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

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

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

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

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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; Platkiwicz 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 (~0.1 pA/μs, lower trace). Representative AP traces from EC layer III PCs are shown (upper traces). Only the first AP was used to estimate the AP threshold (box area). (A) Determination of AP threshold. APs were evoked by a ramp current injection (~0.1 pA/μs, lower trace). Representative AP traces from EC layer III PCs are shown (upper traces). Only the first AP was used to estimate the AP threshold (box area).

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 ± 0.70 Hz, n = 23; KO: 6.12 ± 0.52 Hz, n = 48; p = 0.0082; Figures 1D–1F) and decreased AP threshold (WT: −39.93 ± 0.33 mV; KO: −42.61 ± 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 ~15 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 potential of Fmr1 KO neurons with that in WT mice (Figure 1G). We observed that the resting membrane potential of Fmr1 KO neurons was significantly hyperpolarized compared to WT neurons (~42.61 ± 0.21 mV vs. ~39.93 ± 0.33 mV; p < 0.00001).}

0.82 mV, n = 6; KO: −41.88 ± 0.33 mV, n = 6; p = 0.0096; Figure 1C).

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membrane capacitance in EC layer III PCs of Fmr1 KO mice, since our observations of decreased AP threshold and increased firing in these neurons were made in the intact EC circuits (Figures 1D–1G), in the presence of this compensatory effect of tonic inhibition.

Since sub-threshold currents are believed to have a critical role in controlling RMP and regulating AP initiation and, thus, 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 (Honigsperger 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 2A2). 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 = 0.769; Figure 2B). Together, these results suggest 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 2B2). 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 = 0.0239; Figure 2C2). 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.

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 depolarization 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, V1/2: 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...
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) $I$-$V$ curves (B1) were constructed from the ramp-evoked $I_{\text{NaP}}$ (mean current value over 0.01 mV intervals from averages of four to five trials for each cell to approximate quasi-steady-state current). Currents were normalized to corresponding cell capacitance for better comparison. (B2) $I_{\text{NaP}}$ 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 \( \text{Na}^+ \)-activated \( K^+ \) currents and other \( K^+ \) and \( Ca^{2+} \) 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 \( \text{NaCl} \) and \( \text{CdCl}_2 \) (100 \( \mu \text{M} \)) 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-activation dependence of \( I_{\text{NaP}} \) was not different between genotypes (\( V_{1/2} \): WT, -36.27 ± 0.28 mV; KO, -36.49 ± 0.41 mV; \( p = 0.9738 \); Figure 3D2). We note that \( V_{1/2} \) 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 \( Ca^{2+} \) channel blockers). Finally, using a potent \( I_{\text{NaP}} \) inhibitor riluzole (10 \( \mu \text{M} \)) (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 \( \mu \text{M} \)) 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 \( \mu \text{M} \)) abolished the difference in AP threshold between genotypes (WT, -36.41 ± 2.15 mV, \( n = 6 \); KO, -37.38 ± 0.96, \( n = 6 \); \( p = 0.710 \); Figure 3E3). Moreover, we also found that the \( I_{\text{NaP}} \) opener veratridine (1 \( \mu \text{M} \)) shifted the threshold to a more hyperpolarizing voltage in both genotypes, and, most importantly, it also abolished the difference in AP threshold between genotypes (WT, -44.36 ± 0.98 mV, \( n = 6 \); KO, -44.98 ± 0.45, \( n = 6 \); \( p = 0.613 \); 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 increased \( I_{\text{NaP}} \) causes the increased excitability of EC layer III PCs in \( Fmr1 \) KO mice.

### Hypereexcitability 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 implying dysfunction of mGluR5 or GABA\(_B\)R 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 GABA\(_B\) 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 \( \mu \text{M} \), in combination with fast transmission blockers and CGP55845) abolished the difference in threshold between

(D) \( I_{\text{NaP}} \) evoked by a repolarizing ramp (\( D_1 \): +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. (\( D_2 \)) The difference of I-V curves between WT and \( Fmr1 \) KO neurons. Inset: \( I_{\text{NaP}} \) at -40 mV. (\( D_3 \)) The voltage-dependent activation of \( I_{\text{NaP}} \) in \( Fmr1 \) KO and WT neurons. Inset: \( V_{1/2} \) 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.
In contrast, the contribution of GABA BRs to the AP threshold, we measured I\textsubscript{NaP} in the presence of four blockers of WT neurons in the presence of these four blockers (Figure 3D), while the voltage-dependent activation of I\textsubscript{NaP} was unaltered between genotypes (with MPEP, at \textit{Fmr1} KO, \(\pm 0.15 \text{ pA/pF}, n = 6; \text{KO}, \pm 0.28 \text{ pA/pF}, n = 6; p = 0.871;\) 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 \textit{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 \textit{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 \textit{Fmr1} KO compared with WT neurons (MPEP: WT, \(-1.28 \text{ mV}; \text{KO}, \pm 4.20 \text{ mV};\) fenobam: WT, \(-1.16 \text{ mV}; \text{KO}, \pm 3.69 \text{ mV};\) Figure 4B). In contrast, the contribution of GABA\textsubscript{A}Rs to the AP threshold was very modest in both genotypes (Figure 4B). Taken together, these results indicate that the decreased AP threshold in \textit{Fmr1} KO neurons is caused by the abnormally elevated mGluR5 signaling.

