Increased persistent sodium current causes neuronal hyperexcitability in the entorhinal cortex of Fmr1 knockout mice

Pan-Yue Deng  
Washington University School of Medicine in St. Louis

Vitaly A. Klyachko  
Washington University School of Medicine in St. Louis

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Increased Persistent Sodium Current Causes Neuronal Hyperexcitability in the Entorhinal Cortex of *Fmr1* Knockout Mice

**Graphical Abstract**

- **Wild type**
  - FMRP
  - mGluR5-PLC-PKC
- **Fmr1 KO**
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  - 
  - 

**Highlights**

- AP threshold is decreased in EC layer III pyramidal neurons of *Fmr1* KO mice
- AP threshold changes are caused by increased $I_{NaP}$ in *Fmr1* KO mice
- Abnormal threshold is mediated by increased mGluR5-PLC-PKC pathway signaling

**Authors**

Pan-Yue Deng, Vitaly A. Klyachko

**Correspondence**

klyachko@wustl.edu

**In Brief**

Deng and Klyachko demonstrate that hyperexcitability of cortical excitatory neurons in a mouse model of fragile X syndrome is caused by a reduced action potential threshold. This excitability defect results from dysregulation of sodium channels by an exaggerated mGluR5-PLC-PKC pathway signaling.

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Increased Persistent Sodium Current Causes Neuronal Hyperexcitability in the Entorhinal Cortex of Fmr1 Knockout Mice

Pan-Yue Deng1 and Vitaly A. Klyachko1,2,*

1Departments of Cell Biology and Physiology, Biomedical Engineering, Washington University School of Medicine, St. Louis, MO 63110, USA
2Lead Contact
*Correspondence: klyachko@wustl.edu
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SUMMARY

Altered neuronal excitability is one of the hallmarks of fragile X syndrome (FXS), but the mechanisms underlying this critical neuronal dysfunction are poorly understood. Here, we find that pyramidal cells in the entorhinal cortex of Fmr1 KO mice, an established FXS mouse model, display a decreased AP threshold and increased neuronal excitability. The AP threshold changes in Fmr1 KO mice are caused by increased persistent sodium current (INaP). Our results indicate that this abnormal INaP in Fmr1 KO animals is mediated by increased mGluR5-PLC-PKC (metabotropic glutamate receptor 5/phospholipase C/protein kinase C) signaling. These findings identify Na+ channel dysregulation as a major cause of neuronal hyperexcitability in cortical FXS neurons and uncover a mechanism by which abnormal mGluR5 signaling causes neuronal hyperexcitability in a FXS mouse model.

INTRODUCTION

Fragile X syndrome (FXS), the most common cause of inherited intellectual disability, is typically associated with cognitive, behavioral, and social impairments, as well as a wide range of neurological abnormalities (Santoro et al., 2012). A prominent characteristic of neurological defects in FXS is neuronal hyperexcitability, which is believed to cause a variety of symptoms, including hyperactivity, increased sensitivity to sensory stimuli, and a high incidence of seizures (Contractor et al., 2015). These phenotypes account for a considerable part of the disease pathophysiology. However, the underlying mechanisms responsible for increased neuronal excitability in FXS remain poorly understood.

Altered neuronal excitability has been reported in various brain regions of FXS models (Deng et al., 2013; Gibson et al., 2008; Kalmbach et al., 2015; Myrick et al., 2015; Tang and Alger, 2015; Zhang et al., 2014). Among these regions, the parahippocampal cortices—and, particularly, the entorhinal cortex (EC)—play an essential role in the generation and maintenance of a wide range of seizure syndromes (Chatzikonstantinou, 2014). Anatomically, EC mediates the majority of connections between various brain regions and the hippocampus, which is one of the most heavily investigated brain areas implicated in the pathology of FXS (Santoro et al., 2012). Thus, the EC is regarded as the gateway to the hippocampus and has been implicated as one of the key epileptogenic brain areas (Chatzikonstantinou, 2014). Despite its critical role in cortico-hippocampal network excitability, little is known about pathophysiological changes in the EC that occur in FXS models. Thus, EC represents a highly relevant model system to investigate excitability defects in FXS.

