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Delayed *in vitro* development of Up states but normal network plasticity in Fragile X circuits

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Abstract

A broad range of neurophysiological phenotypes have been reported since the generation of the first mouse model of Fragile X syndrome (FXS). However, it remains unclear which phenotypes are causally related to the cognitive deficits associated with FXS. Indeed, because many of these phenotypes are known to be modulated by experience, a confounding factor in the interpretation of many studies is whether some phenotypes are an indirect consequence of abnormal development and experience. To help diminish this confound we first conducted an *in vitro* developmental study of spontaneous neural dynamics in cortical organotypic cultures. A significant developmental increase in network activity and Up states was observed in both wild-type and *Fmr1*$^{-/y}$ circuits, along with a specific developmental delay in the emergence of Up states in knockout circuits. To determine whether Up state regulation is generally impaired in FXS circuits, we examined Up state plasticity using chronic optogenetic stimulation. Wild-type and *Fmr1*$^{-/y}$ stimulated circuits exhibited a significant decrease in overall spontaneous activity including Up state frequency; however, no significant effect of genotype was observed. These results demonstrate that developmental delays characteristic of FXS are recapitulated during *in vitro* development, and that Up state abnormalities are probably a direct consequence of the disease, and not an indirect consequence of abnormal experience. However, the fact that *Fmr1*$^{-/y}$ circuits exhibited normal homeostatic modulation of Up states suggests that these plasticity mechanisms are largely intact, and that some of the previously reported plasticity deficits could reflect abnormal experience or the engagement of compensatory mechanisms.

Introduction

Fragile X syndrome (FXS) is caused by the loss of the Fragile X mental retardation protein (FMRP) (Verkerk *et al.*, 1991; O'Donnell & Warren, 2002; Santoro *et al.*, 2012). In mice, deletion of the gene *Fmr1*, which codes for FMRP, generates a diverse array of neuronal phenotypes, including abnormalities in dendritic spine morphology and stabilization (Irwin *et al.*, 2000; Nimchinsky *et al.*, 2001; Cruz-Martin *et al.*, 2010; Portera-Cailliau, 2012), metabotropic glutamate receptor (mGluR)-long-term depression (Huber *et al.*, 2002; Bear *et al.*, 2004), synaptic plasticity (Larson *et al.*, 2005; Desai *et al.*, 2006; Meredith *et al.*, 2007; Kim *et al.*, 2013), homeostatic plasticity (Soden & Chen, 2010), short-term synaptic plasticity (Deng *et al.*, 2011, 2013), axonal development (Antar *et al.*, 2006; Bureau *et al.*, 2008), GABAergic inhibition (Curia *et al.*, 2009; Vislay *et al.*, 2013; He *et al.*, 2014), inter-neuronal connectivity (Hanson & Madison, 2007; Gibson *et al.*, 2008), channelopathies (Brown *et al.*, 2010; Gross *et al.*, 2011; Brager *et al.*, 2012; Lee & Jan, 2012; Deng *et al.*, 2013; Routh *et al.*, 2013; Zhang *et al.*, 2014), and imbalanced excitation/inhibition (Gibson *et al.*, 2008; Harlow *et al.*, 2010; Paluszkiewicz *et al.*, 2011a; Goncalves *et al.*, 2013). The sheer diversity of reported neural phenotypes has led some to suggest that FXS may be best understood in the light of abnormal network function (Belmonte & Bourgeron, 2006). Moreover, because cognition and behavior are not the products of isolated neurons, network-level approaches provide an important framework for understanding and treating FXS. Indeed, patients with FXS exhibit increased incidents of epilepsy (Musumeci *et al.*, 1999; Hagerman & Stafstrom, 2009), and hypersensitivity to sensory stimuli (Miller *et al.*, 1999; Frankland *et al.*, 2004; Van der Molen *et al.*, 2012). In addition, several studies in Fmr1 knockout (KO) mice have reported enhanced spontaneous and evoked activity in neural networks from Fmr1 animals (Gibson *et al.*, 2008; Hays *et al.*, 2011; Goncalves *et al.*, 2013).

A further challenge in interpreting the diversity of neural phenotypes observed in the mouse model of FXS is that most of the phenotypes are also associated with experience-dependent plasticity (Buonomano & Merzenich, 1998; Holtmaat & Svoboda, 2009). For example, spine density and stability are altered as a function of environmental enrichment and learning (Greenough *et al.*, 1985; Trachtenberg *et al.*, 2002; Holtmaat & Svoboda, 2009), and the magnitude and direction of synaptic plasticity can be modulated by experience (Kirkwood *et al.*, 1995; Rioult-Pedotti *et al.*, 2000). Indeed, the same plasticity protocol that leads to long-term potentiation in control animals can generate long-term depression in animals deprived of normal sensory experience (Wang *et al.*, 2012). Thus, one must consider the possibility that some of the neural phenotypes

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observed in *Fmr1* KO animals are a consequence of abnormal sensory experience, e.g. impaired sensory perception, learning, and social interactions.

In vitro developmental studies provide one approach towards addressing these confounds as observed phenotypes are more likely to arise from the genotype and not from abnormal experience or development. Thus, we conducted *in vitro* developmental studies of spontaneous neural activity and Up states in cortical organotypic cultures from male KO ($Fmr1^{-/y}$) and wild-type (WT) littermates. We also characterised the network-level plasticity of spontaneous activity in order to study how chronic external stimuli alter activity in Fragile X circuits. We used an optogenetic approach, i.e. cortical slices expressing channelrhodopsin (ChR) 2 were optically stimulated over the course of days to induce a homeostatic down-regulation of network activity. The focus on internal network dynamics and Up states provides an effective approach to study network-level abnormalities, because emergent properties such as Up states ultimately reflect the net interaction of many of the different reported neural phenotypes (Chauvette *et al.*, 2010; Crunelli & Hughes, 2010).

Materials and methods

Experimental animals

All experiments were conducted according to the US National Institutes of Health guidelines for animal research, and approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles. WT male [$Fmr1^{+/y}$ (#4828)] and *Fmr1* KO female [$Fmr1^{-/-}$ (#4624)] mice on the FVB background (FVB.129P2) were obtained from the Jackson Laboratory. A colony was established by breeding heterozygous ($Fmr1^{-/+}$) females and WT ($Fmr1^{+/y}$) males. Post-natal day 5–6 WT ($Fmr1^{+/y}$) and KO ($Fmr1^{-/y}$) male littermates were used in all experiments. Mice were housed in the Division of Laboratory Animal Medicine UCLA vivarium under a 12 h light/dark cycle until 1 h before brain tissue collection.

