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Dysregulation and restoration of homeostatic network plasticity in fragile X syndrome mice

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Abstract

Chronic activity perturbations in neurons induce homeostatic plasticity through modulation of synaptic strength or other intrinsic properties to maintain the correct physiological range of excitability. Although similar plasticity can also occur at the population level, what molecular mechanisms are involved remain unclear. In the current study, we utilized a multielectrode array (MEA) recording system to evaluate homeostatic neural network activity of primary mouse cortical neuron cultures. We demonstrated that chronic elevation of neuronal activity through the inhibition of GABA(A) receptors elicits synchronization of neural network activity and homeostatic reduction of the amplitude of spontaneous neural network spikes. We subsequently showed that this phenomenon is mediated by the ubiquitination of tumor suppressor p53, which is triggered by murine double minute-2 (Mdm2). Using a mouse model of fragile X syndrome, in which fragile X mental retardation protein (FMRP) is absent (*Fmr1* knockout), we found that Mdm2-p53 signaling, network synchronization, and the reduction of network spike amplitude upon chronic activity stimulation were all impaired. Pharmacologically inhibiting p53 with Pifithrin- α or genetically employing *p53* heterozygous mice to enforce the inactivation of p53 in *Fmr1* knockout cultures restored the synchronization of neural network activity after chronic activity stimulation and partially corrects the homeostatic reduction of neural network spike amplitude. Together, our findings reveal the roles of both *Fmr1* and Mdm2-p53 signaling in the homeostatic regulation of neural network activity and provide insight into the deficits of excitability homeostasis seen when *Fmr1* is compromised, such as occurs with fragile X syndrome.

Keywords

Fmr1; FXS; p53; Mdm2; neural network; plasticity

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AUTHORS' CONTRIBUTIONS

KAJ, KYL, SS, DEE and NPT designed and performed the experiments. KAJ, KYL and NPT analyzed the data. KAJ, DEE and NPT wrote and edited the manuscript. All authors read and approved the final manuscript.

1. INTRODUCTION

Homeostatic plasticity, or scaling, describes the cellular processes that act to counteract activity challenges or perturbations in order to maintain neuronal excitability within a physiological range (Frischknecht et al., 2014). Neurons carry out homeostatic plasticity through transcriptional, translational, and post-translational mechanisms to modulate the levels and/or activities of proteins involved in excitability regulation, such as ion channels, vesicle trafficking proteins, and synaptic scaffolding proteins (Schanzenbacher et al., 2016). Subsequently, synaptic or intrinsic excitability is adjusted. Although similar plasticity can also occur at the population level, as demonstrated in multiple recent studies (Hengen et al., 2013; Hengen et al., 2016), the molecular mechanisms underlying homeostatic network plasticity are less understood. Our current study was designed to answer this question through the use of a multielectrode array (MEA) recording system with primary mouse cortical neuron cultures.

Altered or insufficient homeostatic plasticity has been linked to pathological conditions associated with imbalanced circuit excitability, including epilepsies, autism spectrum disorders (ASDs), and schizophrenia (Schanzenbacher et al., 2016). Genome-wide studies on homeostatic plasticity have illustrated the up- or down-regulation of many genes associated with these diseases (Chang et al., 2010; Gao et al., 2010; Jakawich et al., 2010; Lee et al., 2011; Penney et al., 2012; Wang et al., 2013; Wondolowski and Dickman, 2013; Zhong et al., 2012). However, how the dysregulation of these genes disrupt homeostatic plasticity is largely unknown. To assess this question, we studied the *fragile X mental retardation 1 (Fmr1)*. Transcriptional silencing or loss-of-function mutations in the *Fmr1* gene, which encodes the fragile X mental retardation protein (FMRP), leads to fragile X syndrome (FXS) (Ashley et al., 1993). FXS is the most common inherited form of mental retardation and autism; its population prevalence is about 1 in 4,000 among males and 1 in 8,000 among females (Abrahams and Geschwind, 2008; Kelleher and Bear, 2008). Multiple symptoms associated with neuronal and synaptic hyperexcitability are seen in both FXS patients and *Fmr1* knockout mice, including sensory hypersensitivity, anxiety, and seizures (Brennan et al., 2006; Gross et al., 2011; Iacoangeli and Tiedge, 2013; Repicky and Broadie, 2009). This hyperexcitability may also contribute to the developmental delays and learning-associated abnormalities seen in FXS (Brennan et al., 2006). Despite the current understanding of the mechanisms leading to hyperexcitability, it is unclear whether and why homeostatic regulation of neuronal excitability fails in FXS. To this end, we evaluated how disruption of *Fmr1* alters homeostatic regulation of neural excitability, particularly at the network level, and how to correct it.