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

Because our results demonstrate that enhanced I\textsubscript{NaP} in \textit{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\textsubscript{NaP} in \textit{Fmr1} KO neurons. To minimize the network influences from other glutamatergic or GABAergic receptors on I\textsubscript{NaP}, we measured I\textsubscript{NaP} in the presence of four blockers of NMDA, AMPA, GABA\textsubscript{A}, and GABA\textsubscript{B} receptors. First, we found that I\textsubscript{NaP} was still significantly larger in the \textit{Fmr1} KO than in the WT neurons in the presence of these four blockers (I\textsubscript{NaP} at \(-40 \text{ mV}: \text{WT}, -1.40 \pm 0.23 \text{ pA/pF}, n = 7; \text{KO}, -2.33 \pm 0.18 \text{ pA/pF}, n = 7; p = 0.0092;\) Figures S3A and S3B) and was nearly the same as I\textsubscript{NaP} measured without the blockers (Figure 3D), while the voltage-dependent activation of I\textsubscript{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\textsubscript{NaP}. In contrast, when mGluR5 blocker MPEP (10 \textmu M) was combined with the other four blockers, it decreased the I\textsubscript{NaP} in \textit{Fmr1} KO neurons (Figures 4C and 4H), and, most importantly, MPEP abolished the difference in I\textsubscript{NaP} between genotypes (with MPEP, at \(-40 \text{ mV}: \text{WT}, -1.20 \pm 0.15 \text{ pA/pF}, n = 6; \text{KO}, -1.14 \pm 0.28 \text{ pA/pF}, n = 6; p = 0.871;\) Figures 4C and 4H). We note that MPEP decreased I\textsubscript{NaP} predominantly in \textit{Fmr1} KO neurons, with negligible effect in WT neurons. These results indicate that elevated mGluR5 signaling leads to enhanced I\textsubscript{NaP} in \textit{Fmr1} KO neurons.

Rapid normalization of I\textsubscript{NaP} in \textit{Fmr1} KO neurons by acute inhibition of mGluR5 suggests that the effects of elevated mGluR5 signaling on I\textsubscript{NaP} are mediated, at least in part, by the modulation of Na\textsuperscript{+} channel activity. Indeed, pharmacological activation of group I mGluRs has been shown to modulate the activity of Na\textsuperscript{+} channels and, specifically, I\textsubscript{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\textsubscript{NaP} in \textit{Fmr1} KO neurons, we examined the effects of disrupting the mGluR5-PLC signaling pathway using a selective PLC inhibitor, edelfosine. Application of edelfosine (1 \textmu M) via the recording pipette (to avoid network effects) completely abolished the differences in I\textsubscript{NaP} between genotypes (at \(-40 \text{ mV}: \text{WT}, -1.62 \pm 0.20 \text{ pA/pF}, n = 6; \text{KO}, -1.28 \pm 0.21 \text{ pA/pF}, n = 6; p = 0.3089;\) Figures 4D and 4H). Intracellular application of another PLC inhibitor U73122 (1 \textmu M) similarly abolished the difference in I\textsubscript{NaP} between genotypes (at \(-40 \text{ mV}: \text{WT}, -1.10 \pm 0.18 \text{ pA/pF}, n = 6; \text{KO}, -1.27 \pm 0.28 \text{ pA/pF}, n = 6; p = 0.574;\) 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\textsuperscript{+} channel activity (Li et al., 1993). Therefore, we further tested whether PKC activation downstream of PLC mediates the observed changes in I\textsubscript{NaP}, using two potent and selective PKC inhibitors, calphostin C and PKC\textsubscript{19–36} (a pseudosubstrate peptide inhibitor of PKC). Intracellular application of either calphostin C (10 \textmu M) or PKC\textsubscript{19–36} (2 \textmu M) via the recording pipette completely abolished the differences in I\textsubscript{NaP} between genotypes (Figures 4F–4H) (calphostin C at \(-40 \text{ mV}: \text{WT}, -1.16 \pm 0.23 \text{ pA/pF}, n = 6; \text{KO}, -1.42 \pm 0.20 \text{ pA/pF}, n = 6; p = 0.50199; PKC\textsubscript{19–36}: \text{WT}, -1.18 \pm 0.21 \text{ pA/pF}, n = 7; \text{KO}, -1.11 \pm 0.11 \text{ 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\textsubscript{NaP} in \textit{Fmr1} KO neurons, which, in turn, leads to decreased AP threshold and increased neuronal excitability in the EC layer III PCs of \textit{Fmr1} KO mice.