Organotypic slice preparation

Organotypic slices were prepared using the interface method (Stoppini *et al.*, 1991; Buonomano, 2003) from littermate $Fmr1^{-/y}$ and WT male mice (post-natal day 5–6). Mice were anesthetised with isoflurane and decapitated. The brain was removed and placed in chilled cutting media. Coronal slices (400 μm thickness) containing the primary somatosensory and primary auditory cortex were cut using a vibratome and placed on filters (MillicellCM, Millipore, Billerica, MA, USA) with 1 mL of culture media. The culture media were changed at 1 and 24 h after cutting and every 2–3 days thereafter. The cutting media consisted of Eagle's minimum essential medium (catalog number 15-010; MediaTech, Herndon, VA, USA) plus 3 mM $MgCl_2$, 10 mM glucose, 25 mM HEPES, and 10 mM Tris base. The culture media consisted of Eagle's minimum essential medium plus 4 mM glutamine, 0.6 mM $CaCl_2$, 1.85 mM $MgSO_4$, 30 mM glucose, 30 mM HEPES, 0.5 mM ascorbic acid, 20% horse serum, 10 U/I penicillin, and 10 μg/L streptomycin. Slices were incubated in 5% CO_2 at 35 °C for 7–30 days. A tail sample from each mouse was collected immediately after anesthesia for polymerase chain reaction analysis of tail DNA (genotyping by Transnetyx).

Electrophysiology

Whole-cell recordings were made from layer (L)-II/III regular-spiking, supragranular pyramidal neurons using infrared differential interference contrast visualization. Recording were performed at 7–30 days *in vitro* (DIV) in artificial cerebrospinal fluid composed of (in mM): 125 NaCl, 5.1 KCl, 2.6 $MgSO_4$, 26.1 $NaHCO_3$, 1 NaH_2PO_4, 25 glucose, and 2.6 $CaCl_2$. This artificial cerebrospinal fluid was formulated to match the standard culture media (Goel & Buonomano, 2013). The internal solution for whole-cell recordings contained (in mM): 100 K-gluconate, 20 KCl, 4 ATP-Mg, 10 phospho-creatine, 0.03 GTP-Na, and 10 HEPES and was adjusted to pH 7.3 and 300 mOsm. The temperature was maintained at 32 °C, and the artificial cerebrospinal fluid perfusion rate was set to 3–4 mL/min.

Only cells that satisfied the following criteria were accepted for analysis: membrane potential < −60 mV, input resistance between 100 and 300 MΩ, and series resistance of < 25 MΩ. Cells were discarded if the membrane potential changed by more than 10 mV during the course of recording. Intrinsic excitability was measured as the number of action potentials evoked during a 250 ms current step at intensities of 0.05, 0.1 0.15 and 0.2 nA.

Induction of homeostatic plasticity

Organotypic slices were transfected with AAV5-CaMKIIa-ChR2 (H134R)-EYFP (University of North Carolina Vector Core) at 4–6 DIV. For each slice, 1 μL of the viral solution was gently delivered via a glass pipette into four to five different locations in L-II/III. Slices were virally transfected only for the homeostatic plasticity experiments (data presented in Figs 4 and 5). To reduce variability, these experiments relied on 'sister' slices, i.e. derived from the same batch of animals (littermates), maintained with the same culture medium and serum, placed in the same incubator and virally transfected in the same session. In each experiment, two slices (from the same animal) of each genotype (WT or $Fmr1^{-/y}$) were placed in the 'stimulating incubator' on the day of experiment (total of four slices from two mice, i.e. sister slices). One slice per genotype received chronic stimulation via a blue LED (stimulated slice, superbright LEDs), whereas the other was kept in the same incubator but did not receive stimulation. Optical stimulation consisted of a 50 ms flash of blue light (457 nm) delivered every 30 s for either 2 or 4 days.

Quantification of spontaneous activity

A minimum of 6 min of spontaneous activity was recorded for each neuron. Recordings were sampled at 10 kHz for the developmental study (6–12 min of recording; data presented in Figs 2 and 3) and at 5 kHz for the homeostatic study (10 min of recording; data presented in Figs 4 and 5). Data were saved for off-line analysis using custom-written software in Matlab (MathWorks, Natick, MA, USA).

Spontaneous network events and Up states were quantified based on previously defined criteria (Johnson & Buonomano, 2007; Goel & Buonomano, 2013). The criterion for classifying a spontaneous network event was a voltage deflection of 5 mV above the resting membrane potential ('threshold crossing'). This threshold excluded miniature and unitary excitatory post-synaptic potentials from the analyses and captured primarily network events arising from the activity of multiple pre-synaptic neurons (see Fig. 2E). To prevent the counting of a single event that crossed threshold multiple times within a short window as multiple events, a minimum inter-event interval of 100 ms was used; thus, if the voltage fell and crossed threshold again within < 250 ms, it was still classified as being the same event. Up states were defined as an event that remained above threshold for no < 500 ms. During a network activation such as an Up state, the membrane potential would often make multiple brief

passes above and below this threshold before returning to the resting potential. For this reason and in order to prevent counting of a single Up state that crossed threshold multiple times within a short window as multiple Up states, a minimum inter-event interval of 250 ms was used.

We calculated the SD of the voltage (vSTD) (simply the SD of the recorded membrane potential of a cell) to provide an assumption-independent measure of overall spontaneous activity. We also calculated the vSTD excluding Up state segments.

Statistics

Data are represented by the mean ± SEM. Statistical significance was determined using two-way ANOVAS for Figs 3 and 5, and two-way ANOVAS with repeated-measures *t*-tests for intrinsic excitability data. To contrast differences between specific groups, *post-hoc* tests were performed using *F*-tests for simple effects (Brunning & Kintz, 1970). For reporting the statistical analyses, the value n refers to the number of cells.