Action potential (AP) threshold is one of the key determinants of neuronal excitability (Bean, 2007). The threshold determines when an AP is initiated, sets the neuron’s firing rate, and shapes neuronal computations, including coincidence detection, temporal coding, and feature selectivity (Bean, 2007). AP threshold is governed predominately by Na+ channel availability and activation propensity near the threshold, whereas K+ channels and other conductances can dynamically modulate AP threshold in an adaptive way (Carter and Bean, 2009; Hu et al., 2009; Platkiewicz and Brette, 2010). In addition to the fast transient Na+ current (INaT) underlying the AP rising phase, the Na+ channels can give rise to a noninactivating persistent Na+ current (INaP) that activates at subthreshold voltages (Crill, 1996). Although the amplitude of INaP is generally small, relative to the INaT, it is highly functionally significant and may strongly influence transduction of synaptic inputs into AP generation (Crill, 1996; Hu et al., 2009). Here, we demonstrate that INaP is abnormally increased in the EC layer III excitatory pyramidal neurons of Fmr1 KO mice, leading to decreased AP threshold and increased neuronal excitability. Our results suggest that this enhanced INaP is caused by exaggerated metabotropic glutamate receptor 5 (mGluR5) signaling acting via phospholipase C (PLC) and protein kinase C (PKC), a signaling mechanism distinct from the well-established mGluR5 signaling cascade affecting local translation in Fmr1 knockout (KO) animals. These findings identify Na+ channel dysregulation as a major cause of neuronal hyperexcitability in cortical FXS neurons and uncover a previously unrecognized mechanism by which abnormal mGluR5 signaling causes neuronal hyperexcitability in an FXS mouse model. Our findings may, thus, provide a therapeutic strategy to ameliorate neuronal excitability defects in FXS.
**RESULTS**

**Increased Pyramidal Cell Excitability in the EC of Fmr1 KO Mice**

Superficial layers (layers II–III) of the EC serve as an information gateway to the hippocampus. Accordingly, we first asked whether the excitability of the principal neurons in EC superficial layers (i.e., layer II stellate cells and layer III pyramidal cells [PCs]) is altered in Fmr1 KO mice. APs were evoked by a ramp current injection (Yamada-Hanff and Bean, 2013) (Figure 1A). Only the first APs were used to estimate the AP threshold (box area).

No changes in AP threshold was observed in the stellate cells of Fmr1 KO mice (wild-type [WT]: \(-36.50 \pm 0.56\) mV, \(n = 7\); KO: \(-36.65 \pm 0.69\) mV, \(n = 11\); \(p = 0.517\); Figure 1C). In contrast, we found that the threshold potential of layer III PCs had a significant hyperpolarizing shift in Fmr1 KO neurons (WT: \(-38.31 \pm 0.82\) mV, \(n = 6\); KO: \(-41.88 \pm 0.33\) mV, \(n = 6\); \(p = 0.0096\); Figure 1C).

We further examined the excitability of EC layer III PCs by setting the resting membrane potential at \(-51\) mV through automatic current injection to induce AP firing spontaneously. At this membrane potential, about 70% of WT neurons and all tested Fmr1 KO neurons fired spontaneously (data not shown). Our results confirmed the increased excitability of these neurons in Fmr1 KO mice, as evident by the increased mean firing frequency (WT: \(3.75 \pm 0.70\) Hz, \(n = 23\); KO: \(6.12 \pm 0.52\) Hz, \(n = 48\); \(p = 0.0082\); Figures 1D–1F) and decreased AP threshold (WT: \(-39.93 \pm 0.33\) mV; KO: \(-42.61 \pm 0.21\) mV; \(p < 0.00001\); Figure 1G). We further verified the increased excitability of Fmr1 KO neurons by examining the distribution of instantaneous AP firing frequency in the tested neurons (Figure 1E). Specifically, we noted that, in Fmr1 KO neurons, the first peak of firing frequency shifted from 2.3 to 3.9 Hz, and an additional third peak appeared at \(-24\) Hz (Figure 1E). Therefore, we used the layer III PCs as a model to elucidate the underlying mechanisms of this defect.

**Abnormal Persistent Na+ Current Causes Increased Excitability in EC Layer III PCs of Fmr1 KO Mice**

Because the intrinsic membrane properties play a major role in setting neuronal excitability, we compared the resting
membrane capacitance in EC layer III PCs of Fmr1 KO mice, we recorded I_{NaP} in Fmr1 KO neurons, but it failed to abolish the difference in AP threshold between genotypes (XE991: WT, −40.02 ± 0.71 mV, n = 7; KO, −44.01 ± 1.25 mV, n = 6; p = 0.0051; Figure 2A). Unlike XE991, the HCN channel blocker ZD7288 (10 μM) markedly hyperpolarized the RMP, both in WT and Fmr1 KO neurons to the same extent (ZD7288: WT, −79.20 ± 0.73 mV, n = 6; KO, −79.83 ± 1.80 mV, n = 6; p = 0.768; Figure 2B), suggesting a high activity of HCN channels at the potentials around RMP in both genotypes. However, ZD7288 failed to abolish the difference in AP threshold between genotypes (ZD7288: WT, −37.63 ± 0.97 mV; KO, −43.69 ± 0.66 mV; p = 0.00047; Figure 2B). Finally, low concentrations of tetrodotoxin (TTX) (20 nM) to block I_{NaP} (Hammarstrom and Gage, 1998) had a small but significant hyperpolarizing effect on RMP in both genotypes (basal: WT, −66.45 ± 0.78 mV, n = 11; KO, −66.12 ± 1.11 mV, n = 7; p = 0.816; TTX: WT, −70.0 ± 0.89 mV; KO, −68.57 ± 1.13 mV; p = 0.336; basal versus TTX within genotype: WT, p = 0.0014; KO, p = 0.0023; Figure 2C). Most importantly, TTX (20 nM) abolished the difference in AP threshold between genotypes (WT: −29.75 ± 0.87 mV; KO, −31.75 ± 1.48 mV; p = 0.029; Figure 2C). These results point to abnormal I_{NaP} in Fmr1 KO neurons, but not I_{M} or I_{h}, as a potential cause of the decreased AP threshold in Fmr1 KO neurons.

