Clustered Burst Firing in \textit{FMR1} Premutation Hippocampal Neurons: Amelioration with Allopregnanolone

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ABSTRACT
Premutation CGG repeat expansions (55–200 CGG repeats; preCGG) within the fragile X mental retardation 1 gene (FMR1) cause fragile X-associated tremor/ataxia syndrome (FXTAS). Defects in neuronal morphology and migration have been described in a preCGG mouse model. Mouse preCGG hippocampal neurons (170 CGG repeats) grown in vitro develop abnormal networks of clustered burst (CB) firing, as assessed by multielectrode array (MEA) recordings and clustered patterns of spontaneous Ca\(^{2+}\) oscillations, neither typical of wild type (WT) neurons. PreCGG neurons have reduced expression of vesicular GABA and glutamate (Glu) transporters (VGAT and VGLUT1, respectively) and preCGG hippocampal astrocytes display a rightward shift on Glu uptake kinetics, compared to WT. These alterations in preCGG astrocytes and neurons are associated with 4- to 8-fold elevated Fmr1 mRNA, and occur despite consistent expression of fragile X mental retardation protein (FMRP) levels at approximately 50% of WT levels. Abnormal patterns of activity observed in preCGG neurons are pharmacologically mimicked in WT neurons by addition of Glu or the mGluR1/5 agonist, DHPG, to the medium; or by inhibition of astrocytic Glu uptake with DL-threo-β-benzyloxyaspartic acid (TBOA), but not by the ionotropic Glu receptor agonists, AMPA or NMDA. The mGluR1 (CPCCOEt) or mGluR5 (MPEP) antagonists reversed clustered burst firing. Importantly, the acute addition of the neurosteroid allopregnanolone mitigated functional impairments observed in preCGG neurons in a reversible manner. These results demonstrate abnormal mGluR1/5 signaling in preCGG neurons that is ameliorated by mGluR1/5 antagonists or augmentation of GABA\(_A\) receptor signaling, and identify allopregnanolone as a candidate therapeutic lead.
INTRODUCTION

Fragile X syndrome (FXS) is the most common inherited form of cognitive impairment and a leading single-gene disorder associated with a high rate of autism (1, 2). FXS is caused by trinucleotide (CGG) repeat expansions of >200 repeats (termed full mutation) within the 5’ non-coding region of the fragile X mental retardation 1 (FMR1) gene located on the X chromosome. Expansion CGG repeats greater than 200 generally result in hypermethylation of FMR1 gene, which leads to transcriptional silencing and absence of fragile X mental retardation protein (FMRP) (3-5). Individuals with intermediate length CGG expansions, between 55-200 repeats (premutation), are typically unaffected by FXS but can display a range of clinical features including behavioral and cognitive abnormalities in children (6-9). Premutation carriers have a higher rate of primary ovarian insufficiency (Fragile X-associated Primary Ovarian Insufficiency; FXPOI) (10), and a substantial proportion experience a late-adult-onset neurodegenerative disorder, fragile X-associated tremor/ataxia syndrome (FXTAS) (11-13).

Premutation alleles of the FMR1 gene are quite common in general population. Around 1:250-810 males and 1:130-250 females carry premutation alleles (14-16). In FXS families, around 46% of male premutation carriers and 16% of female carriers over 50 years of age will develop clinical features of FXTAS, with phenotypic penetrance increasing with age (16, 17). Core clinical features of FXTAS include progressive gait ataxia and intention tremor with associated cognitive decline and executive dysfunction, peripheral neuropathy, dysautonomia and Parkinsonism (11, 12, 18, 19). The absence of FXPOI and FXTAS symptoms in full mutation patents implies that FMRP deficiency per se is not responsible for premutation disorders and FXTAS. Instead, evidence from both human and animal studies suggests a direct toxic gain-of-function of premutation CGG (preCGG) alleles due to an increase in the CGG-repeat-containing FMR1 mRNA (20-22). Consistent with this hypothesis, characteristic intranuclear inclusions found in neuronal and glia cells of FXTAS cases (23, 24) have been
demonstrated to contain FMR1 mRNA (25). Additionally, the expanded CGG repeat-RNA is sufficient to form the intranuclear inclusions in both primary neural progenitor cells and established neural cell lines (26), and expression of expanded CGG repeats in Purkinje neurons produces intranuclear inclusions, neurodegeneration and motor deficits (27).

