Enhanced Endocannabinoid Signaling Elevates Neuronal Excitability in Fragile X Syndrome

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Fragile X syndrome (FXS) results from deficiency of fragile X mental retardation protein (FMRP). FXS is the most common heritable form of mental retardation, and is associated with the occurrence of seizures. Factors responsible for initiating FXS-related hyperexcitability are poorly understood. Many protein-synthesis-dependent functions of group I metabotropic glutamate receptors (Gp1 mGluRs) are exaggerated in FXS. Gp1 mGluR activation can mobilize endocannabinoids (eCBs) in the hippocampus and thereby increase excitability, but whether FMRP affects eCBs is unknown. We studied Fmr1 knock-out (KO) mice lacking FMRP to test the hypothesis that eCB function is altered in FXS. Whole-cell evoked IPSCs (eIPSCs) and field potentials were recorded in the CA1 region of acute hippocampal slices. Three eCB-mediated responses were examined: depolarization-induced suppression of inhibition (DSI), mGluR-initiated eCB-dependent inhibitory short-term depression (eCB-iSTD), and eCB-dependent inhibitory long-term depression (eCB-ILTD). Low concentrations of a Gp1 mGluR agonist produced larger eCB-mediated responses in Fmr1 KO mice than in wild-type (WT) mice, without affecting DSI. Western blots revealed that levels of mGlur1, mGlur5, or cannabinoid receptor (CB1R) were unchanged in Fmr1 KO animals, suggesting that the coupling between mGluR activation and eCB mobilization was enhanced by FMRP deletion. The increased susceptibility of Fmr1 KO slices to eCB-ILTD was physiologically relevant, since long-term potentiation of EPSP–spike (E–S) coupling induced by the mGluR agonist was markedly larger in Fmr1 KO mice than in WT animals. Alterations in eCB signaling could contribute to the cognitive dysfunction associated with FXS.
cognitive deficits may result from disordered CB1-mediated signa-
ing, suggests that FMRP deficiency could alter Gp1 mGluR-
dependent eCB mobilization. We have tested this hypothesis in
mouse hippocampal slices and find that FMRP deficiency does
not affect Ca^{2+}-dependent release of eCB, or CB1Rs, but en-
hances the coupling between Gp1 mGluRs and eCB mobilization.
The results may have significant implications for understanding
both FXS and eCB signaling.

Materials and Methods

Animals. We used tissue from 2- to 4-month-old male Fmr1 KO and
age-matched wild-type (WT) mice on the identical background strain,
C57BL/6J (kindly provided by M. McKenna, University of Mary-
land, Baltimore, MD), and mGluR1^{−/−} (from F. Conquet, University of Lau-
sanne, Lausanne, Switzerland) and mGluR5^{−/−} (from Jackson Labora-
tory) mice. To test the generality of the (−)3,5-dihydroxyphenylglycine
(DHPG) sensitivity, in a number of experiments we also used 2- to
4-month-old mice (from Charles River) of the C57BL/6N (males), 129Sv
C57BL/6J (kindly provided by M. McKenna, University of Maryland,
Baltimore, MD), and mGluR1^{−/−} C57BL/6J WT mice. All experimental protocols were
reviewed and approved by the University of Maryland School of Medi-
cine Institutional Animal Care and Use Committee, and all animal han-
dling was conducted in accordance with national and international
guidelines. The number of animals used was minimized, and all neces-
sary precautions were taken to mitigate pain or suffering.

Preparation of slices. Mice were deeply sedated with isoflurane and
decapitated. Slices, 400 μm thick, were cut on a Vibratome (model
VT1200s, Leica Microsystems) in an ice-cold extracellular recording so-
lution. Slices were stored in a holding chamber on filter paper at the
interface of this solution and a moist, oxygenated atmosphere at room
temperature for ≥1 h before transfer to the recording chamber (RC-27L,
Warner Instruments) and warmed to 30°C. The extracellular solution contained the following (in mM): 120 NaCl, 3 KCl, 2.5 CaCl₂, 2 MgSO₄, 1
NaH₂PO₄, 25 NaHCO₃, and 20 glucose, and was bubbled with 95% O₂,
5% CO₂, pH 7.4.