The ubiquitin E3 ligase murine double minute-2 (Mdm2) and one of its well-known substrates, the tumor suppressor p53, are both critical players in cell cycle regulation and programmed cell death (Oliner et al., 2016; Wade et al., 2010). The role in which Mdm2 and p53 play in terminally differentiated neuronal cells is just starting to be uncovered. Our previous studies have shown that Mdm2 is dysregulated in *Fmr1* KO mice, leading to impaired excitatory synapse elimination (Tsai et al., 2017; Tsai et al., 2012). Whether p53 is also affected in *Fmr1* KO mice was unclear until a recent study revealed a role for p53 in *Fmr1*-associated neural stem cell differentiation and proliferation (Li et al., 2016). However,

its functions in *Fmr1*-dependent neural plasticity remain unknown. Our current study revealed that in cultured cortical neurons, chronic neuronal activity induces synchronization of network activity and reduction of spontaneous spike amplitude, which requires Mdm2-mediated p53 ubiquitination. Importantly, these phenomena are absent in *Fmr1* KO cultures. When pharmacologically inhibiting p53 in *Fmr1* KO cultures or genetically crossing *Fmr1* KO mice with *p53* heterozygous mice to reduce p53 expression, chronic neuronal activity-induced synchronization of network activity is restored while homeostatic reduction of spike amplitude is partially corrected. In summary, our work demonstrates novel roles of *Fmr1* and Mdm2-p53 signaling in the homeostatic regulation of neural network activity. Because neuronal excitability imbalance is a common phenotype in many ASDs (Amaral et al., 2008; Levitt et al., 2004), and the Mdm2 signaling pathway is functionally associated with multiple ASD-linked genes such as Pten and MEF2 (Tsai et al., 2017; Tsai et al., 2012; Williams et al., 2015), our results may provide mechanistic insights into the excitability imbalances observed in FXS and other ASDs.

2. MATERIALS AND METHODS

2.1 Animal use:

All animal experiments were performed following the guidelines of Animal Care and Use as provided by the Illinois Institutional Animal Care and Use Committee (IACUC) and the guidelines for the Euthanasia of Animals as provided by the American Veterinary Medical Association (AVMA) in order to minimize animal suffering and the number of animals used. This study was performed under approved IACUC animal protocols from the University of Illinois at Urbana-Champaign (#14139 and #17075 to N.-P. Tsai.)

2.2 Primary cortical neuron cultures

All mice used in this study were in a C57BL/6 genetic background. Primary cortical neuron cultures were made from mice aged at p0-p1 as described previously (Tsai et al., 2012) and maintained in NeuralQ basal medium (GlobalStem) supplemented with B27 supplement (Invitrogen), GlutaMax (final concentration at 2 mM; Invitrogen), and Cytosine β -D-arabinofuranoside (AraC, final concentration at 2 μ M; Sigma). Half the culture media was replaced with fresh media on days-in-vitro (DIV) 2 and every 3-4 days thereafter until the experiments on DIV 13-14. Previous studies have demonstrated that the synaptic connection is mature in primary neuron cultures on DIV 13-14 (Niere et al., 2012; Soden and Chen, 2010; Tsai et al., 2012). Each experiment in this study was performed with at least 3 independent litters and cultures.

2.3 Reagents

Picrotoxin and poly-D-lysine were from Santa Cruz Biotechnology. Pifithrin- α was from Adipogen Corporation. Nutlin-3 was from Cayman Chemical. Dimethyl sulfoxide (DMSO) was from Fisher Scientific. The antibodies used in this study were purchased from Santa Cruz Biotechnology (anti-p53 and IgG controls), GenScript Corporation (anti-Gapdh), and Cell Signaling (anti-Ubiquitin and anti-p53). HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology and Sigma.

2.4. Immunoprecipitation and western blotting

For immunoprecipitation, 80 µg of total protein lysate was mixed with 1 µg of primary antibody for 1 hr at 4 °C with constant rotation. Then, 20 µl of Protein A/G beads (Santa Cruz Biotechnology) were added into the mixture for 1 hr. At the end of the incubation, beads were washed with a buffer (50 mM Tris, pH 7.4, 120 mM NaCl, 0.5% Nonidet P-40) 3 times before western blotting. After subjecting the samples to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the gel was transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking with 1% bovine serum albumin dissolved in TBST buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated with a primary antibody overnight at 4 °C, followed by a 30-min wash with TBST. The membrane was then incubated with an HRP-conjugated secondary antibody for 1 hr, followed by another 30-min wash with TBST. The membrane was then developed with an ECL (enhanced chemiluminescence) solution as described (Waung et al., 2008) and imaged with X-ray films (Denville Scientific Inc) in a darkroom.

2.5 MEA recording and analysis

All MEA recordings were performed using an Axion Muse 64-channel system with single well MEAs (M64-GL1-30Pt200, Axion Biosystems) inside a humidified incubator with 5% CO₂ at 37°C. Field potentials (voltage) at each electrode relative to the ground electrode were recorded with a sampling rate of 25 kHz. After 30 min of baseline recording (0-hr time point), the drug(s) or vehicles indicated in each experiment were added, and the MEA dish was immediately put back into the incubator for an additional 24 or 48 hours before another 30 min of recording. Due to changes in network activity caused by physical movement of the MEA, only the last 15 min of each recording were used in data analyses (Jewett et al., 2016). AxIS software (Axion Biosystems) was used for the extraction of spikes (i.e. action potentials). After filtering, a threshold of ± 6 standard deviations was independently set for each channel; activity exceeding this threshold was counted as a spike.