Knock-in (KI) mouse models have been developed. In one mouse model, native 9-10 CGG repeat allele in the homologous Fmr1 gene was replaced with CGG expansion repeats that can vary from 100 to >300 in size from generation to generation (28). Another KI mouse model was developed wherein CGG-CCG repeats were serially ligated in exon 1 of the endogenous mouse Fmr1 gene (29). Similar to human premutation carriers, hippocampus of premutation mice exhibits elevated Fmr1 mRNA and normal to 50% reductions in FMRP compared to WT, even in mice with large (150-190) repeats (20, 21, 29). The premutation mouse models do not fully recapitulate human FXTAS (28); however, they do show progressive deficits in processing spatial and temporal information, cognitive deficits (30), motor deficits (31), and hyperactivity (32). Ex vivo studies also showed ubiquitin-positive intranuclear inclusions in neurons and astrocytes are neuropathological hallmarks of FXTAS in both humans (23, 24, 25, 33) and mouse brain (20, 29, 34). These ubiquitin-positive intranuclear inclusions were found in both neurons and astrocytes in preCGG mice in vivo. PreCGG mice exhibit altered embryonic neocortical development with migration defects in the neocortex and altered expression of neuronal lineage markers (35). Hippocampal neurons cultured from preCGG mice show impairments in dendritic complexity and altered architecture of synapsin puncta prior to neurodegeneration (36), however the functional consequences of early onset morphological alterations in preCGG neurons have not been investigated. PreCGG mice display upregulated mRNA levels of many components of GABAergic system in cerebellum but not in cortex (37) suggesting altered inhibitory neuronal transmission in preCGG mice model.

Here we report that hippocampal neurons cultured from hemizygous male preCGG mice (170 CGG repeats) have deficiencies in neuronal vesicular GABA and glutamate (Glu)
transporters (VGAT and VGLUT1) and develop abnormal burst-firing electrical activity consisting of clustered patterns of spontaneous synchronized Ca$^{2+}$ oscillations, not typical of neurons cultured from male wild type (WT) mice. Treatment of WT neuronal cultures with pharmacological agents that inhibit Glu uptake, activate mGlu R1/5, or inhibit GABA$_A$ receptors, all result in clustered burst firing behavior that phenocopies preCGG neurons. These data provide functional evidence of imbalanced excitatory-inhibitory neurotransmission in preCGG neurons. The mGluR1 (CPCCOEt) or mGluR5 (MEPP) antagonists reversed clustered burst firing. Furthermore, treatment of preCGG neurons with the neurosteroid allopregnanolone, a positive allosteric modulator of GABA$_A$ receptors, mitigate the functional electrical impairments observed in preCGG neurons in a reversible manner, identifying a possible therapeutic strategy for ameliorating abnormal neuronal activity in individuals with the premutation.

RESULTS

PreCGG neurons express elevated $Fmr1$ mRNA and intermediate levels of FMRP

Western blotting with a chicken monoclonal antibody detects FMRP (38) in the lysate of astrocyte cultures and astrocyte-neuronal co-cultures with the major band at 72 kDa (Fig. 1A), a band absent in brain lysate of FMRP knock-out mice, a model of FXS (data not shown). When normalized to the intensity of $\beta$-actin, hippocampal neurons cultured from preCGG mice with ~170 CGG expansion express 46.5±3.2% and 51.4 ±0.1% of the FMRP levels found in respective WT neurons measured at 14 days in vitro (DIV) and 21 DIV. Compared to WT, preCGG astrocytes express 55.8±6.6% the level of FMRP (Fig. 1B). Results from RT–PCR analyses show that premutation cultures (mean expansion 175 CGG repeats) show 4.1, 7.6 and 8.4-fold higher $Fmr1$ mRNA levels than the corresponding WT astrocyte and 14 DIV as well as 21 DIV hippocampal neuronal cultures, respectively (Fig. 1C).
Premutation hippocampal neurons display prominent clustered burst (CB)-firing behavior

Simultaneous extracellular recordings of electrical activity from multiple sites within the neuronal cultures with a high spatial resolution provide a robust measure of network activity and connectivity (39). Electrical firing activity in cultures isolated from WT and preCGG hippocampus are measured using 64-electrode MEAs by converting electrical field potential recordings to raster plots as described in Methods and Supplemental Figure S1. By culture day 7 DIV, both WT and preCGG hippocampal neurons display similar patterns of spontaneous electric activity comprised of infrequent synchronized bursts of field potentials, mixed with desynchronized random spiking (Fig. 2A&B). Neither spike frequency nor burst duration differs between WT and preCGG cultures at 7 DIV (Fig. 2C&D). Synchronized firing activity increases in frequency and duration as neuronal networks matured by 21 DIV. At 21 DIV, preCGG hippocampal neurons display a distinct firing pattern composed of intense clustered bursts (CB) interspersed with brief periods of quiescence (Fig. 2A&B). The percentage of MEAs reporting the CB firing pattern is significantly higher in the preCGG neuronal cultures than in WT neurons. With WT hippocampal neurons, <10% of the MEAs recorded (n=37) show CB firing patterns lasting 10 sec or longer, compared to >60% (n=26) of the MEAs with preCGG neurons displaying CB firing lasting 10 sec or longer (Supplemental Fig. S2, black bars). PreCGG neurons (21 DIV) display neuronal network activity having higher spike frequency (WT = 1.55±0.22 spikes s⁻¹, n=26 vs. preCGG = 4.35±0.64 spikes s⁻¹, n=37, p<0.01) and longer mean burst duration (WT = 0.43± 0.10 s, n=26 vs. preCGG = 1.67±0.24 s, n=37, p<0.01).