Results

Up states are highly correlated across neurons

Spontaneous neural activity occurs at both the synaptic (e.g. miniature excitatory post-synaptic potentials) and network level. Network-level spontaneous activity reflects the orchestrated regulation of numerous synaptic and cellular properties, and it provides an important indicator of normal network function (Thompson, 1997; Johnson & Buonomano, 2007; Destexhe, 2011; Runfeldt *et al.*, 2014), e.g. connectivity, synaptic strength, the balance of excitation and inhibition, and intrinsic excitability. Spontaneous network activity can take on at least two qualitatively different forms, i.e. brief events that reflect bouts of simultaneous activity in a subset of neurons, and Up states.

Up states are network-wide events, as demonstrated by the fact that, during an Up state, the vast majority of neurons within a network participate in the Up state. Furthermore, there is a high degree of correlation between voltage waveforms recorded in different neurons during Up states (MacLean *et al.*, 2005; Johnson & Buonomano, 2007; Paluszkiewicz *et al.*, 2011b; Poskanzer & Yuste, 2011; Runfeldt *et al.*, 2014). Thus, the spontaneous voltage activity recorded from a single neuron provides an excellent readout of network dynamics. Figure 1A–C shows three examples of dual current-clamp recordings performed in three different organotypic slices from WT animals. Each pair shows a 40 s recording of spontaneous voltage activity. The voltage activity depicted in any two simultaneously recorded neurons was highly correlated, and when an Up state occurred in one neuron it also occurred in the second neuron. We never observed an Up state in one neuron but not in the other. The high correlation held true even when neurons were more than 1000 μm apart (Fig. 1C). Figure 1D shows that the mean correlation between cells, as measured over the entire trace or a window around an Up state, was above 0.75. These results are in accordance with previous data (MacLean *et al.*, 2005; Poskanzer & Yuste, 2011) and confirm that intracellular recordings from a single pair of cells provided an accurate measure of network-level Up state activity.

Fmr1$^{-/y}$ circuits exhibit a developmental delay of the emergence of spontaneous activity and Up states

We characterised the *in vitro* development of spontaneous activity in cortical organotypic slices from *Fmr1*$^{-/y}$ and WT male littermate mice to examine potential network-level abnormalities in FMRP KO circuits. Whole-cell recordings were performed from L-II/III pyramidal neurons from slices cultured for 7–30 days. The voltagegrams shown in Fig. 2A and B indicate that neurons of both WT and *Fmr1*$^{-/y}$ circuits exhibited very little spontaneous activity at early ages (6–10 DIV) as indicated by the low incidence of deflections from resting membrane potentials. Interestingly, mature circuits (21–30 DIV) exhibited significantly more and richer forms of spontaneous activity (Fig. 2C and D). Each voltagegram shows data from 10 randomly selected neurons and each row represent a 40 s recording from one neuron.

To quantify the development of spontaneous activity, we used the following measures illustrated in Fig. 2E: vSTD, Up state frequency, event frequency, and Up state duration. The SD provides an assumption-independent measure of spontaneous activity, whereas the other measures are dependent on the choice of voltage and duration thresholds (see Materials and methods). In order to disentangle spontaneous Up states from short-lasting bouts of spontaneous activity, we also measured the SD of membrane voltage after Up segments were removed. A two-way ANOVA analysis revealed a significant increase in vSTD with development ($F_{3,151} = 13.89$, $P < 10^{-7}$; Fig. 3A) as well as a significant interaction between the age and genotype factors ($F_{3,151} = 3.55$, $P = 0.016$; Fig. 3A). *Post-hoc* analyses of this interaction revealed that, in WT circuits, vSTD increased significantly between 6–10 DIV and 11–15 DIV ($F_{1,30} = 22.34$, $P < 10^{-5}$, *F*-test for simple effects), whereas no significant increase was observed in *Fmr1*$^{-/y}$ circuits during this same period ($P = 0.10$). However, although vSTD did not increase in the WT circuits between 11–15 DIV and 16–20 DIV ($P = 0.78$), it did increase in the *Fmr1*$^{-/y}$ circuits ($F_{1,29} = 14.86$, $P = 0.001$, *F*-test for simple effects). Additionally, at 11–15 DIV, the vSTD of WT circuits was significantly higher than in *Fmr1*$^{-/y}$ circuits ($F_{1,32} = 13.68$, $P = 0.011$, *F*-test for simple effects), whereas at 16–20 DIV, the vSTD of WT circuits was marginally lower than in *Fmr1*$^{-/y}$ circuits ($F_{1,35} = 3.65$, $P = 0.06$, *F*-test for simple effects). These results show that, during *in vitro* development, there was a significant increase in network activity in both WT and *Fmr1*$^{-/y}$ circuits. However, the emergence of spontaneous activity in *Fmr1*$^{-/y}$ circuits was developmentally delayed. The vSTD measure, however, did not discern whether this delay reflected a general abnormality in the development of all types of spontaneous network activity, or of specific forms of spontaneous activity, such as spontaneous Up states.

The quantification of spontaneous Up states (see Materials and methods) revealed a strong interaction effect between the age and genotype factors ($F_{3,151} = 4.48$, $P = 0.004$; Fig. 3B) as well as the expected increase with development ($F_{3,151} = 16.18$, $P < 10^{-8}$; Fig. 3B). *Post-hoc* analyses revealed that, between 6–10 DIV and 11–15 DIV, the Up state frequency of WT circuits increased dramatically ($F_{1,30} = 21.59$, $P < 10^{-5}$, *F*-test for simple effects), whereas in the *Fmr1*$^{-/y}$ circuits, the Up state frequency only increased when circuits reached 16–20 DIV ($F_{1,29} = 12.43$, $P = 0.001$, *F*-test for simple effects).