### Increased Persistent Sodium Current Underlies AP Threshold Changes in Fmr1 KO Mice

To verify the aforementioned observations and examine changes in I_{NaP} in Fmr1 KO neurons, we recorded I_{NaP} evoked by a slow depolarizing ramp (20 mV/s) to measure its quasi-steady-state voltage dependence (Yamada-Hanff and Bean, 2015), using the same internal and external solutions as those used in AP recordings (as described earlier). The TTX-sensitive current was first evident at approximately −65 mV (Figure 3A). AP currents that escaped voltage-clamp control were present in most neurons. As expected, I_{NaP} was significantly increased in Fmr1 KO neurons (I_{NaP} at −40 mV: WT, −1.88 ± 0.55 pA/pF, n = 5; KO, −3.71 ± 0.52 pA/pF, n = 5; p = 0.0297; Figure 3B), while the voltage-dependent activation of I_{NaP} was not altered in Fmr1 KO neurons (I_{NaP} half activation voltage, V_{1/2}: WT, −41.1 ± 0.33 mV, n = 5; KO, −40.7 ± 0.19 mV, n = 5; p = 0.5638; Figure 3C).

To further confirm these observations, we modified our protocol to avoid generation of escaped AP currents and achieve more reliable I_{NaP} recordings. We also modified our recording solutions to include blockers of K⁺ and Ca²⁺ channels to setting neuronal excitability, we then probed whether changes in sub-threshold currents play a role in the increased excitability in Fmr1 KO neurons. There are three major types of sub-threshold currents in central neurons: M current (I_{M}, carried by Kv7 channels), H current (I_{h}, carried by hyperpolarization-activated cyclic nucleotide–gated (HCN) channels) and I_{NaP} (Hönigsperger et al., 2015; Stafstrom, 2007; Yamada-Hanff and Bean, 2013, 2015). Therefore, we used specific inhibitors of I_{M}, I_{h}, or I_{NaP} to probe their role in regulating neuronal excitability in EC layer III PCs. The Kv7 channel inhibitor XE991 (10 μM) had no detectable effect on RMP (Figure 2A), suggesting a low activity of these channels at the potentials around RMP in EC layer III PCs. XE991 slightly decreased the threshold potential in both WT and Fmr1 KO neurons, but it failed to affect the differences in threshold between genotypes (XE991: WT, −40.02 ± 0.71 mV, n = 7; KO, −44.01 ± 1.25 mV, n = 6; p = 0.0051; Figure 2A). Unlike XE991, the HCN channel blocker ZD7288 (10 μM) markedly hyperpolarized the RMP, both in WT and Fmr1 KO neurons to the same extent (ZD7288: WT, −79.20 ± 0.73 mV, n = 6; KO, −79.83 ± 1.80 mV, n = 6; p = 0.768; Figure 2B), suggesting a high activity of HCN channels at the potentials around RMP in both genotypes. However, ZD7288 failed to abolish the difference in AP threshold between genotypes (ZD7288: WT, −37.63 ± 0.97 mV; KO, −43.69 ± 0.66 mV; p = 0.00047; Figure 2B). Finally, low concentrations of tetrodotoxin (TTX) (20 nM) to block I_{NaP} (Hammarstrom and Gage, 1998) had a small but significant hyperpolarizing effect on RMP in both genotypes (basal: WT, −66.45 ± 0.78 mV, n = 11; KO, −66.12 ± 1.11 mV, n = 7; p = 0.816; TTX: WT, −70.0 ± 0.89 mV; KO, −68.57 ± 1.13 mV; p = 0.336; basal versus TTX within genotype: WT, p = 0.0014; KO, p = 0.0023; Figure 2C). Most importantly, TTX (20 nM) abolished the difference in AP threshold between genotypes (WT: −29.75 ± 0.87 mV; KO, −31.75 ± 1.48 mV; p = 0.029; Figure 2C). These results point to abnormal I_{NaP}, but not I_{M} or I_{h}, as a potential cause of the decreased AP threshold in Fmr1 KO neurons.

### Figure 2. Abnormal Persistent Na⁺ Current in EC Layer III PCs of Fmr1 KO Mice

(A) Effect of Kv7 channel inhibitor XE991 on RMP (A1) and AP threshold (A2).

(B) Same as in (A), but for the HCN channel blocker ZD7288.