The settings for the detection of burst activity were set at a minimum of 5 spikes with a maximum inter-spike interval of 0.1 sec (Jewett et al., 2016). The synchrony index was computed through AxIS software based on a previously published algorithm (Eggermont, 2006) by taking the cross-correlation between two spike trains, removing the portions of the cross-correlogram that are contributed by the auto-correlations of each spike train, and reducing the distribution to a single metric. A value of 0 corresponds to no synchrony and a value of 1 corresponds to perfect synchrony (Jewett et al., 2016).

The spikes detected in each electrode could be heterogeneous due to the number of neurons within close proximity to an electrode and/or the synchrony of those neurons. Thus, when an electrode detects spikes from a large number of neurons or from a group of neurons that are not well synchronized, the detected spikes may appear to be more heterogeneous. In contrast, when an electrode detects spikes from a smaller number of neurons or from a group of neurons that are well synchronized, the detected spikes may appear less heterogeneous. To minimize the variability between cultures and electrodes, the recording from each MEA culture at the 24-hr or 48-hr time point was compared to the 0-hr time point from that same culture.

2.6 Patch-clamp electrophysiology

Whole-cell patch-clamp recordings were made as described previously (Liu et al., 2017). In brief, the recordings were performed at room temperature (23-25°C) in a submersion chamber continuously perfused with artificial cerebrospinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 4 CaCl₂, 4 MgCl₂, 1 NaH₂PO₄, 26 NaHCO₃ and 11 D-Glucose, saturated with 95% O₂/5% CO₂ (pH 7.4, 310 mOsm). aCSF was supplemented with 1 μM TTX and 100 μM picrotoxin for miniature excitatory postsynaptic currents (mEPSCs) measurements. Whole cell recording pipettes (~4-6 MΩ) were filled with intracellular solution containing (in mM): 130 K-gluconate, 6 KCl, 3 NaCl, 10 HEPES, 0.2 EGTA, 4 Mg-ATP, 0.4 Na-GTP, 14 Tris-phosphocreatine, 2 QX-314 (pH 7.25, 285 mOsm). Membrane potential was clamped at -60 mV. All recordings were performed with Clampex 10.6 and Multiclamp 700B amplifier interfaced with Digidata 1440A data acquisition system (Molecular Devices). Recordings were filtered at 1 kHz and digitized at 10 kHz. mEPSCs were analyzed with Mini Analysis Program (Synaptosoft) with a 5-pA threshold level.

2.7 Measurement of p53 transcriptional activity

The reagents for the p53 transcriptional activity assay were obtained from Cayman Chemical. Primary cortical neurons treated with DMSO or Picrotoxin were lysed and subjected to an enzyme-linked immunosorbent assay (ELISA)-based approach to measure endogenous p53 binding to the surface of 96-well plates pre-coated with a p53 response element (Jewett et al., 2015).

2.8 Reverse transcription and real-time PCR

After drug treatment, the total RNA from cortical neurons in cultures was obtained with TRIzol reagent (Life Technologies). Reverse transcription was performed with Photoscript Reverse Transcriptase (New England Biolab) and the real-time PCR was performed with Thermo Scientific Maxima SYBR Green reagent. The primers used in this study were: *Gapdh*, 5'-GGT ATT CAA GAG AGT AGG GAG-3' and 5'-GGG TGC AGC GAA CTT TAT TG-3'; *p53*, 5'-AGA GTC TAT AGG CCC ACC CC-3' and 5'-GCT CGA CGC TAG GAT CTG AC-3'.

2.9 Statistical Analysis

For multiple comparisons, a two-way ANOVA and Tukey *post-hoc* test were performed. A student *t*-test was used in Figs 2A, 7B and 7C. Each “n” indicates an independent culture in MEA or biochemistry experiments, and a cell in patch-clamp experiments. Differences are considered significant at the level of $p < 0.05$.

3. RESULTS

3.1 Mdm2-p53 signaling is required for chronic activity-induced reduction of neural network spike amplitude.

Chronic neuronal activity stimulation elicits homeostatic reduction of synaptic strength or intrinsic excitability. Although similar plasticity can also occur at the population level, what molecular mechanisms are involved remain unclear. To answer these questions, we

employed an MEA system to record extracellular spontaneous spikes (action potentials) of wild-type (WT) cultured primary cortical neurons. To induce neuronal activity, we treated cultures with the GABA(A) receptor antagonist Picrotoxin (PTX, 100 μ M) or vehicle (DMSO) starting on day-in-vitro (DIV) 13-14, as previously described (Jewett et al., 2016; Jewett et al., 2015; Mihalas et al., 2013; Seeburg et al., 2008). The recordings were performed immediately before and 24, or 48 hours after treatment. When comparing the recordings from the same culture before and after treatment, we found that PTX significantly reduced the average amplitude of spontaneous spikes after 48 hours of PTX administration (Fig. 1A and Supplementary Fig. 1A). Based on these results, as well as previous studies demonstrating homeostatic downscaling after PTX administration in cultures for two days (Fu et al., 2011; Hu et al., 2010; Turrigiano et al., 1998), we chose to measure the change following 48-hours of treatment for the rest of our experiments. In summary, these results indicate that chronic neuronal activity perturbations trigger a homeostatic response at the network level.