In addition, there was no significant genotype or interaction effect on spontaneous activity as measured by event frequency, although there was again a significant developmental increase in event frequency ($F_{3,151} = 8.73$, $P < 10^{-4}$; data not shown). We also measured the SD of the voltage after Up state segments were excluded to obtain an assumption-independent measure of non-Up state spontaneous activity. Again, no genotype or interaction effect was found on spontaneous activity as measured in Fig. 3C. These results indicate that the abnormalities found in *Fmr1*$^{-/y}$ circuits did not reflect

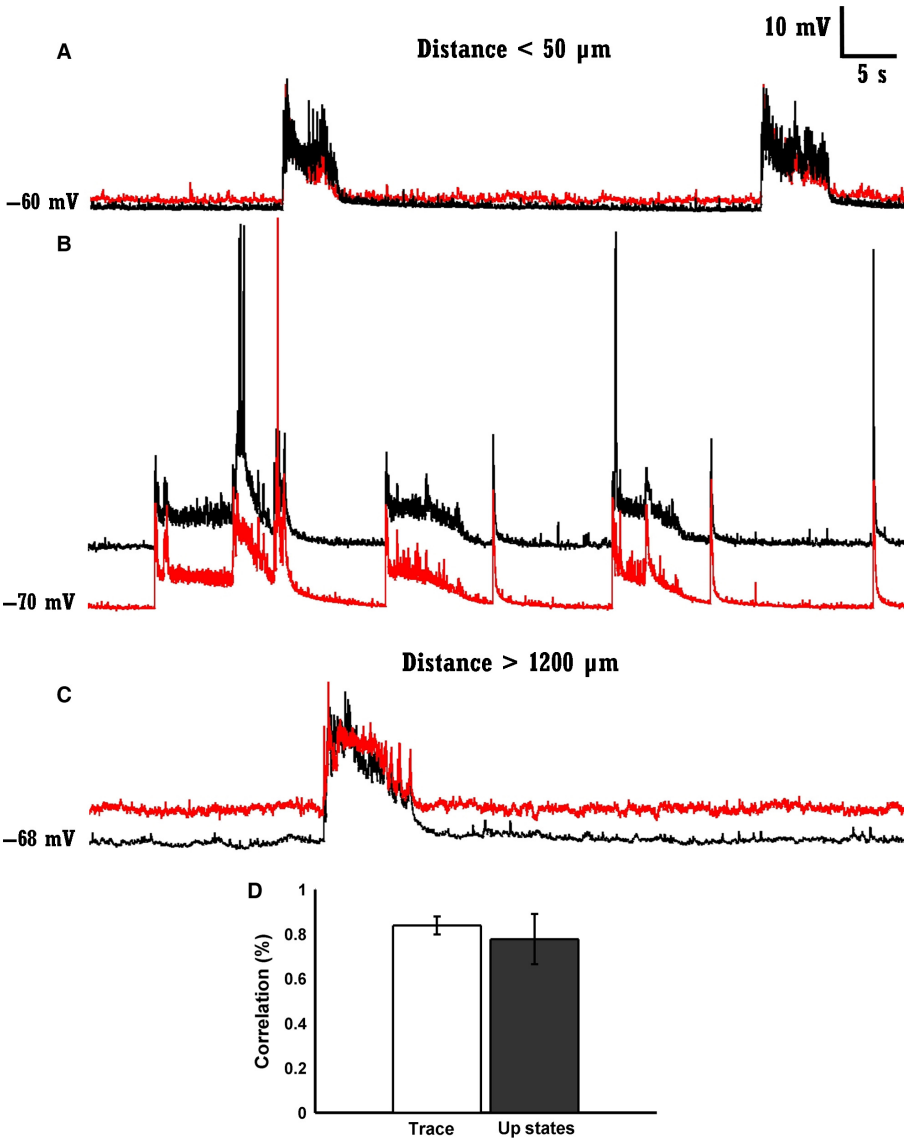

FIG. 1. Network activity. Up states are highly correlated across neurons. (A) Voltage traces from two simultaneously recorded neurons in WT circuits (< 50 μm apart). Up states in both neurons occurred synchronously. (B) Same as A. (C) Same as A and B but from two neurons that were more than 1200 μm apart. (D) Mean correlation of entire traces (white bar) and mean correlation of Up states (dark bar). Data were collected from seven pairs of neurons from seven different slices of WT circuits.

a non-specific alteration of all forms of spontaneous network activity, but rather that these effects were specific to the development of Up states.

Previous results, both *in vitro* (acute slices) and *in vivo*, have found that the durations of spontaneously occurring Up states are altered in *Fmr1* KO mice (Gibson *et al.*, 2008; Hays *et al.*, 2011). We thus analysed the Up state duration as a function of age and genotype. Because circuits at 6–10 DIV exhibited few Up states, we excluded this time point from our analysis (Fig. 3D). The analysis of Up state duration did not reveal a significant genotype or genotype and age interaction effect, although there was again a significant increase in Up state duration with development ($F_{3,151} = 6.05$, $P = 0.003$).

Together, these results show that, in both WT and *Fmr1*$^{-/y}$ circuits, spontaneous activity increases with development, further confirming previous results showing a progressive transition from mostly single events occurring during the first week *in vitro* to the presence of more frequent and longer-duration Up states at older ages *in vitro* (Johnson & Buonomano, 2007). However, *Fmr1*$^{-/y}$ circuits exhibited a significant developmental delay in the emergence of spontaneous network activity that was specific to the occurrence of Up states.

Abnormal intrinsic excitability in Fmr1$^{-/y}$ *circuits*

In vivo and *ex-vivo* studies have previously reported that cortical networks from *Fmr1* KO mice are hyperexcitable (Gibson *et al.*, 2008; Curia *et al.*, 2009; Olmos-Serrano *et al.*, 2010; Testa-Silva *et al.*, 2012; Goncalves *et al.*, 2013). Such hyperexcitability could be attributed to a number of different synaptic, cellular, and network mechanisms, including intrinsic neuronal excitability (Gibson *et al.*, 2008; Zhang *et al.*, 2014). Therefore, we also measured intrinsic excitability as the number of action potentials elicited by 250 ms current steps at intensities between 0.05 and 0.2 nA. As expected, a