(C) Same as in (A), but for the I_{NaP} blocker, 20 nM TTX.

**p < 0.01; ns, not significant. All data are means ± SEM.

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Figure 3. Enhanced Persistent Na⁺ Current Underlies AP Threshold Changes in Fmr1 KO Neurons

(A) $I_{\text{NaP}}$ evoked by a depolarizing voltage ramp (−100 to −20 mV, 20 mV/s) before (traces a) and during (traces b) the application of TTX. TTX-sensitive current (traces c) was obtained by subtraction. Note that the large escaped AP currents were truncated to emphasize the $I_{\text{NaP}}$.

(B) $I_{\text{NaP}}$ at −40 mV was significantly larger in Fmr1 KO neurons.

(C) Voltage-dependent activation curve of $I_{\text{NaP}}$ (C1). Data were fitted by Boltzmann function. Summarized data of half activation voltage, $V_{1/2}$ (C2).

(legend continued on next page)
minimize contamination of $I_{\text{NaP}}$ measurements from Na⁺-activated K⁺ currents and other K⁺ and Ca²⁺ conductances. Briefly, we used a Cs⁺-based internal solution supplemented with 4-AP (2 mM) and TEA (10 mM) and also included TEA (20 mM), to replace equimolar NaCl and CdCl₂ (100 μM) in external solution. Under these conditions and using a repolarizing ramp voltage (+30 to −65 mV, −50 mV/s) to evoked $I_{\text{NaP}}$, we could record $I_{\text{NaP}}$ without any AP currents out of voltage-clamp control. In these measurements, we focused on the voltage in the range from −65 to −20 mV for better comparison with the aforementioned results. It is noteworthy that, within a certain voltage range, activation of $I_{\text{NaP}}$ has been shown to be independent of polarity of the voltage ramp (Astman et al., 2006). Indeed, consistent with the aforementioned findings, we found that $I_{\text{NaP}}$ was significantly larger in Fmr1 KO than WT neurons ($I_{\text{NaP}}$ at −40mV: WT, −1.29 ± 0.23 pA/pF, n = 7; KO, −2.21 ± 0.32 pA/pF, n = 6; p = 0.0066; Figures 3D1 and 3D2). As in the experiments described earlier, the voltage-dependent activation of $I_{\text{NaP}}$ was not different between genotypes (V1/2: WT, −36.27 ± 0.28 mV; KO, −36.49 ± 0.41 mV; p = 0.9738; Figure 3D2). We note that V1/2 was shifted to a more depolarizing voltage in both genotypes, compared to the aforementioned measurements (Figure 3C), presumably due to the changes in internal/external solutions (Na⁺ and K⁺ gradients, K⁺ and Ca²⁺ channel blockers). Finally, using a potent $I_{\text{NaP}}$ inhibitor riluzole (10 μM) (Spadoni et al., 2002; Urbani and Belluzzi, 2000), we further verified that the observed differences between Fmr1 KO and WT are due to altered $I_{\text{NaP}}$. Indeed, riluzole (10 μM) eliminated differences in ramp-evoked current between genotypes in (riluzole at −40 mV: WT, −0.83 ± 0.16 pA/pF, n = 6; KO, −1.06 ± 0.24 pA/pF, n = 6; p = 0.195; Figures 3E1 and 3E2). Together, these results suggest that $I_{\text{NaP}}$ is abnormally increased in Fmr1 KO neurons. Our measurements further indicate that, within the voltage range used in our recordings, depolarizing and repolarizing ramp measurements of $I_{\text{NaP}}$ are largely equivalent. Therefore, to avoid contamination from escaped AP currents, in the following experiments, we use the repolarizing ramps to record $I_{\text{NaP}}$.

If reduced AP threshold in Fmr1 KO neurons results from increased $I_{\text{NaP}}$, then inhibition of $I_{\text{NaP}}$ should abolish the differences in threshold between genotypes. Indeed, we found that riluzole (10 μM) abolished the difference in AP threshold between genotypes (WT, −36.41 ± 2.15 mV, n = 6; KO, −37.38 ± 0.96 mV, n = 6; p = 0.710; Figure 3E2). Moreover, we also found that the $I_{\text{NaP}}$ opener veratridine (1 μM) shifted the threshold to a more hyperpolarizing voltage in both genotypes, and, most importantly, it also abolished the difference in threshold between genotypes (WT, −44.36 ± 0.98 mV, n = 6; KO, −44.98 ± 0.45 mV, n = 6; p = 0.613; Figure 3E3). The evidence that both $I_{\text{NaP}}$ inhibitor riluzole and $I_{\text{NaP}}$ opener veratridine abolished the difference in threshold between genotypes suggests that the abnormal $I_{\text{NaP}}$ is unlikely to be caused by altered expression of Na⁺ channels in Fmr1 KO neurons. Taken together, these results suggest that an abnormally enhanced $I_{\text{NaP}}$ causes the increased excitability of EC layer III PCs in Fmr1 KO mice.