We have previously identified that chronic neuronal activity stimulation induces the ubiquitination and inactivation of the tumor suppressor p53 by Mdm2 (Jewett et al., 2015). To determine whether Mdm2-p53 signaling participates in the homeostatic reduction of spontaneous spikes, we employed a validated small molecule inhibitor, Nutlin-3 (1 μ M), which blocks the interaction of Mdm2 and p53, thereby preventing p53 inactivation through Mdm2-mediated ubiquitination (Secchiero et al., 2011; Shen and Maki, 2011; Van Maerken et al., 2014; Vassilev et al., 2004). While treatment with Nutlin-3 alone does not affect the amplitude of spontaneous spikes, it inhibits the reduction of spontaneous spike amplitude induced by chronic PTX treatment (Fig. 1A). Together, our data showed that chronic neuronal activity stimulation elicits a Mdm2-p53 signaling-dependent reduction of neural network spike amplitude.

3.2 Chronic activity-induced reduction of neural network spike amplitude is altered in *Fmr1* KO cultures.

We next asked whether chronic activity-induced homeostatic reduction of spontaneous spike amplitude requires *Fmr1*, a massive regulator for neurodevelopment and excitability homeostasis (Davis and Broadie, 2017). To this end, we employed primary cortical neuron cultures made from *Fmr1* KO mice and treated the cultures with the vehicle or PTX (100 μ M). We observed that the cultures treated with PTX exhibited elevated spontaneous spike amplitude after PTX treatment for 48 hours (Fig. 1B and Supplementary Fig. 1B). This contrasts with the previous data in WT cultures, in which PTX treatment leads to a reduction of spontaneous spike amplitude. These data suggest that the Mdm2-p53 signaling-dependent reduction of neural network spike amplitude is impaired in *Fmr1* KO neurons. This conclusion is further supported by our data showing that Nutlin-3, the small molecule which inhibits the Mdm2-p53 interaction, did not produce a significant effect on neural network spike amplitude in *Fmr1* KO neurons, even in conjunction with PTX treatment (Fig. 1B).

Because *Fmr1* KO neurons are known to exhibit alterations in synaptic connection and excitability (Berry-Kravis et al., 2010; Brennan et al., 2006; Luscher and Huber, 2010), we examined whether improper neural network spike amplitude in *Fmr1* KO neurons resulted

from impaired synaptic connections or an insufficient response to chronic PTX treatment. To do so, we first performed whole-cell patch-clamp recordings to measure miniature excitatory post-synaptic current (mEPSC) from WT and *Fmr1* KO neurons. Our data show that *Fmr1* KO neurons exhibit similar, and perhaps even slightly stronger synaptic connections, than WT neurons as demonstrated by comparable mEPSC frequency and slightly elevated mEPSC amplitude (Fig. 2A). We next determined whether WT and *Fmr1* KO cultures respond to PTX treatment differently by analyzing burst activity, which is defined as a series of spontaneous spikes occurring within a short period of time. Burst activity in cultures is known to be elevated by PTX treatment and serves as an indicator for elevated neuronal activity (Jewett et al., 2016; Schmitt et al., 2004). We defined a minimum of 5 spontaneous spikes with a maximum inter-spike interval of 0.1 second as a burst, as previously reported (Jewett et al., 2016), and confirmed that PTX treatment for 48 hours induced burst activity, as demonstrated by an increased number of spikes per burst, which occurred at a similar level in both WT and *Fmr1* KO cultures (Fig. 2B). In summary, our data confirm that, despite normal synaptic connection and a successful induction of neuronal activity by PTX, homeostatic reduction of neural network spike amplitude is altered in *Fmr1* KO cultures. Based on the results from Fig. 1, we hypothesized that this deficit in *Fmr1* KO cultures was a result of altered Mdm2-p53 signaling following chronic activity stimulation.

3.3 *Fmr1* KO cultures fail to elicit activity-induced p53 ubiquitination and inactivation.

We aimed to test the hypothesis that altered p53 down-regulation and ubiquitination is responsible for impaired homeostatic network activity in *Fmr1* KO cultures. We first measured the total level and ubiquitination of p53 in total brain lysates from WT and *Fmr1* KO mice. As shown (Supplementary Fig. 2), we did not observe significant differences between WT and *Fmr1* KO, suggesting that the down-regulation and ubiquitination of p53 may be seen only after chronic activity stimulation. We then measured the level of p53 in cortical neuron cultures following treatment with vehicle or PTX for 48 hours. As shown in Fig. 3A, PTX significantly reduced the level of p53 in WT cultures, but not in *Fmr1* KO cultures. Using an ELISA-based approach to measure the transcriptional activity of p53 (Jewett et al., 2015), we obtained results consistent with the notion that PTX reduces the activity of p53 in WT cultures, but not in *Fmr1* KO cultures (Fig. 3B). The mRNA analysis using real-time RT-PCR did not find significant changes in p53 mRNA levels (Fig. 3C), further supporting the hypothesis that the downregulation of p53 occurs at the post-transcriptional level. These results confirm a deficit of p53 inactivation in *Fmr1* KO cultures upon chronic activity stimulation.