Electrophysiology. Whole-cell pipettes were pulled from thin wall glass
capillaries (1.5 O.D., World Precision Instruments). Electrode resist-
tances in the bath were 3–6 MΩ with internal solution containing the
following (in mM): 90 CsCH₃SO₄, 1 MgCl₂, 50 CsCl, 2 MgATP, 0.2
Ca₄-BAPTA, 10 HEPES, 0.3 Tris GTP, and 5 QX314. If the series resis-
tances changed by ≥20%, the data were discarded. Data were collected
with an Axopatch 1C amplifier (Molecular Devices), filtered at 1 kHz,
digitized at 5 kHz using a Digidata 1200 (Molecular Devices) and
acquired using Clampex 8 software (Molecular Devices). 2,3-Dihydroxy-6-nitro-7-
chloroquinoxaline-2,3-dione (NBQX) (10 μM) and α-AP5 (20 μM) were present in all whole-cell experiments to block glutamater-
gic EPSCs. Monosynaptic evoked IPSCs (eIPSCs) were elicited by 100-
μs-long extracellular stimuli delivered at 0.25 Hz with concentric bipolar
stimulating electrodes placed in stratum radiatum (s. radiatum).

Slices were pretreated with α-agatoxin GVIA (agatoxin, 300 nM
317x151)
to reduce the contribution of eCB-insensitive eIPSCs (Lenz et al., 1998;
Wilson et al., 2001). Stimulation in s. radiatum elicited eIPSCs every 4 s,
and at 90 s intervals, the pyramidal cell was depolarized to 0 mV for 1 s
and at 90 s intervals, the pyramidal cell was depolarized to 0 mV for 1 s
open voltage-gated calcium channels, increase [Ca^{2+}]_
218x141)) was calculated as the

Figure 1. Fmr1 KO neurons are more sensitive than C57BL/6J WT neurons to DHPG-induced
eCB mobilization. A, Representative traces showing eIPSC suppression caused by DHPG in Fmr1
KO and WT mice. Calibration: 200 pA, 50 ms. B, Concentration–response curves of DHPG-
duced eIPSC suppression in Fmr1 KO and WT mice. At 10 μM the differences were greatest (WT
mice: 72.5 ± 3.9%, n = 23; Fmr1 KO mice: 45.2 ± 4.6%, n = 15, p < 0.01). Pretreatment and
bath application of the GP1 mGluR antagonists MPEP (10 μM) and YM258198 (4 μM) blocked
the suppressive effect of 10 μM DHPG in Fmr1 KO mice (gray triangle, 101.0 ± 3.9%, n = 4).
C, Cumulative probability plot of eIPSC suppression induced by 10 μM DHPG (difference be-
tween groups significant by K–S test; p < 0.01).
Results

mGlRs mobilize eCBs in Fmr1 KO mice more effectively than in C57BL/6J WT mice

To determine whether mGlR-dependent eCB-ISTD is different in CA1 pyramidal neurons from Fmr1 KO and WT mice, both C57BL/6J, we bath applied the selective Gp1 agonist, DHPG, at concentrations from 1 to 50 μM for 3–4 min (Fig. 1A). At doses of >2 μM, DHPG reduced eIPSCs in both WT and Fmr1 KO mice. The responses were similar at the highest doses; however, at intermediate concentrations, DHPG suppressed eIPSCs more effectively in Fmr1 KO mice.

Figure 2. FMRP deficiency does not alter Ca^{2+}-dependent eCB mobilization, CB1R responsiveness, or protein expression levels of mGluR1, mGluR5, or CB1R. A, Representative traces showing DSI induced by a 1 s step depolarization in WT (left) and Fmr1 KO (right) mice. Calibration: WT, 200 pA, 0.5 min; Fmr1 KO, 150 pA, 0.5 min. B, Cumulative probability plot of DSI magnitudes recorded in WT (black circles) and Fmr1 KO (white circles) mice; difference n.s. by K–S test. Inset, Mean DSI magnitudes: WT mice: 56.0 ± 1.5%, n = 69; Fmr1 KO mice: 55.2 ± 2.2%, n = 51 (difference n.s. by t test). C, The time course of decay of DSI (n = 24 trials, 1 trial each in 24 cells). D, DSI integral shaded area (see Materials and Methods). Inset, Mean DSI integrals did not differ between Fmr1 KO and WT mice. WT: 967.1 ± 82.6% s, n = 24; Fmr1 KO: 936.8 ± 127.7% s, p > 0.1. E–G, Representative photomicrographs and grouped data of Western blots from hippocampal tissue in all sections. Blot densities measured with NIH Image. The densitometry values were normalized to their respective β-actin values. E, mGluRs from WT (n = 5), mGluR5−/− (n = 2), and Fmr1 KO (n = 6) mice. F, mGluR1 from WT (n = 5), mGluR1−/− (n = 3), and Fmr1 KO (n = 4) mice. G, CB1R from WT (n = 4) and Fmr1 KO (n = 4) mice.