**Hyperexcitability of EC Layer III PCs in Fmr1 KO Mice Is Mediated by Exaggerated mGluR5 Signaling**

Na⁺ channel activity is continuously and extensively modulated by a variety of signaling pathways, including metabotropic neurotransmitter receptors. Given a number of studies implicating dysfunction of mGluR5 or GABAB receptor signaling pathways in FXS models (Bear et al., 2004; Pacey et al., 2009; Wahlstrom-Helgren and Klyachko, 2015), both of which are known to modulate Na⁺ channel properties (Crill, 1998), we examined whether these signaling pathways play a role in the changes of AP threshold in Fmr1 KO neurons. We first pharmacologically isolated the cells from glutamatergic and GABAergic transmission networks by using a cocktail containing both fast and slow synaptic transmission blockers (in micromolar: 50 APV, 10 DNQX, 5 gabazine, 2 CGP55845, and 10 MPEP to block NMDA, AMPA, GABA_A, GABA_B, and mGluR5 receptors, respectively). Surprisingly, the cocktail of these five blockers completely abolished the difference in AP threshold between genotypes (WT, −41.82 ± 0.15 mV, n = 6; KO, −42.01 ± 0.17 mV, n = 7; p = 0.462; Figure 4A), indicating that changes in AP threshold in Fmr1 KO neurons are mediated by the activation of one or several signaling pathways coupled to these receptors. In line with our aforementioned findings that increased excitability could not be attributed to network changes in fast synaptic transmission, we found that inhibition of fast synaptic transmission alone (with APV, DNQX, and gabazine) failed to abolish the difference in threshold between genotypes (WT, −41.75 ± 0.48 mV, n = 12; KO, −43.71 ± 0.49 mV, n = 13; p = 0.0079; Figure 4A), pointing to abnormal metabotropic signaling pathways as mediators of AP threshold changes in Fmr1 KO neurons. We further found that inhibition of GABAB receptors with CGP55845, in combination with fast transmission blockers, shifted the threshold in a hyperpolarizing direction for both genotypes, but more importantly, it failed to reduce differences in threshold between genotypes (WT, −43.10 ± 0.69 mV, n = 6; KO, −46.21 ± 0.61 mV, n = 6; p = 0.032; Figure 4A). In contrast, the mGluR5 blocker MPEP in combination with the fast transmission blockers abolished differences in AP threshold between genotypes (WT, −41.43 ± 0.53 mV, n = 7; KO, −42.11 ± 0.50 mV, n = 6; p = 0.914; Figure 4A). These results single out the dysfunction of mGluR5 signaling as a mediator of AP threshold defect in EC layer III PCs in Fmr1 KO mice. To verify this finding, we used another specific mGluR5 antagonist fenobam. As expected, fenobam (10 μM, in combination with fast transmission blockers and CGP55845) abolished the difference in threshold between

(D) $I_{\text{NaP}}$ evoked by a repolarizing ramp (D1; +30 to −65 mV, −50 mV/s). For better comparison with (A), the traces are presented in the same direction as in (A) in a range from −65 to −20 mV. Arrow indicates the time direction. (D2) The difference of I-V curves between WT and Fmr1 KO neurons. Inset: $I_{\text{NaP}}$ at −40 mV. (D3) The voltage-dependent activation of $I_{\text{NaP}}$ in Fmr1 KO and WT neurons. Inset: V1/2 of $I_{\text{NaP}}$ activation.

(E) Effects of $I_{\text{NaP}}$ inhibitor riluzole on $I_{\text{NaP}}$ and the AP threshold in Fmr1 KO and WT neurons. (E1) Sample $I_{\text{NaP}}$ traces. (E2) I-V curves. Inset: $I_{\text{NaP}}$ at −40 mV. (E3) Effects of the $I_{\text{NaP}}$ inhibitor riluzole and $I_{\text{NaP}}$ opener veratridine on the AP threshold.

*p < 0.05; **p < 0.01; ns, not significant. All data are means ± SEM.
Figure 4. Exaggerated mGluR5-PLC-PKC Signaling Causes Enhanced Persistent Na⁺ Current in Fmr1 KO Neurons

(A) Effects of various combinations of blockers on AP threshold.
(B) Estimates for receptor contributions to cell excitability from (A).
(C) Sample INaP traces (upper panel) and summarized data (lower panel) for INaP measured in the presence of mGluR5 inhibitor MPEP in combination with four other blockers (against NMDA, AMPA, GABA_A, and GABA_B receptors).
(D) Same as in (C) but for INaP measured with intracellular application of selective PLC inhibitor edelfosine.
(E) Same as in (C) but for INaP measured with intracellular application of PLC inhibitor U73122.
(F) Same as in (C) but for INaP measured with intracellular application of PKC inhibitor calphostin C.
(G) Same as in (C) but for INaP measured with intracellular application of PKC inhibitor PKC19–36.
(H) INaP values at −40 mV summarized from (C)–(G).