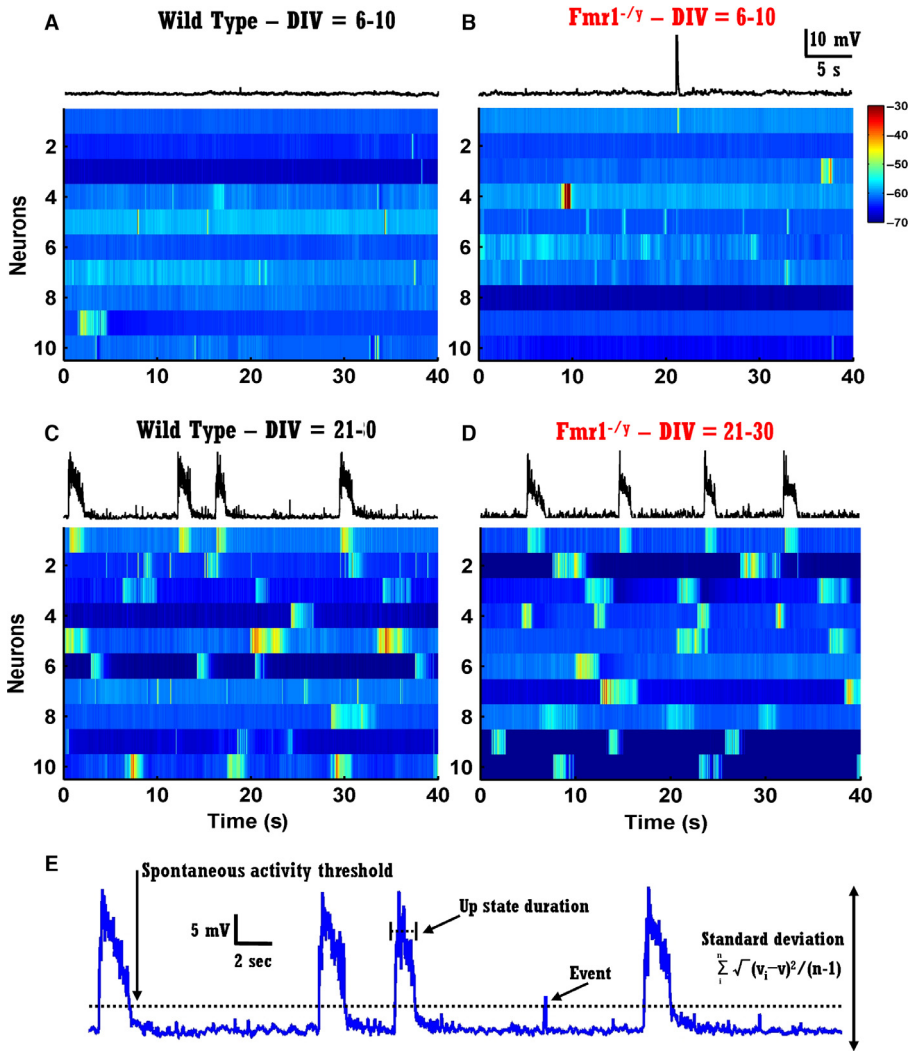

FIG. 2. Development of spontaneous network activity *in vitro*. (A and B) Each voltagegram represents 10 randomly selected neurons recorded at an early developmental age (6–10 DIV). Each row represents a 40 s recording from one neuron. Left panels: neurons from WT circuits; right panels: neurons from *Fmr1*$^{-/y}$ circuits. The trace above voltagegrams presents the same trace as the first row of the voltagegram. (C and D) Same as in A and B but from mature circuits (21–30 DIV). (E) Different quantitative measures of spontaneous network activity. A 40 s voltage trace of spontaneous activity of a pyramidal neuron.

three-way ANOVA (age, genotype, current intensity; repeated measures) revealed a significant decrease in excitability with development for both genotypes ($F_{3,151}$ = 6.94; $P < 10^{-4}$, data not shown) (Johnson & Buonomano, 2007), as well as a significant difference between *Fmr1*$^{-/y}$ and WT circuits as revealed by the main genotype effect ($F_{1,151}$ = 10.67; P = 0.001) and the significant interaction between genotype and age ($F_{3,151}$ = 9.53; $P < 10^{-6}$). *Post-hoc* analyses revealed that *Fmr1*$^{-/y}$ neurons were significantly more excitable compared with WT neurons at 6–10 DIV ($F_{1,23}$ = 9.28, P = 0.006) and 16–20 DIV ($F_{1,34}$ = 20.4, $P < 10^{-4}$). In contrast, at 11–15 DIV, *Fmr1*$^{-/y}$ circuits exhibited reduced excitability compared with WT circuits ($F_{1,32}$ = 58.24, P = 0.017). Although intrinsic excitability could contribute to the differences seen in Up state frequency it could not account for the developmental delay in network activity for a number of reasons, including the absence of any change in excitability in WT cells during the period of the largest increase in Up state frequency (between 6–10 DIV and 11–15 DIV), raising the possibility that the increase in excitability in *Fmr1*$^{-/y}$ could be a compensatory mechanism triggered by the delay in Up state development (see Discussion).

Homeostatic down-regulation of spontaneous activity by optogenetic stimulation

The developmental delay of Up state emergence in *Fmr1*$^{-/y}$ circuits, together with the absence of a delay in the development of general features of spontaneous activity, suggests potential abnormalities in one or more of the mechanisms governing Up state dynamics. To determine whether this is a general and long-lasting abnormality, we next investigated whether homeostatically-induced plasticity of Up states is also altered in *Fmr1*$^{-/y}$ circuits.

Previous studies using chronic electrical stimulation (Goel & Buonomano, 2013) demonstrated a homeostatic down-regulation of spontaneous Up state frequency. Here we examined the effects of long-term optical stimulation on spontaneous activity and Up states in WT and *Fmr1*$^{-/y}$ circuits. We used an optogenetic approach to stimulate slices, and to emulate the increase in externally driven activity that occurs during development. Slices were transfected with AAV5-CaMKIIa-ChR2(H134R)-EYFP at 4–6 DIV (Fig. 4A) and optically stimulated for 2 or 4 days at > 22 DIV (see Materials and methods). Two types of light-evoked responses were elicited in L-II/