**DISCUSSION**

Here, we demonstrate that PCs in the EC layer III of \textit{Fmr1} KO mice have a decreased AP threshold and increased excitability caused by dysregulation of Na\textsuperscript{+} channels. Our results indicate that this Na\textsuperscript{+} channel dysregulation is mediated by exaggerated mGluR5-PLC-PKC signaling that markedly increases persistent Na\textsuperscript{+} current in \textit{Fmr1} KO neurons. These findings suggest that Na\textsuperscript{+} 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\textsubscript{NaP} eliminates differences in the AP threshold between \textit{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\textsuperscript{+} channels (Brown et al., 2010; Deng et al., 2013; Gross et al., 2011;
Fmr1 signaling cascade by which abnormal mGluR5 activation impacts a signaling mechanism distinct from the well-established signal activity of mGluR5 acting via the PLC-PKC pathway. This is agreement with this notion, we found that reduced AP threshold absolutely contribute to regulation of RMP and the AP threshold. Ih; in contrast, sub-threshold hyperpolarization dampens IM and INaP but enhances I NaP. 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 INaP but not IM or Ih. We note that our results do not exclude a possibility that, in addition to INaP, IM 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 (p = 0.00368; data not shown), which might indicate a change in INaP.

Our data further indicate that changes in both INaP and the AP threshold are mediated by exaggerated mGluR5 signaling in the absence of FMRF. It is well accepted that loss of FMRF causes abnormally elevated mGluR5 activity (Santoro et al., 2012). PLC is a well-defined effector of mGluR5 signaling (Kettunen et al., 2002) and is well known to modulate activity of voltage-gated ion channels and, specifically, INaP (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 INaP 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 INaP (Carlert et al., 2006; D’Ascenzo et al., 2009). Our results suggest that the increased INaP 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 INaP are mediated, in part, by altered Na+ channel expression, our results indicate that the functional regulation of INaP 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 INaP inhibitors or the INaP 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 (p = 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 INaP as a potential target to alleviate these hyperexcitability defects. Indeed, although the amplitude of INaP 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 INaP particularly susceptible to hyperexcitability defects leading to seizures (Stafstrom, 2007), a common dysfunction in FXS. Indeed, pro-epileptic conditions have been associated with elevated INaP (Azouz et al., 1996; Somjen and Müller, 2000), and mutations in Na+ channels that cause increases in INaP have been found in patients with epilepsy (Meisler and Kearney, 2005; Vreugdenhil et al., 2004; Rhodes et al., 2005). Moreover, INaP inhibitors have been effective in treating both partial and generalized tonic-clonic seizures in humans (Stafstrom, 2007). A number of anti-epileptic drugs are INaP 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 INaP 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. INaP 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 INaP recordings, cell capacitance was compensated. Series resistance compensation for treating hyperexcitability defects in FXS.

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.
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rent in layer 5 neocortical neurons is primarily generated in the proximal axon.


REFERENCES

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AUTHOR CONTRIBUTIONS

P.-Y.D. and V.A.K. conceived and designed the study, P.-Y.D. conducted the

and analyzed the data. P.-Y.D. and V.A.K. wrote the manuscript.


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Carlier, E., Sourdet, V., Boudkkazi, S., Dégilde, P., Ankri, N., Fronzaroli-Moli-
nieres, L., and Debanne, D. (2006). Metabotropic glutamate receptor subtype 1

regulates sodium currents in rat neocortical pyramidal neurons. J. Physiol. 577, 141–154,

Carlier, E., Sourdet, V., Boudkkazi, S., Dégilde, P., Ankri, N., Fronzaroli-Molini-

eries, L., and Debanne, D. (2006). Metabotropic glutamate receptor subtype 1

regulates sodium currents in rat neocortical pyramidal neurons. J. Physiol. 577, 141–154,

Carlier, E., Sourdet, V., Boudkkazi, S., Dégilde, P., Ankri, N., Fronzaroli-Molin-

eries, L., and Debanne, D. (2006). Metabotropic glutamate receptor subtype 1

regulates sodium currents in rat neocortical pyramidal neurons. J. Physiol. 577, 141–154,

Carlier, E., Sourdet, V., Boudkkazi, S., Dégilde, P., Ankri, N., Fronzaroli-Molini-

eries, L., and Debanne, D. (2006). Metabotropic glutamate receptor subtype 1

regulates sodium currents in rat neocortical pyramidal neurons. J. Physiol. 577, 141–154,

Carlier, E., Sourdet, V., Boudkkazi, S., Dégilde, P., Ankri, N., Fronzaroli-Molini-

eries, L., and Debanne, D. (2006). Metabotropic glutamate receptor subtype 1

regulates sodium currents in rat neocortical pyramidal neurons. J. Physiol. 577, 141–154,