*p < 0.05; **p < 0.01; ns, not significant. All data are mean ± SEM.
genotypes (WT, $-41.94 \pm 0.58$ mV, $n = 7$; KO, $-42.53 \pm 0.26$ mV, $n = 8$; $p = 0.786$; Figure 4A).

To further clarify this issue, we estimated the relative contributions of the aforementioned receptors to neuronal excitability through comparison of the changes in AP threshold by their specific blockers. In the WT neurons, the relative contribution of combined activation of these five receptors to the AP threshold is about $-2$ mV, as evident by subtracting threshold values obtained with and without all five blockers (Figure 4B). This can be interpreted to indicate that the network activity normally maintains the excitatory/inhibitory (E/I) balance via these five receptors with slight inhibitory dominance. In contrast, in Fmr1 KO neurons, the net effect of the same five receptors is only about $+0.06$ mV, suggesting that the E/I balance in the Fmr1 KO network shifts toward increased excitability. We note that the addition of any blocker alters not only the individual cells we recorded from but also the entire network excitability; therefore, the contribution of a specific receptor to cell excitability can only be determined while all other receptors are blocked. Thus, by subtracting threshold values obtained with and without MPEP (or fenobam) in the presence of four other blockers, this analysis demonstrates the markedly increased contribution of mGluR5 signaling to AP threshold in Fmr1 KO compared with WT neurons (MPEP: WT, $-1.28$ mV; KO, $-4.20$ mV; fenobam: WT, $-1.16$ mV; KO, $-3.69$ mV; Figure 4B).

In contrast, the contribution of GABA_BRs to the AP threshold was very modest in both genotypes (Figure 4B). Taken together, these results indicate that the decreased AP threshold in Fmr1 KO neurons is caused by the abnormally elevated mGluR5 signaling.

**Exaggerated mGluR5 Signaling Acting via PLC-PKC Pathway Causes Increased Persistent Na⁺ Current in Fmr1 KO Neurons**

Because our results demonstrate that enhanced $I_{\text{NaP}}$ in Fmr1 KO neurons decreases AP threshold, and that the abnormal AP threshold is also attributed to the elevated mGluR5 activity, we then asked whether the elevated mGluR5 signaling causes enhanced $I_{\text{NaP}}$ in Fmr1 KO neurons. To minimize the network influences from other glutamatergic or GABAergic receptors on $I_{\text{NaP}}$, we measured $I_{\text{NaP}}$ in the presence of four blockers of NMDA, AMPA, GABA_A, and GABA_B receptors. First, we found that $I_{\text{NaP}}$ was still significantly larger in the Fmr1 KO than in the WT neurons in the presence of these four blockers ($I_{\text{NaP}}$ at $-40$ mV: WT, $-1.40 \pm 0.23$ pA/pF, $n = 7$; KO, $-2.33 \pm 0.18$ pA/pF, $n = 7$; $p = 0.0092$; Figures S3A and S3B) and was nearly the same as $I_{\text{NaP}}$ measured without the blockers (Figure 3D), while the voltage-dependent activation of $I_{\text{NaP}}$ was unaffected in both genotypes (Figure S3C). These results support the notion that these four receptors modulate AP threshold through mechanisms other than $I_{\text{NaP}}$. In contrast, when mGluRs blocker MPEP (10 μM) was combined with the other four blockers, it decreased the $I_{\text{NaP}}$ in Fmr1 KO neurons (Figures 4C and 4H), and, most importantly, MPEP abolished the difference in $I_{\text{NaP}}$ between genotypes (with MPEP, at $-40$ mV: WT, $-1.20 \pm 0.15$ pA/pF, $n = 6$; KO, $-1.14 \pm 0.28$ pA/pF, $n = 6$; $p = 0.871$; Figures 4C and 4H). We note that MPEP decreased $I_{\text{NaP}}$ predominantly in Fmr1 KO neurons, with negligible effect in WT neurons.

These results indicate that elevated mGluR5 signaling leads to enhanced $I_{\text{NaP}}$ in Fmr1 KO neurons.