Figure 3. Low dose of DHPG induces eCB-ILTD in Fmr1 KO but not in WT mice. A, Representative traces. Calibration: 200 pA, 3 min. B, Grouped data obtained from WT (black circles) and Fmr1 KO (white circles) mice. Inset, Summary of mean eIPSC depression measured 20–25 min after DHPG washout. WT mice: 94.8 ± 3.2%, n = 4; Fmr1 KO mice: 68.5 ± 3.0%, n = 5, p < 0.01.
Fmr1 KO mice seem to have heightened responsiveness to DHPG, but alternatively, C57BL/6J WT mice might have abnormally low responsiveness that is absent in the KO. To test this possibility, we applied 10 μM DHPG to pyramidal cells in slices from WT C57BL/6N male mice, or female WT 129-Sv or CD1 mice. DHPG-induced eIPSC suppressions among all WT mice were indistinguishable, and significantly smaller than responses of the KO mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The time to peak (t_{1/2}) of the KO mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), showing that DHPG does not have bet-

**Figure 4.** E–S coupling potentiation in Fmr1 KO and WT mice. **A1,** DHPG (50 μM) applied for 10 min induced E–S coupling potentiation in both WT and Fmr1 KO mice. **A2,** DHPG (10 μM) applied for 10 min induced strong E–S coupling potentiation in Fmr1 KO mice, but had only a modest effect in WT mice. **A3,** Pretreatment and continuous presence of 5 μM SR141716A blocked E–S coupling potentiation in Fmr1 KO mice. **B,** Representative traces. Calibration: 1 mV, 5 ms. **C,** Summary of E–S coupling potentiation in WT and Fmr1 KO mice. 50 μM DHPG strongly potentiated E–S coupling 35–45 min after DHPG application in both WT (n = 23.7%, p = 0.01) and Fmr1 KO mice (n = 23.7%, p = 0.01). However, 20 min after washout of DHPG, the eIPSCs returned to baseline (Fig. 2E–G). Hence, increased receptor number does not explain the increased sensitivity to DHPG in Fmr1 KO mice; rather, coupling between the mGluRs and eCB mobilization could be responsible.

**Protein expression of mGluR1, mGluR5, or CB1R is unchanged in Fmr1 KO mice**

Several mechanisms could underlie the increased ability of mGluRs to induce eCB-mediated responses in Fmr1 KO mice, including enhanced CB1R expression or downstream effectors, or increases in mGluR1, mGluR5, or coupling between mGluRs and eCB mobilization. Western blot analyses revealed no differences between Fmr1 KO and WT mice in expression of any of these receptors (Fig. 2E–G). Hence, increased receptor number does not explain the increased sensitivity to DHPG in Fmr1 KO mice; rather, coupling between the mGluRs and eCB mobilization could be responsible.

**mGluR-dependent eCB-iLTD is enhanced in Fmr1 KO mice**

Prolonged activation (~10 min) of Gp1 mGluRs with 50 μM DHPG produces eCB-iLTD (Chevaleyre and Castillo, 2003; Edwards et al., 2006); lower DHPG concentrations generally do not. If coupling between mGluRs and eCB mobilization is enhanced in Fmr1 KO mice, then lower DHPG concentrations might be able to induce eCB-iLTD in these mice. To test this prediction, we applied 10 μM DHPG to slices of both Fmr1 KO and WT mice for 10 min. The eIPSCs were suppressed to 62.1 ± 4.6% of baseline in WT mice and 36.3 ± 3.9% of baseline in Fmr1 KO mice during DHPG application (Fig. 3A, B) (p < 0.01). However, 20 min after washout of DHPG, the eIPSCs returned to baseline (n = 4) in WT mice, but remained depressed (n = 5, p < 0.01) (Fig. 3B) in Fmr1 KO mice.