To determine whether impaired p53 inactivation in *Fmr1* KO cultures occurs as a result of reduced ubiquitination, we measured p53 ubiquitination through immunoprecipitation of endogenous p53 followed by western blotting with anti-ubiquitin antibody 48 hours after vehicle or PTX treatment in WT and *Fmr1* KO cultures. As shown in Fig. 3D, the ubiquitination of p53 is elevated after PTX treatment in WT cultures. In *Fmr1* KO cultures, despite a trend toward basally elevated p53 ubiquitination, which is likely a result of basally elevated phosphorylation of Mdm2 (Tsai et al., 2017), chronic PTX fails to further promote p53 ubiquitination. Altogether, our results demonstrate that chronic neuronal activity-induced p53 ubiquitination and that inactivation of p53 require *Fmr1*.

3.4 Chronic activity-induced synchronization of neural network activity is reduced in *Fmr1* KO cultures.

It has been shown that chronic activity stimulation through the inhibition of GABAergic signaling can elevate the synchrony of neuronal firing events (Jewett et al., 2016; Khazipov and Holmes, 2003), which could be a contributing factor for the homeostatic control of neuronal excitability (Frohlich et al., 2008; Lazarevic et al., 2013). Impaired homeostatic reduction of spontaneous network spike amplitude in *Fmr1* KO cultures suggest that the network synchronization may also be impaired in *Fmr1* KO cultures. Furthermore, we have previously shown that Mdm2-p53 signaling is involved in the synchronization of neural network activity upon activity stimulation (Jewett et al., 2016). Because our current data showed that Mdm2-p53 signaling is altered in *Fmr1* KO cultures (Fig. 3), we hypothesized that chronic activity-induced neural network synchrony requires *Fmr1* and is therefore impaired in *Fmr1* KO cultures. To this end, we compared the neural network synchrony after the treatment of vehicle or PTX for 48 hours (Fig. 4), shown in representative traces from two designated electrodes more than 250 μm away from each other on a single MEA (Fig. 4A, 4B) and by quantification of the synchrony index, which is based upon the synchrony of spike firing between electrode pairs throughout the entire MEA (Fig. 4C). As expected, PTX significantly elevated synchrony in WT cultures (Fig. 4A, 4C). However, synchrony is only slightly, but not significantly, elevated in *Fmr1* KO cultures after PTX treatment (Fig. 4B, 4C). These data indicate that chronic activity-induced neural network synchronization is impaired in *Fmr1* KO cultures. We then aimed to determine whether restoration of Mdm2-p53 signaling or p53 inactivation in *Fmr1* KO cultures could correct neural network synchronization and allow for the homeostatic reduction of neural network spike amplitude.

3.5 Inhibition of p53 restores chronic activity-induced synchronization of neural network activity.

In an effort to elicit p53 inactivation in *Fmr1* KO cultures upon chronic activity stimulation, we employed a widely used p53 transcriptional inhibitor Pifithrin- α (1 μM) (Ehrnhoefer et al., 2014; Gowran and Campbell, 2008; Hoshino et al., 2014). As demonstrated previously, treatment with Pifithrin- α efficiently reduces p53 activity without affecting cell viability in cultures (Jewett et al., 2016). We first tested the effect of Pifithrin- α on neural network synchronization in WT cultures. As shown in Fig. 5A, Pifithrin- α alone does not affect neural network synchronization, indicating that inhibition of p53 is not sufficient to alter synchronization. When we applied PTX for 48 hours to WT cultures pretreated with DMSO or Pifithrin- α , we observed a similar elevation of neural network synchronization (Fig. 5A). The effect of PTX on cultures pretreated with Pifithrin- α is slightly smaller than those pretreated with DMSO, possibly due to the fact that inhibition of p53 can reduce or delay network synchronization at an earlier time point (Jewett et al., 2016). Together, these results suggest that inhibition of p53 does not alter chronic activity-induced neural network synchronization in WT cultures.

We next evaluated how inhibition of p53 could affect neural network synchronization in *Fmr1* KO cultures. As in WT cultures, treatment of Pifithrin- α alone in *Fmr1* KO cultures does not trigger significant effect on neural network synchronization (Fig. 5B). Although PTX alone does not elevate neural network synchronization in *Fmr1* KO cultures,

pretreatment with Pifithrin- α significantly promotes PTX-induced neural network synchronization (Fig. 5B). These data confirmed our hypothesis that inhibition of p53 is able to restore PTX-induced neural network synchronization in *Fmr1* KO cultures.