Rapid normalization of $I_{\text{NaP}}$ in Fmr1 KO neurons by acute inhibition of mGluR5 suggests that the effects of elevated mGluR5 signaling on $I_{\text{NaP}}$ are mediated, at least in part, by the modulation of Na⁺ channel activity. Indeed, pharmacological activation of group I mGluRs has been shown to modulate the activity of Na⁺ channels and, specifically, $I_{\text{NaP}}$ (Carlier et al., 2006; D’Ascenzo et al., 2009) in a PLC-dependent manner (D’Ascenzo et al., 2009). To probe the signaling pathway mediating mGluR5 actions on $I_{\text{NaP}}$ in Fmr1 KO neurons, we examined the effects of disrupting the mGluR5-PLC signaling pathway using a selective PLC inhibitor, edelfosine. Application of edelfosine (1 μM) via the recording pipette (to avoid network effects) completely abolished the differences in $I_{\text{NaP}}$ between genotypes (at $-40$ mV: WT, $-1.62 \pm 0.20$ pA/pF, $n = 6$; KO, $-1.28 \pm 0.21$ pA/pF, $n = 6$; $p = 0.3089$; Figures 4D and 4H). Intracellular application of another PLC inhibitor U73122 (1 μM) similarly abolished the difference in $I_{\text{NaP}}$ between genotypes (at $-40$ mV: WT, $-1.10 \pm 0.18$ pA/pF, $n = 6$; KO, $-1.27 \pm 0.28$ pA/pF, $n = 6$; $p = 0.574$; Figures 4E and 4H). PKC is the major downstream effector of PLC activation (Mochly-Rosen et al., 2012) and is a well-known modulator of Na⁺ channel activity (Li et al., 1993). Therefore, we further tested whether PKC activation downstream of PLC mediates the observed changes in $I_{\text{NaP}}$, using two potent and selective PKC inhibitors, calphostin C and PKC19-36 (a pseudosubstrate peptide inhibitor of PKC). Intracellular application of either calphostin C (10 μM) or PKC19-36 (2 μM) via the recording pipette completely abolished the differences in $I_{\text{NaP}}$ between genotypes (Figures 4F-4H) (calphostin C at $-40$ mV: WT, $-1.16 \pm 0.23$ pA/pF, $n = 6$; KO, $-1.42 \pm 0.20$ pA/pF, $n = 6$; $p = 0.5019$; PKC19-36: WT, $-1.18 \pm 0.21$ pA/pF, $n = 7$; KO, $-1.11 \pm 0.11$ pA/pF, $n = 6$; $p = 0.8308$).

Taken together, our results suggest that an exaggerated activity of mGluR5 acting via the PLC-PKC signaling pathway enhances $I_{\text{NaP}}$ in Fmr1 KO neurons, which, in turn, leads to decreased AP threshold and increased neuronal excitability in the EC layer III PCs of Fmr1 KO mice.

**DISCUSSION**

Here, we demonstrate that PCs in the EC layer III of Fmr1 KO mice have a decreased AP threshold and increased excitability caused by dysregulation of Na⁺ channels. Our results indicate that this Na⁺ channel dysregulation is mediated by exaggerated mGluR5-PLC-PKC signaling that markedly increases persistent Na⁺ current in Fmr1 KO neurons. These findings suggest that Na⁺ channel dysregulation plays a major role in neuronal hyperexcitability in an FXS mouse model. Our results also reveal an important mechanism by which abnormal mGluR5 signaling causes neuronal hyperexcitability in the absence of FMRP. The finding that inhibition of $I_{\text{NaP}}$ eliminates differences in the AP threshold between Fmr1 KO and WT neurons suggests an avenue for development of therapeutic strategies to normalize neuronal hyperexcitability in FXS.

Emerging evidence links hyperexcitability in FXS with dysfunction in a number of ion channels, predominately K⁺ channels (Brown et al., 2010; Deng et al., 2013; Gross et al., 2011;
local translation in Fmr1

...cascade by which abnormal mGluR5 activation impacts...activity of mGluR5 acting via the PLC-PKC pathway. This is...activity (Hönigsperger et al., 2015; Yamada-Hanff and Bean, 2013, 2015). However, these three conductances have distinct pattern of voltage dependence: sub-threshold depolarization enhances \( I_{NaP} \) and \( I_{NaP} \) but dampens \( I_{h} \); in contrast, sub-threshold hyperpolarization dampens \( I_{NaP} \) and \( I_{NaP} \) but enhances \( I_{h} \). As a result, these three conductances differentially contribute to regulation of RMP and the AP threshold. In agreement with this notion, we found that reduced AP threshold of EC layer III PCs in Fmr1 KO mice is caused by enhanced \( I_{NaP} \) but not \( I_{NaP} \) or \( I_{h} \). We note that our results do not exclude a possibility that, in addition to \( I_{NaP} \), \( I_{NaP} \) is also altered in Fmr1 KO neurons. Indeed, the maximal rise speed of AP, a \( Na^{+} \) channel-dependent parameter, is increased in Fmr1 KO neurons (\( \rho = 0.00368 \); data not shown), which might indicate a change in \( I_{NaP} \).