**CB1R-dependent E–S coupling potentiation is enhanced in Fmr1 KO mice**

A lasting decrease GABAergic inhibition underlies the form of LTP called E–S coupling potentiation (Chevaleyre and Castillo, 2003); thus, E–S coupling should be facilitated in Fmr1 KO mice. A 10 min application of 50 μM DHPG strongly potentiated E–S coupling 35–45 min after DHPG application in both WT (n = 5)
and Fmr1 KO (n = 7) mice (Fig. 4A1, typical traces in Fig. 4B). However, 10 μM DHPG applied for 10 min induced strong E–S coupling potentiation in Fmr1 KO (Fig. 4A2) (n = 6) but only a slight effect in WT (n = 7) mice (p < 0.05). SR141716A prevented E–S coupling potentiation in both groups (Fig. 4A3) (n = 6, Fmr1 KO; n = 5, WT).

Discussion

Our results reveal that FMRP deficiency in C57BL/6J mice leads to increased neuronal excitability mediated by eCBs. The ability of Gp1 mGluRs to mobilize eCBs is heightened in Fmr1 KO animals, resulting in more pronounced eCB-iLTD, as well as greater susceptibility to eCB-iLTD, and E–S coupling potentiation. By altering mechanisms of synaptic plasticity, these factors could contribute to the cognitive dysfunctions associated with FXS.

eCBs are mobilized by two kinds of cellular stimulation: a strong rise in [Ca2+]i, or activation of certain G-protein-coupled receptors, such as Gp1 mGluRs (Maejima et al., 2001; Varma et al., 2001). Activation of Gp1 mGluRs stimulates local translation of synaptic mRNAs (Weiler and Greenough, 1993; Merlin et al., 1998; Huber et al., 2000; Bear et al., 2004), including the mRNA that encodes FMRP (Weiler and Greenough, 1999). Our data suggest that FMRP is involved in regulating mGluR-dependent mobilization of eCB. Similarities in magnitude and duration of DSI in Fmr1 KO and WT mice suggested that FMRP did not affect Ca2+-induced mobilization of eCBs, CB1Rs, or the effector mechanisms that inhibit GABA release downstream of CB1R. Maximal responses produced by DHPG were unaffected by FMRP deficiency, implying that the coupling between mGluRs and eCB mobilization may be quantitatively, but not qualitatively, altered. Data from Western blots of mGluRs and CB1Rs, together with the increased capacity of moderate concentrations of DHPG to initiate eCB-dependent responses, support the conclusion that the coupling between mGluRs and eCB mobilization is modulated by FMRP.

The mechanism of FMRP modulation of eCB mobilization cannot be determined without more information on the pathway between mGluRs and the signaling pool of eCBs (probably 2-AG) (e.g., Hashimoto et al., 2005). eCBs are derived directly from membrane lipids (Piomelli, 2003), and it is unlikely that FMRP is immediately involved in this process. Nevertheless, cellular proteins regulated by FMRP may play some role. Diacylglycerol lipase mediates 2-AG synthesis (Piomelli, 2003), and gene knock-out experiments show that PLCβ1 is upstream of eCB mobilization in the hippocampus (Hashimoto et al., 2005), although obligatory activation PLCβ1 before brief, phasic release of eCBs has not been demonstrated (Hashimoto et al., 2005; Edwards et al., 2008). A presently uncharacterized transporter participates in eCB release in some circumstances (Adermark and Lovinger, 2007; Edwards et al., 2008), and might constitute another potential target for FMRP regulation. Understanding the connections between FMRP and eCB mobilization will be an important goal of future studies.

Bear and colleagues (Bear et al., 2004; Dolen et al., 2007) have put forward an “mGluR theory of fragile X mental retardation,” which holds that dysfunction of Gp1 mGluR effector mechanisms stemming from FMRP deficiency may cooperate in shaping the FXS phenotype. Because eCB mobilization is downstream of these mGluRs, our study effectively tested a prediction of the theory, and our results are broadly consistent with it. Importantly, though less obviously, our data may also help account for some poorly understood details of FXS-related phenomena. For instance, enhancement of eCB-iLTD (Fig. 3) could foster the “hyperplasticity” represented by enhanced LTD in the CA1 (Huber et al., 2002). Approximately 25% of FXS patients suffer from epilepsy during development (Sabaratnam et al., 2001), and seizure activity is often increased by suppression of GABA inhibition. Prolonged epileptiform discharges can be mediated by altered activation of Gp1 mGluRs in Fmr1 KO mice (Chuang et al., 2005; Bianchi et al., 2009); however, in these experiments bicuculline is usually used to block GABA responses and induce epileptiform discharges. Perhaps the disinhibition represented by eCB-iLTD contributes to initial changes of pyramidal cell excitability, and thus sets the stage for the prolonged seizure states. The endocannabinoid system could represent another target for intervention in the treatment of FXS.

References


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