3.6 Inhibition of p53 partially corrects homeostatic reduction of neural network spike amplitude.

We asked whether inhibition of p53 is also able to restore the homeostatic reduction of neural network spike amplitude in *Fmr1* KO cultures. We again first tested the effect of Pifithrin- α on neural network spike amplitude in WT cultures. As shown in Fig. 6A, Pifithrin- α does not affect basal neural network spike amplitude or chronic PTX-induced reduction of neural network spike amplitude in WT cultures, consistent with our observation that Pifithrin- α does not have an effect on neural network synchronization in WT cultures. Importantly, when we applied Pifithrin- α in *Fmr1* KO cultures (Fig. 6B), although Pifithrin- α alone did not have an effect, it reduced the abnormally elevated neural network spike amplitude induced by PTX treatment. These data suggest that the inhibition of p53 partially rescues the chronic activity-induced homeostatic reduction of neural network spike amplitude in *Fmr1* KO cultures.

To validate that the effects of Pifithrin- α are specific to p53 and to provide a secondary method to confirm the role of p53 in neural network plasticity, we crossed *Fmr1* KO (*Fmr1*^{-/-}) mice with WT (*p53*^{+/+}) or *p53* heterozygous (*p53*^{+/-}) littermate mice to genetically reduce p53 in *Fmr1* KO. Using this approach, we were able to reduce p53 levels by approximately 41.5% in cultured *Fmr1* KO cortical neurons (Fig. 7A). We then evaluated chronic activity-induced neural network synchronization and homeostatic reduction of neural network spike amplitude in *Fmr1*^{-/-} *p53*^{+/+} and *Fmr1*^{-/-} *p53*^{+/-} cultures. As shown in Figs. 7B and 7C, the cultures prepared from *Fmr1*^{-/-} *p53*^{+/+} mice continue to show impaired neural network synchronization and abnormally elevated neural network spike amplitude after PTX treatment as previously observed (Fig. 4B and 4C). However, when we employed the cultures prepared from *Fmr1*^{-/-} *p53*^{+/-} mice, we observed results similar to those from *Fmr1* KO (*Fmr1*^{-/-}) cultures pretreated with Pifithrin- α (Figs. 5B and 6B). Specifically, we observed that PTX-induced neural network synchronization is restored (Fig. 7B) and the abnormally elevated neural network spike amplitude is reduced (Fig. 7C).

In an effort to evaluate whether a previously identified p53 target gene, neural precursor cell expressed developmentally down-regulated gene 4-2, *Nedd4-2*, is involved in activity-induced homeostatic reduction of neural network spike amplitude, we utilized cultures prepared from *Nedd4-2*^{andi} mice in which the major form of Nedd4-2 in cortical neurons is deficient (Jewett et al., 2015). However, our data show that the homeostatic reduction of neural network spike amplitude remains intact in cultures prepared from *Nedd4-2*^{andi} cultures (Fig. 8), suggesting that other mechanisms induced by p53 inactivation are involved in this homeostatic network plasticity.

Altogether, our results suggest that impaired p53 inactivation contributes to the alterations of neural network synchronization and homeostatic reduction of neural network spike amplitude in *Fmr1* KO cultures. Importantly, the inhibition of p53, either pharmacologically or genetically, partially corrects these deficits.

4. DISCUSSION

Homeostatic regulation of neuronal excitability is crucial to allow the circuit to accurately determine the baseline for long-term plasticity events and maintain a physiological range of excitability (Davis, 2013). Our current study demonstrated that such homeostatic regulation can occur at the population level, demonstrated by a reduction of spontaneous neural network spike amplitude upon chronic activity stimulation in mouse cortical neuron cultures. Further, this study demonstrated that such a phenomenon requires *Fmr1* and activity-induced p53 inactivation. Two factors may potentially contribute to the effects seen on the amplitude of spontaneous neural network spikes. The first factor is the intrinsic excitability of individual neurons, which affects the strength of individual action potentials and subsequently affects the strength of a network spike. The second factor is the number of inputs contributing to a firing event, which can be affected by the functional connectivity of neurons in a population or the number of neurons that fire above the detecting thresholds. Both of the factors described above can be modulated by chronic activity perturbations (Bartley et al., 2008; Cingolani and Goda, 2008; Gasselin et al., 2015) and could contribute to the homeostatic regulation of spike amplitude that we observed in cultured cortical neuron networks. To dissect the proportional contribution of these factors, future experiments with a cell-autonomous manipulation of p53 (such as through sparse knockdown/knockout of *p53* or through an injection of p53 inhibitor into single neurons) or *Fmr1* (through sparse knockdown/knockout of *Fmr1*) followed by chronic activity stimulation at the population level and whole-cell recording of action potentials at the single-cell level would be essential.