Altered patterns of spontaneous Ca²⁺ oscillation in preCGG hippocampal neurons.

Cultured hippocampal neurons display spontaneous synchronous Ca²⁺ oscillations (40). We simultaneously evaluated the spontaneous Ca²⁺ dynamics in WT and preCGG neurons cultured on 96-well plates using the FLIPR TETRA® high throughput cellular imaging system.
Similar to the clustered burst-firing behavior observed in MEA measurements, preCGG hippocampal neurons (14 DIV) display temporally clustered Ca\(^{2+}\) oscillations interspersed with quiescent periods in nearly 90\% of the wells measured (n=93), a pattern seen in only 23.9\% of the wells (n=71) containing WT hippocampal neurons (Fig. 3).

**PreCGG hippocampal neurons express less VGLUT1 and VGAT**

VGLUT and VGAT are transporters that are expressed primarily in excitatory and inhibitory neurons (41, 42) and are responsible for the uptake of Glu and GABA into presynaptic vesicles (43, 44). Disruption in the balance between excitatory and inhibitory transmission has been shown to influence overall neuronal activity, leading to abnormal burst-firing in neurons (45). We measured the expression levels of VGLUT1 and VGAT using western blot analysis. Figures 4A & B show that at 7 DIV both VGLUT1 and VGAT expression are slightly higher in preCGG compared to WT neurons. However, by 21 DIV preCGG neurons display significantly lower expression of both VGLUT1 and VGAT than WT, whereas the ratios of VGAT to VGLUT1 do not differ between genotypes. However, the VGAT/VGLUT1 ratio declined in both genotypes from 7 to 21 DIV (Fig. 4C). The trajectories of in vitro expression for VGAT and VGLUT1 diverge. VGAT decreases 27.0\% in preCGG but only 6.0\% in WT between 7 and 21 DIV (Fig. 4D). By contrast, VGLUT1 increases 280.1\% and 203.3\% in WT and preCGG neurons, respectively, during this DIV period (Fig. 4D).

**PreCGG hippocampal astrocytes display lower affinity of glutamate uptake**

Astrocyte transporters remove Glu from the synapse (46). We compared Glu uptake kinetics of hippocampal astrocytes cultured from WT and preCGG male pups. Figure 5A shows similar Glu uptake in WT (126.1±1.7 nmol/mg protein) and preCGG astrocytes (126.9±2.2 nmol/mg protein) over 30 min. Preincubation (25 min) of astrocytes with DL-threo-β-benzyloxyaspartic acid (TBOA) and L-trans-pyrrolidine-2,4-dicarboxylic acid (L-trans-PDC), non-
selective inhibitors of Glu uptake, inhibited Glu uptake 50% and >90%, respectively regardless of genotype. Initial experiments indicate that 100 µM Glu is sufficient for linear rates of uptake for at least 10 min (data not shown). Therefore for the kinetics analysis, the incubation time was shortened to 10 min. Figure 5B shows that the $V_{\text{max}}$ for Glu does not differ between WT and preCGG hippocampal astrocytes, although preCGG astrocytes display a significant lower affinity for Glu (preCGG $K_m = 28.4\pm2.2$ µM, Mean±SEM; WT $K_m = 20.0\pm2.8$ µM, Mean±SEM, n=4, $p<0.01$).

**Modulation of Glu and GABA signaling produces CB firing pattern in WT neurons**

Since preCGG astrocytes may have impaired Glu clearance, we investigated the influence of glutamate on the neuronal firing activity in WT neurons. Acute application of 100 µM of glutamate to the WT neurons produced a CB firing pattern. Glu significantly increased the spike rates from $1.44\pm0.24$ to $6.77\pm1.24$ spikes s$^{-1}$ as well as the mean burst duration from $0.23\pm0.01$ to $4.52\pm0.76$ s (Fig. 6C). To test if the rightward shift of Glu uptake observed in preCGG astrocyte cultures affects neuronal firing, we mimicked the Glu transport impairment in WT neurons by introducing TBOA, a non-selective, competitive glutamate uptake antagonist. Figure 6D&E shows that application of TBOA (100 µM) produced a CB firing pattern in WT hippocampal neurons, increasing spike rate from $1.05\pm0.10$ to $2.42\pm0.29$ spikes s$^{-1}$ as well as the mean burst duration from $0.42\pm0.03$ to $0.84\pm0.07$ s. Considered together, these data demonstrate that pharmacologically mimicking the dysfunction of Glu signaling could reproduce the CB firing pattern observed in the premutation neurons.

