; Antar et al., 2004, 2005; Hou et al., 2006). In the dorsal horn, mGluR1/5 are required for the induction of LTP (Azkue et al., 2003), and mGluR5 is strongly implicated in nociceptive sensitization (Fisher and Coderre, 1996; Karim et al., 2001; Adwanikar et al., 2004). Hence, we propose that FMRP-regulated translational mechanisms play a previously unappreciated role in regulating mGluR1/5-mediated nociception. Our results in the *Fmr1* KO mouse and with mTOR inhibition in wild-type mice are consistent with the hypothesis that spinal LTP is disrupted in *Fmr1* KO mice or in the presence of rapamycin; however, we cannot discount the possibility that spinal LTD is enhanced in *Fmr1* KO mice, as in other brain regions (Huber et al., 2002; Koekkoek et al., 2005). LTD can be induced in spinal neurons by mGluR1/5 stimulation (Heinke

and Sandkuhler, 2005), and spinal LTD has been associated with antinociception (Sandkuhler et al., 1997). Hence, an enhancement of spinal LTD in *Fmr1* KO mice could yield a similar behavioral phenotype to decreased LTP. The resolution of these questions will require spinal electrophysiological recordings in *Fmr1* KO mice.

In addition to LTP and LTD, a shorter-term form of nociceptive sensitization, called wind-up, is normally expressed in the spinal cord and is characterized by a gradual increase in C-fiber-evoked spike activity in dorsal horn neurons and their ascending fibers (Baranauskas and Nistri, 1998; Herrero et al., 2000). Our findings demonstrate a clear reduction of wind-up in ascending fibers of the contralateral VLF in *Fmr1* KO mice compared with wild-type mice, which adds additional weight to the hypothesis that spinal nociceptive sensitization is decreased in *Fmr1* KO mice.

Our experiments also suggest that FMRP and mTOR contribute to the peripheral sensitization of nociceptors. Several experiments have demonstrated that dorsal root ganglion axons are translationally competent (Zheng et al., 2001; Willis et al., 2005) and that axonal translation is important for growth cone guidance and collapse (Verma et al., 2005; Wu et al., 2005) and for injury- or depolarization-induced hyperexcitability of *Aplysia* sensory axons (Weragoda et al., 2004). We have previously demonstrated that FMRP is transported to the peripheral axons of rat nociceptors (Price et al., 2006). It is also known that FMRP localizes to axonal growth cones, where it is involved in regulating axonal growth (Antar et al., 2006), and that FMRP is associated with RNAi machinery in sensory axons (Murashov et al., 2007). Here, we have shown that intraplantar rapamycin inhibited second-phase formalin responses in wild-type but not KO mice, demonstrating a role for peripheral mTOR-mediated translation in this model. Moreover, mGluR1/5-mediated thermal hyperalgesia is completely absent in the *Fmr1* KO, suggesting that FMRP regulates mGluR1/5-mediated translation locally in peripheral nociceptors and that this process plays a role in thermal hyperalgesia, at least in response to DHPG. Finally, we have shown that the development of neuropathic allodynia is delayed in *Fmr1* KO mice, which supports the notion that FMRP regulates peripheral nociceptor hyperexcitability, a role that could be linked to axonal regeneration, in the case of peripheral nerve injury (Willis and Twiss, 2006). Interestingly, in male fragile X premutation carriers, in which there is an increase in *FMR1* gene transcription, peripheral neuropathy is a common clinical feature (Jacquemont et al., 2003; Berry-Kravis et al., 2007). These clinical findings have led to the hypothesis that an increase in *FMR1* mRNA causes neuropathy (Tassone et al., 2007), a hypothesis supported by experiments in *Drosophila* models (Jin et al., 2007; Sofola et al., 2007). In *Fmr1* KO animals, which lack *Fmr1* mRNA and FMRP protein, we have observed the opposite: a delay in the development of neuropathic allodynia.

Finally, a major behavioral feature of fragile X mental retardation is self-injurious behavior (Symons et al., 2003). The connection between self-injurious behavior and pain sensitivity is not fully understood (Symons and Danov, 2005), but there are indications that self-injurious behaviors can be reduced by increasing pain sensitivity with opioid antagonist treatment (Osman and Loschen, 1992; Symons et al., 2004). Although deficits in nociceptive sensitization are unlikely to precipitate self-injurious behavior, this hypothesis does provide a rationale for the persistence of such behavior. We are not aware of clinical studies that address the notion that self-injurious behavior is related to decreased nociceptor sensitization in experimental pain models (and there are obvious ethical concerns in conducting such studies), but it is nonetheless interesting that self-injurious behavior is a common behavioral feature of many developmental disorders characterized by deficits in synaptic plasticity (Osman and Loschen, 1992; Percy, 2002; Symons et al., 2003), including fragile X mental retardation and Rett syndrome (Moretti et al., 2006). Here, in a mouse model of fragile X mental retardation, we have provided behavioral and electrophysiological evidence for marked decreases in plasticity of the nociceptive system. Deficits in wind-up in ascending nociceptive pathways that accompany fragile X mental retardation may be particularly significant, in that spinal wind-up is associated with temporal summation and "second pain" responses in humans and nonhuman primates (Price et al., 1977, 1978). More work is needed to establish a connection between altered nociceptive sensitization and self-injurious behavior; however, our results demonstrate that in a mouse model of a human disease in which self-injurious behavior is prominent, there are clear indications of decreased nociceptive sensitization linked to deficits in signaling pathways that regulate neuronal plasticity.

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