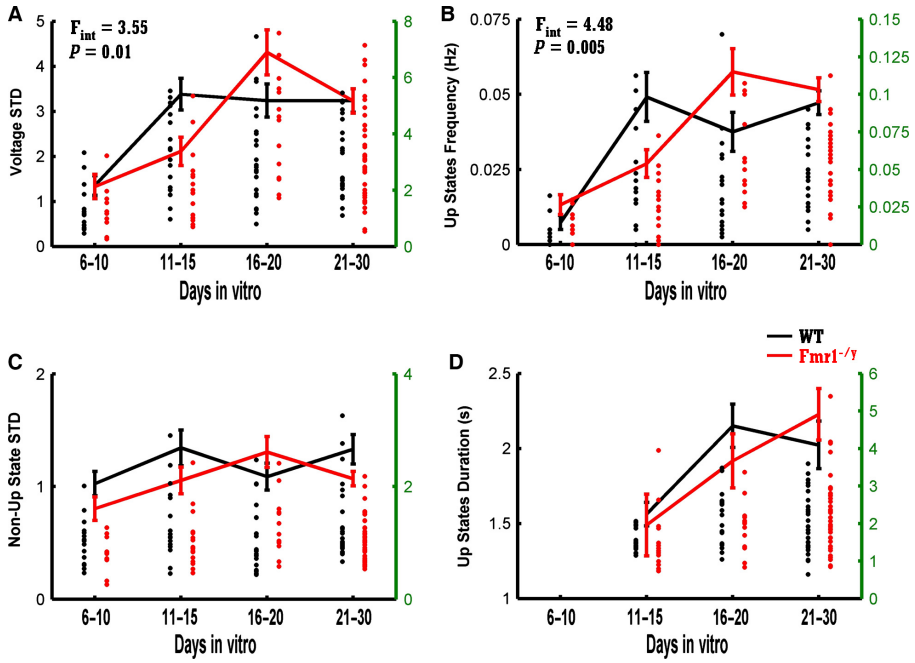

FIG. 3. Developmental delay of Up states in *Fmr1*$^{-/y}$ circuits. (A) Littermate WT (black) and *Fmr1*$^{-/y}$ (red) circuits exhibit a developmental increase in the vSTD ($F_{3,151}$ = 13.89, $P < 10^{-7}$). There was a developmental delay in the *Fmr1*$^{-/y}$ circuits as revealed by a significant interaction between genotype and age ($F_{3,151}$ = 3.55, P = 0.016). Dots represent single neurons and are plotted against the green y-axes (right side; some dots are overlapping). The total number of cells recorded from WT circuits was 80, i.e. 15 (6–10 DIV), 17 (11–15 DIV), 22 (16–20 DIV) and 26 (21–30 DIV). The total number of cells recorded from *Fmr1*$^{-/y}$ circuits was 79, i.e. 10 (6–10 DIV), 17 (11–15 DIV), 14 (16–20 DIV) and 38 (21–30 DIV). (B) Up state frequency increased significantly with development for WT and *Fmr1*$^{-/y}$ circuits ($F_{3,151}$ = 16.18, $P < 10^{-8}$). Neurons from *Fmr1*$^{-/y}$ circuits exhibited a significant delay in the emergence of Up states as indicated by the significant interaction between genotype and age ($F_{3,151}$ = 4.48, P = 0.004). Up state frequency was significantly reduced in *Fmr1*$^{-/y}$ circuits compared with WT circuits at 11–15 DIV ($F_{1,32}$ = 5.59, P = 0.02). Number of cells is as in A. (C) STD excluding Up states. There was no significant effect of genotype, development, or interaction between them. Number of cells is as in A. (D) Mean Up states duration increased significantly with development for both WT and *Fmr1*$^{-/y}$ circuits ($F_{3,151}$ = 6.05, P = 0.003). No difference between the two genotypes was observed. Number of cells is as in A without the cells at 6–10 DIV.

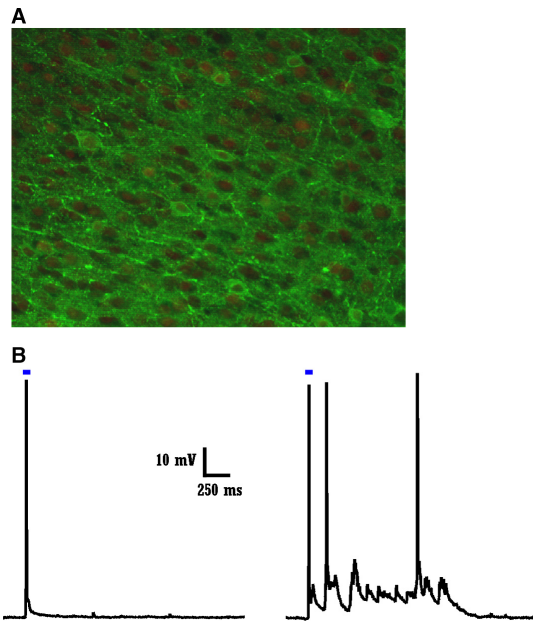

FIG. 4. Optogenetic transfection and chronic stimulation of organotypice cultures. (A) Confocal image of a transfected slice containing cells expressing ChR2-EYFP. Slice was counterstained with anti-Green fluorescent protein and anti-Neuronal Nuclei. Overlay of green channel (GFP, ChR-positive cells) and red channel (anti-NeuN) to label all neurons. (B) Range of light-evoked responses in two ChR-positive cells. Left cell shows a direct light-evoked response, whereas the cell on the right shows both direct and indirect light-evoked responses.

III pyramidal neurons: a short-latency depolarization (Fig. 4B, left cell) or a slow-latency prolonged depolarization, produced by the recurrent activation of light-responsive neurons (Fig. 4B, right cell). Cells that exhibited a short-latency (< 15 ms) depolarization of at least 5 mV were designated as ChR-positive cells.

The voltagegrams shown in Fig. 5A–D reveal that chronic optical stimulation of both WT and *Fmr1*$^{-/y}$ circuits resulted in a significant reduction of network spontaneous activity. Each voltagegram represents 10 randomly selected neurons from four conditions: unstimulated WT slices (Fig. 5A), unstimulated *Fmr1*$^{-/y}$ slices (Fig. 5B), stimulated WT slices (2 or 4 days) (Fig. 5C) and stimulated *Fmr1*$^{-/y}$ slices (Fig. 5D). A two-way ANOVA of sister slices (see Materials and methods) revealed a significant reduction in vSTD following chronic optical stimulation for both WT and *Fmr1*$^{-/y}$ circuits ($F_{2,46}$ = 18.76, $P < 10^{-5}$, Fig. 6A), and no significant difference between the two genotypes. Quantifications of Up state frequency also revealed a significant stimulation effect, indicating that Up state frequency was significantly reduced following chronic optical stimulation ($F_{2,46}$ = 25.42, $P < 10^{-7}$), and no genotype effect. Additionally, no significant difference was found between 2 or 4 days of stimulation, meaning that 2 days of optical stimulation was enough to induce a significant reduction in spontaneous activity. Chronic optical stimulation also induced a significant decrease in intrinsic excitability, and the degree of this plasticity was normal in the *Fmr1*$^{-/y}$ circuits (data not shown).