It remains to be determined which downstream pathways or target genes of p53 are involved in our observed network plasticity mechanism. Our current study has suggested that *Nedd4-2* is unlikely to be a key target gene of p53 in this mechanism (Fig. 8). We therefore suspect that other mechanisms induced by p53 inactivation are involved in this homeostatic network plasticity. Although unbiased screening of p53 genes has been performed previously using various tissues or cells (Tebaldi et al., 2015; Wei et al., 2006), a unique transcriptome of p53 in neurons is still lacking. It is expected that such analysis in neurons upon chronic activity perturbations would likely reveal essential targets of p53 involved in network plasticity. Furthermore, despite our observation that PTX treatment reduced total activity levels of p53 (Fig. 3), it remains unclear whether the specific activity of p53 is modulated upon activity stimulation by other mechanisms, such as phosphorylation (Fraser et al., 2010; Kodama et al., 2010). Because different regulatory mechanisms could differentially affect the function of p53 toward its target gene expression, a systematic analysis on post-translational modifications and interactome of p53 will be necessary to provide a broad picture of p53 regulation in network plasticity.

Another important result uncovered by our study is the alteration of homeostatic network plasticity in cultures made from *Fmr1* KO mice, the model of FXS. Our data showed that, instead of a reduction of neural network spike amplitude, the amplitude was elevated in the *Fmr1* KO cultures upon chronic PTX treatments. This gain-of-function deficit in *Fmr1* KO suggests that, in addition to impaired Mdm2-p53 signaling and p53 inactivation, chronic activity stimulation likely triggers additional mechanisms in *Fmr1* KO cultures, and subsequently strengthens the network activity. This prediction may also be applicable in

explaining the network synchronization that, although Mdm2-mediated p53 inactivation could delay or slightly reduce chronic activity-induced network synchronization in WT cultures (Jewett et al., 2016), inhibition of p53 promotes that in *Fmr1* KO cultures (Figs. 5 and 7). Although dysregulated excitability is commonly observed in FXS patients and animal models, such an impairment of chronic activity-induced network plasticity has not been experimentally described specifically in the context of FXS to our knowledge. It is unclear at this moment how this deficit is linked to other known mechanisms underlying excitability dysregulation in FXS, including exaggerated metabotropic glutamate receptor signaling (Ronesi et al., 2012; Volk et al., 2007), dysfunction in GABAergic transmission (Martin et al., 2014; Paluszkiwicz et al., 2011) and altered properties of large-conductance calcium-activated potassium channels (also called BK channels) (Deng and Klyachko, 2016). It also remains to be determined whether our mechanism can be applied to homeostatic plasticity *in vivo*, such as after chronic sensory deprivation/enhancement (Teichert et al., 2017). As our data showed extremely low level of basal p53 ubiquitination in total brain lysates (Supplementary Fig. 2), it raised a possibility that activity-induced p53 ubiquitination may not be observed homogeneously *in vivo* and could only be detected in a small population of cells. This suggests a future need to evaluate the role of Mdm2-p53 signaling and homeostatic network plasticity *in vivo*, and to determine whether inhibition of p53 reverses any excitability deficits at the circuit level in *Fmr1* KO mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chronic neuronal activity-induced homeostatic reduction of spontaneous spike amplitude requires Mdm2-p53 signaling and is impaired *Fmr1* KO cortical neuron cultures. Quantification of average spontaneous spike amplitude (left) and representative average traces of 1-min recording (right) of (A) WT and (B) *Fmr1* KO cortical neuron cultures treated with DMSO, PTX, Nutlin-3, or PTX + Nutlin-3 starting at DIV 13-14 for 48 hours. In the traces, the black lines represent the average of all the spikes within representative 1-min recordings. Traces are from the same designated electrode at 0- and 48-hr time points.

Data are represented as mean $\pm$ SEM with * $p < 0.05$, ns: non-significant (n = 5-7 independent cultures).

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Figure 2. *Fmr1* KO cultures exhibit mature synaptic connection and proper burst activity upon PTX treatment.

(A) Patch-clamp recording from WT and *Fmr1* KO cortical neurons at DIV 14.

Representative mEPSC traces, and quantification of mEPSC amplitude and frequency are shown ($n = 15$ and 20 for WT and *Fmr1* KO, respectively).

(B) Quantification of relative number of spikes per burst (left) and representative traces from the same designated

electrode at 0- and 48-hr time points (right) of WT and *Fmr1* KO cortical neuron cultures.

Data are represented as mean $\pm$ SEM with $*p < 0.05$, ns: non-significant ($n = 5-7$ independent cultures).

Figure 3. Chronic neuronal activity-induced p53 ubiquitination and inactivation are impaired in *Fmr1* KO cortical neuron cultures.

(A) Representative western blots of p53 and Gapdh, and quantification of p53 normalized to Gapdh from WT and *Fmr1* KO cortical neuron cultures treated with DMSO or PTX for 48 hours starting at DIV 14 ($n = 7$). (B) Transcriptional activity of p53 from WT and *Fmr1* KO cortical neuron cultures treated with DMSO or PTX for 48 hours starting at DIV 14 ($n = 4$). (C) Quantitative real-time RT-PCR of *p53* mRNA normalized to *Gapdh* mRNA from WT or *Fmr1* KO cortical neuron cultures treated with DMSO or PTX for 48 hours starting at DIV 14 ($n = 4$). (D) Western blots of Ubiquitin (Ub) or p53 after p53 or control IgG immunoprecipitation from DMSO- or PTX-treated WT and *Fmr1* KO cortical neuron cultures ($n = 3$). While IgG serves as an antibody control, cultures made from p53 KO (*p53*^{-/-}) serve as a secondary negative control for p53 signal. Proteasome inhibitor MG-132 (10 μ M) was added 30 minutes prior to the harvest of cells and during the preparation of cell lysates to stabilize ubiquitinated proteins. The quantification of ubiquitin signal was done by first measuring the entire area of smear, from slightly above 50 KDa to 250 KDa, followed by subtracting the average signal of IgG controls (to remove signal from non-specific bands). The intensity was then normalized to precipitated p53 followed by normalization to DMSO-treated WT group. Data are represented as mean $\pm$ SEM with * $p < 0.05$, ** $p < 0.01$, ns: non-significant.