Our data further indicate that changes in both \( I_{NaP} \) and the AP threshold are mediated by exaggerated mGluR5 signaling in the absence of FMRP. It is well accepted that loss of FMRP causes abnormally elevated mGluR5 activity (Santoro et al., 2012). PLC is a well-defined effector of mGluR5 (Kettunen et al., 2002) and is well known to modulate activity of voltage-gated ion channels and, specifically, \( I_{NaP} \) (D’Ascenzo et al., 2009). The receptor-dependent activation of PLC results in the generation of diacylglycerol and IP3. IP3, in turn, triggers the release of calcium from the ER, causing a rise in cytosolic calcium concentration, which then activates PKC (Kettunen et al., 2002). Activation of PKC has been shown to increase neuronal excitability by enhancing \( I_{NaP} \) at subthreshold voltages in neocortical neurons (Astman et al., 2006). In addition, activation of mGluR5 by DHPG in WT mice has been shown to upregulate \( I_{NaP} \) (Carlier et al., 2006; D’Ascenzo et al., 2009). Our results suggest that the increased \( I_{NaP} \) in Fmr1 KO neurons is caused by the exaggerated activity of mGluR5 acting via the PLC-PKC pathway. This is a signaling mechanism distinct from the well-established signaling cascade by which abnormal mGluR5 activation impacts local translation in Fmr1 KO animals (Santoro et al., 2012). While we cannot rule out the possibility that changes in \( I_{NaP} \) are mediated, in part, by altered \( Na^{+} \) channel expression, our results indicate that the functional regulation of \( I_{NaP} \) by mGluR5 is sufficient to account for the changes in the AP threshold in Fmr1 KO neurons. This notion is supported by the findings that either \( I_{NaP} \) inhibitors or the \( I_{NaP} \) opener is sufficient to abolish the differences in the AP threshold between genotypes. This is further supported by the observation that, in PCs isolated from circuit activity (via blockade of both ionic and metabotropic transmission receptors), the maximal rise speed of AP is no longer different between genotypes (\( \rho = 0.758 \); data not shown), suggesting that, in the absence of circuit activity, \( Na^{+} \) channel properties are similar in Fmr1 KO and WT animals. Taken together, these results uncover a distinct mechanism by which abnormal mGluR5 signaling causes hyperexcitability in cortical neurons of Fmr1 KO mice.

Effects of excessive mGluR5 signaling on circuit excitability are reflected in the prolongation of persistent cortical activity, or UP states, in Fmr1 KO mice (Hays et al., 2011), and our findings point to \( I_{NaP} \) as a potential target to alleviate these hyperexcitability defects. Indeed, although the amplitude of \( I_{NaP} \) is small, relative to the transient \( Na^{+} \) current, this persistent component is highly functionally significant because it is activated ~10 mV negative to the threshold potential and is characterized by steep voltage dependence at subthreshold potentials, thus providing strongly positive feedback for further depolarization (Bean, 2007; Crill, 1996). These features make neurons with increased \( I_{NaP} \) particularly susceptible to hyperexcitability defects leading to seizures (Stafstrom, 2007), a common dysfunction in FXS. Indeed, pro-epileptic conditions have been associated with elevated \( I_{NaP} \) (Azouz et al., 1996; Somjen and Müller, 2000), and mutations in \( Na^{+} \) channels that cause increases in \( I_{NaP} \) have been found in patients with epilepsy (Meisler and Kearney, 2005; Vreugdenhil et al., 2004; Rhodes et al., 2005). Moreover, \( I_{NaP} \) inhibitors have been effective in treating both partial and generalized tonic-clonic seizures in humans (Stafstrom, 2007). A number of anti-epileptic drugs are \( I_{NaP} \) inhibitors and have no effects on transient \( Na^{+} \) current at therapeutic concentrations (Segal and Douglas, 1997; Spadoni et al., 2002; Taverna et al., 1998). Given that hyperexcitability-associated phenotypes are common in FXS, our finding that enhanced \( I_{NaP} \) has a profound effect on neuronal excitability in EC layer III PCs of Fmr1 KO mice suggests that it may play an important role in the pathophysiology of FXS. \( I_{NaP} \) may, thus, represent a therapeutic target for treating hyperexcitability defects in FXS.

**EXPERIMENTAL PROCEDURES**

**Animals and Slice Preparation**

Fmr1 KO and WT control mice on FVB background were obtained from The Jackson Laboratory. Slices were prepared as previously described (Deng et al., 2013). All animal procedures were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and conformed to Washington University Animal Studies Committee guidelines.

**Electrophysiology**

Whole-cell recordings using a Multiclamp 700B amplifier (Molecular Devices) were made from PCs or stellate cells of EC superficial layers. For \( I_{NaP} \) recordings, cell capacitance was compensated. Series resistance compensation was enabled with 80%–90% correction and 16-μs lag. \( I_{NaP} \) was isolated by subtracting current in 1 μM TTX from that before TTX. All recordings were conducted at near-physiological temperature (33°C–34°C).

**Statistical Analysis**

Data are presented as means ± SEM. Student’s paired or unpaired t test was used for statistical analysis as appropriate; significance was set at \( p < 0.05 \). The n was number of cells tested. Additional details are available in the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.046.
AUTHOR CONTRIBUTIONS

P.-Y.D. and V.A.K. conceived and designed the study, P.-Y.D. conducted the experiments and analyzed the data. P.-Y.D. and V.A.K. wrote the manuscript.

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