These results demonstrate that chronic optical stimulation resulted in a significant reduction in all measures of spontaneous network activity, and that this form of homeostatic plasticity was normal in *Fmr1*$^{-/y}$ circuits. To the best of our knowledge, these are the first

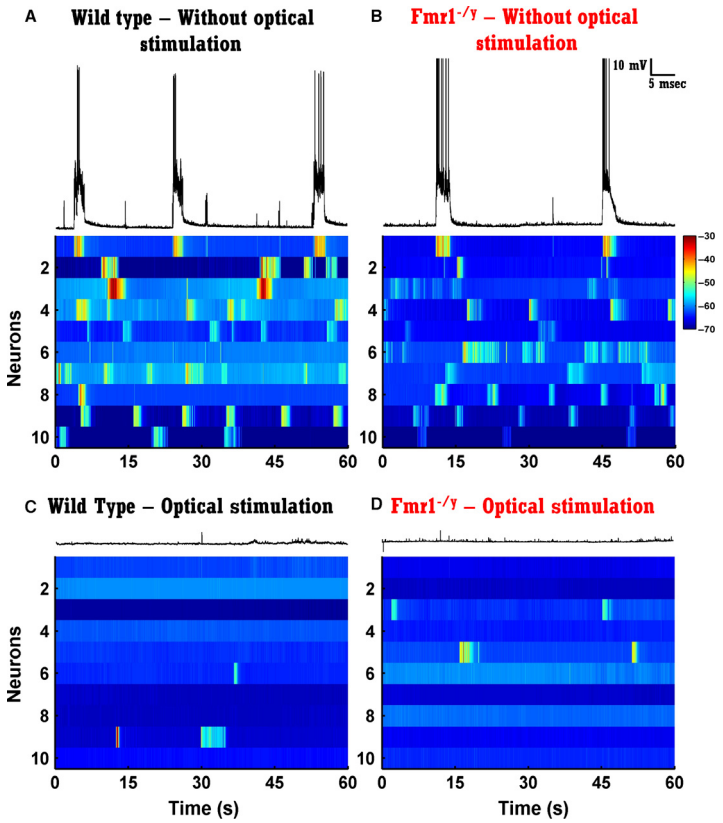

FIG. 5. Plasticity of spontaneous network activity *in vitro*. (A and B) Each voltagegram represents one trace from 10 randomly selected neurons recorded from slices that did not receive optical stimulation. Left panels: neurons from WT circuits; right panels: neurons from *Fmr1*$^{-/y}$ circuits. The trace above voltagegrams presents the same trace as the first row of the voltagegram. (C and D) Same as A and B but from slices that received optical stimulation for 2 or 4 days.

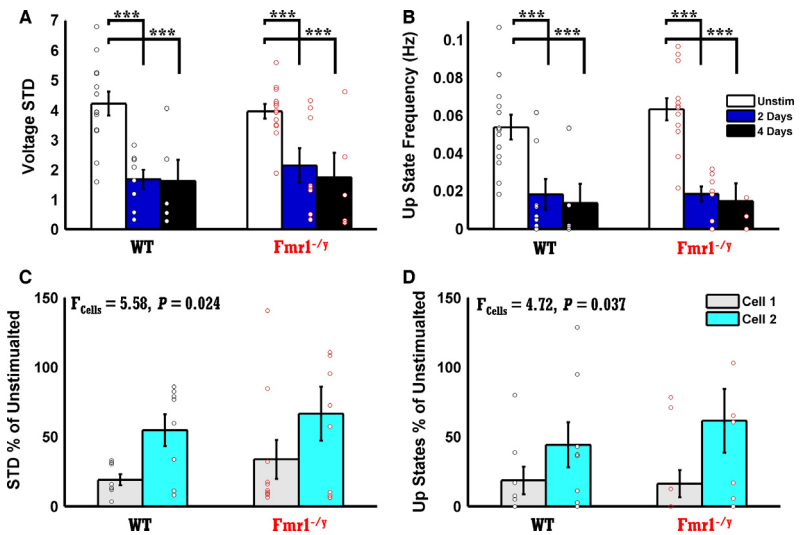

FIG. 6. Normal homeostatic plasticity of Up states in *Fmr1*$^{-/y}$ circuits. (A) Chronic optical stimulation resulted in a significant reduction in vSTD in both WT and *Fmr1*$^{-/y}$ circuits ($F_{2,46}$ = 18.76, $P < 10^{-5}$), with no significant difference between 2 and 4 days of stimulation. There was no difference in homeostatic plasticity of vSTD between WT and *Fmr1*$^{-/y}$ circuits. White bars, no optical stimulation; blue bars, 2 days of optical stimulation; black bars, 4 days of optical stimulation; black dots, neurons from WT circuits; red dots, neurons from *Fmr1*$^{-/y}$ circuits. Number of neurons in WT and *Fmr1*$^{-/y}$ groups are the same due to sister-slice design. Unstimulated slices, n = 13; 2 days of stimulation, n = 8; 4 days stimulation, n = 5. Note that the number of neurons is higher than the number of dots due to overlapping data. (B) Same as in A but for Up state frequency. Stimulation induced a decrease in Up state frequency ($F_{2,46}$ = 25.42, $P < 10^{-7}$), and no difference between genotypes. Same number of neurons as in A. (C) Same data from A but analysed according to the order in which the cells were recorded, and collapsed across 2 and 4 days of stimulation. Data are normalised to the sister unstimulated slice. A significant difference in vSTD was found between cell order, i.e. neurons from the first recorded cell (Cell 1) exhibited reduced values compared with the second cell (Cell 2) ($F_{1,35}$ = 5.58, P = 0.024). Activity-induced plasticity was the same in WT and *Fmr1*$^{-/y}$ circuits. Gray bars, Cell 1; Cyan bars, Cell 2. Number of cells in each group was the same (n = 8). (D) Same as C but for Up state frequency. There was a significant effect of stimulation on Up state frequency ($F_{1,35}$ = 4.72, P = 0.037). Number of cells in each group was the same (n = 10).

experiments to show that chronic optical stimulation can induce homeostatic down-regulation of spontaneous network activity and Up states, although other laboratories have used optogenetic approaches to induce synaptic homeostatic plasticity (Goold & Nicoll, 2010).