Figure 4. *Fmr1* KO cultures exhibit reduced network synchronization after chronic neuronal activity stimulation.

(A-B) Representative traces of recordings from two electrodes that are over 250 μ m away from each other, and (C) quantification of synchrony index from entire MEA of WT and *Fmr1* KO cortical neuron cultures treated with DMSO or PTX for 48 hours starting at DIV 14. Selected electrodes are marked with “1” and “2” on the electrode map. Traces from the same electrodes at 0- and 48-hr time points are shown on the right. Data are represented as mean $\pm$ SEM with * $p < 0.05$, ns: non-significant ($n = 8$ independent cultures).

Figure 5. Pifithrin- α restores network synchronization after chronic neuronal activity stimulation in *Fmr1* KO cultures.

(A-B) Representative traces of recordings from two electrodes that are over 250 μ m away from each other, and quantification of synchrony index from entire MEA of (A) WT and (B) *Fmr1* KO cortical neuron cultures treated with DMSO, PTX, Pifithrin- α , or PTX + Pifithrin- α starting at DIV 13-14 for 48 hours. Selected electrodes are marked with “1” and “2” on the electrode map (left). Traces from the same electrodes at 0- and 48-hr time points are shown on the right. Data are represented as mean $\pm$ SEM with * p <0.05, ** p <0.01, ns: non-significant (n = 5-8 independent cultures).

Figure 6. Pifithrin- α partially rescues homeostatic response of spontaneous spike amplitude after chronic neuronal activity stimulation in *Fmr1* KO cultures.

(A-B) Quantification of average spontaneous spike amplitude (left) and representative average traces of 1-min recording (right) of (A) WT and (B) *Fmr1* KO cortical neuron cultures treated with DMSO, PTX, Pifithrin- α , or PTX + Pifithrin- α starting at DIV 13-14 for 48 hours. In the traces, the black lines represent the average of all the spikes within representative 1-min recordings. Traces are from the same designated electrode at 0- and 48-

hr time points. Data are represented as mean $\pm$ SEM with * $p < 0.05$, ** $p < 0.01$, ns: non-significant (n = 7-9 independent cultures).

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Figure 7. Genetically reducing p53 in *Fmr1* KO restores network synchronization and partially rescues homeostatic response of spontaneous spike amplitude after chronic neuronal activity stimulation in cortical neuron cultures.

(A) Representative western blots of p53 and Gapdh from *Fmr1* KO x *p53*^{+/+} and *Fmr1* KO x *p53*^{+/-} cortical neuron cultures at DIV 14. The level of p53 in *Fmr1* KO x *p53*^{+/-} was 58.4% $\pm$ 4.1% comparing to *Fmr1* KO x *p53*^{+/+} (repeated with 3 cultures). (B) Quantification of synchrony index from entire MEA (left) and representative traces of recordings from two electrodes that are over 250 μ m away from each other (right) of *Fmr1* KO x *p53*^{+/+} and *Fmr1* KO x *p53*^{+/-} cortical neuron cultures treated with PTX starting at DIV 13-14 for 48 hours. Selected electrodes are marked with “1” and “2” on the electrode map (left). Traces from the same electrodes at 0- and 48-hr time points are shown on the right. (C) Quantification of average spontaneous spike amplitude (left) and representative average traces of 1-min recording (right) of *Fmr1* KO x *p53*^{+/+} and *Fmr1* KO x *p53*^{+/-} cortical neuron cultures treated with PTX at DIV 13-14 for 48 hours. In the traces, the black lines represent the average of all the spikes within representative 1-min recordings. Traces are from the same designated electrode at 0- and 48-hr time points. Data are represented as mean $\pm$ SEM with *p<0.05 (n = 4-8 for B, and 6-8 for C).

Figure 8. *Nedd4-2* is not required for chronic neuronal activity-induced homeostatic reduction of spontaneous spike amplitude.

Quantification of average spontaneous spike amplitude (left) and representative average traces of 1-min recording (right) of *Nedd4-2^{andi}* cortical neuron cultures treated with DMSO or PTX starting at DIV 13-14 for 48 hours. In the traces, the black lines represent the average of all the spikes within representative 1-min recordings. Traces are from the same designated electrode at 0- and 48-hr time points. Data are represented as mean $\pm$ SEM with * $p < 0.05$ ($n = 5-6$ independent cultures).