Although the induction of homeostatic plasticity was normal, it is possible that, in *Fmr1*$^{-/y}$ circuits, a difference was present in the reversibility of this plasticity once stimulation had ceased. Therefore, we analysed vSTD and Up state frequency according to the order in which cells were recorded. The first cell of a slice (Cell 1) was recorded up to 20 min after the end of chronic stimulation, whereas the second cell (Cell 2) was recorded over the next 20–40 min (Fig. 6C and D). The decrease in spontaneous Up state frequency was significantly larger in Cell 1 compared with Cell 2 (vSTD: $F_{1,35} = 5.58$, $P = 0.024$; Up state frequency: $F_{1,35} = 4.72$, $P = 0.037$), but there was no difference between WT and *Fmr1*$^{-/y}$ circuits. These results clearly indicate that the mechanisms that control homeostatic plasticity of network activity and Up state modulation were intact in *Fmr1*$^{-/y}$ circuits, even though these circuits exhibited a developmental delay in the emergence of Up states.

Discussion

The diversity of neural phenotypes that have been reported in animal models of Fragile X highlight the challenge in determining which phenotypes are a primary consequence of the disease, and those that arise as a secondary consequence of abnormal development and experience. To help overcome this challenge we used organotypic slices, which undergo a developmental process that recapitulates (Bolz, 1994; Echevarria & Albus, 2000; De Simoni *et al.*, 2003; Johnson & Buonomano, 2007) many aspects of *in vivo* development (Hubel & Wiesel, 1963; Colonnese *et al.*, 2010).

Our results revealed an *in vitro* developmental delay of Up state emergence in *Fmr1*$^{-/y}$ circuits. Critically, this delay did not reflect a generalised delay of all forms of spontaneous network activity. The alterations in Up state dynamics are consistent with previous reports of Up state abnormalities in acute slices and *in vivo* (Gibson *et al.*, 2008; Hays *et al.*, 2011; Goncalves *et al.*, 2013). We did not observe any abnormalities in Up state duration or firing frequency during Up states, as in some previous studies. Such differences are probably in part a consequence of the use of *in vivo*/acute preparations vs. organotypic slices. However, the previous studies, together with the current study, clearly indicate that, across preparations, Up states represent a significant neural phenotype in FXS. Furthermore, the nature of the current *in vitro* developmental study suggests that the alterations in Up state development are not a secondary consequence of experience or developmental abnormalities but a direct result of FMRP loss. Thus, our data confirm previous results and show significant network abnormalities in FXS KO circuits that are more likely to arise from the absence of the FMRP and not as an indirect result of abnormal development or experience.

Consistent with previous studies, we also observed abnormalities in intrinsic excitability (Gibson *et al.*, 2008; Zhang *et al.*, 2014). Although intrinsic excitability in WT neurons decreased throughout development, excitability in *Fmr1*$^{-/y}$ neurons increased from 11–15 DIV to 16–20 DIV, before decreasing again. These results are the first to show that FXS circuits are not always more excitable than WT circuits and that the intrinsic excitability of neurons depends greatly on the time of test. Previous work in rat organotypic slices (Johnson & Buonomano, 2007) suggests that the developmental increase in Up states is not produced by changes in intrinsic excitability. Indeed, in the current study there was no change in excitability in the WT circuits during the period of the largest increase in Up state frequency (between 6–10 DIV and 11–15 DIV), nor was there a correlation between excitability and Up state frequency (data not shown). Additionally, we did not find any genotypic difference in firing frequency during Up states. Together, these observations raise the possibility that the abnormal developmental increase in intrinsic excitability in *Fmr1*$^{-/y}$ networks (16–20 DIV) reflected a compensatory mechanism in response to the delayed increase in Up states at 11–15 DIV.

The homeostatic plasticity of spontaneous network activity in response to global pharmacological manipulations and electrical stimulation has been established previously (Ramakers *et al.*, 1990; Johnson & Buonomano, 2007; Goel & Buonomano, 2013). However, it was not known whether spontaneous activity of *Fmr1*$^{-/y}$ circuits is sensitive to chronic stimulation. We examined this issue using an optogenetic approach in which slices expressing ChR were stimulated while in the incubator. Both WT and *Fmr1*$^{-/y}$ circuits exhibited reduced network activity in response to chronic stimulation and no difference was found between the two circuits. In addition, both WT and *Fmr1*$^{-/y}$ circuits exhibited a significant but similar reduction in intrinsic excitability following stimulation. Together, these results indicate that mature *Fmr1*$^{-/y}$ circuits exhibit normal homeostatic plasticity of Up states.

A number of studies have reported developmental delays of neural phenotypes in FXS (Nimchinsky *et al.*, 2001; Bureau *et al.*, 2008; Cruz-Martin *et al.*, 2010; Harlow *et al.*, 2010; He & Portera-Cailliau, 2013; Padmashri *et al.*, 2013). Here we report, for the first time, a delay of a neural phenotype during *in vitro* development, thus providing strong evidence that Up state abnormalities are a direct consequence of the absence of FMRP, rather than an indirect product of altered development or experience. Importantly, despite abnormal development of Up states, a fairly complex network-level form of plasticity (homeostatic plasticity of Up state frequency) was normal in mature *in vitro* circuits. This was unexpected because many of the neural phenotypes associated with FXS, including abnormal long-term potentiation/long-term depression, spike timing dependent plasticity, homeostatic plasticity, and the balance of excitation and inhibition, would be expected to alter the ability of networks to homeostatically adjust Up state dynamics (Haider *et al.*, 2006; Mann *et al.*, 2009; Chen *et al.*, 2013). Thus, the observation that chronic stimulation appears to produce normal homeostatic plasticity of Up state frequency raises the interesting possibility that mature cortical circuits may have largely normal forms of plasticity in place. These results also suggest that some reported neural phenotypes could indeed reflect compensatory mechanisms, and that *in vitro* studies could provide an approach to address such confounds.

Abbreviations

ChR, channelrhodopsin; DIV, days *in vitro*; FMRP, Fragile X mental retardation protein; FXS, Fragile X syndrome; KO, knockout; vSTD, SD of the voltage; WT, wild-type.

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