We next examined the roles of ionotropic (NMDA and AMPA) glutamate receptors and type I metabotropic glutamate receptors (mGluRs) on the producing CB firing pattern by application of specific agonists. As shown in Supplemental Figure S3A, application of NMDA produced a robust increase on the spike rate, however, rather than forming a CB firing pattern, these spikes were evenly distributed over the recording period. Application of a low concentration of AMPA (100 nM) produced a slight increase of spike rate. These spikes were
mostly asynchronous and randomly spaced and did not form a CB pattern. A higher concentration of AMPA (1 µM) eliminated most electric activity (Fig. S3B). The actions of NMDA and AMPA observed here are similar to those observed previously in cortical neurons (47).

**Figure 7** shows that application of type I mGluR activator, DHPG (10 µM), produced a CB firing pattern in WT hippocampal neurons increasing the spike frequency from 0.85±0.08 to 21.79±1.09 spike/s as well as the mean burst duration from 0.31±0.02 to 1.05±0.02 s. These data demonstrate that the type I mGluRs play a key role in generation of the CB firing pattern.

Reduced expression levels of VGAT could decrease GABA levels in presynaptic stores and lead to reduced strength and efficacy of GABA release. To assess the role of GABA<sub>A</sub> receptors, we pharmacologically suppressed GABA<sub>A</sub> receptor function to mimic the reduced GABA signaling observed in premutation hippocampal neurons using picrotoxin, a specific noncompetitive antagonist of GABA<sub>A</sub> receptors. **Figure 8** shows that application of picrotoxin (100 µM) did produce CB firing pattern in WT hippocampal neurons although the duration of the cluster is shorter than those observed in preCGG hippocampal neurons. Picrotoxin alone increased the spike rate from 0.61±0.06 to 1.83±0.02 spike/s and the mean burst duration from 0.30±0.02 to 0.64±0.01 s, respectively. Application of picrotoxin (100 µM) and DHPG (10 µM) together in WT hippocampal neurons produced a robust CB firing pattern similar to the one observed in premutation hippocampal neurons with increased spike rate (from 0.61±0.06 to 7.7±0.57 spikes s<sup>-1</sup>) as well as longer mean burst duration (from 0.30±0.02 to 1.27±0.06 s). These data together demonstrate that manipulation of either Glu or GABA signaling in WT cultures recapitulates CB firing patterns in preGCC neurons thereby implicating an altered excitatory-inhibitory balance in preCGG hippocampal neuronal transmission.
Suppression of type I mGluRs function or enhancement of GABA<sub>A</sub> receptors activity rescue the CB phenotype in preCGG hippocampal neurons

Given the pivotal roles of type I mGluRs and GABA<sub>A</sub> receptors signaling on the generation of the CB firing pattern, we next evaluated the neuronal firing activity after suppression of type I mGluR activity or enhancement of GABA<sub>A</sub> receptor activity in preCGG hippocampal neurons. Figure 9A&B shows that application of MPEP, an antagonist of mGluR5 receptors, inhibited spike rate and mean burst duration in a concentration-dependent manner reversing CB firing patterns to a randomly distributed burst-firing pattern seen in WT cultures. Similarly, CPCCOEt, an mGluR1 antagonist also suppressed the spike firing rate as well as mean burst duration thereby mitigating the CB firing pattern to a randomly distributed burst-firing pattern observed in WT neurons.

We next evaluated the influence of allopregnanolone, a positive allosteric modulator of the postsynaptic GABA<sub>A</sub> receptors (48), on the electric firing pattern of preCGG hippocampal neurons. Figure 10A&B shows that allopregnanolone suppressed the CB firing pattern of preCGG hippocampal neurons (reducing spike rate as well as mean burst duration) in a concentration-dependent and reversible manner.

DISCUSSION

In this study, we have demonstrated that hippocampal neurons cultured from preCGG mice display a high penetrance for CB electrical spiking activity and abnormal patterns of spontaneous Ca<sup>2+</sup> oscillations under basal culture conditions. Functional differences in preCGG neurons are observed in the presence of significantly elevated Fmr1 mRNA and moderate levels of FMRP expression that are consistent with the previous findings in brain lysates and neuronal cultures prepared from KI mice expressing CGG repeats in the premutation range (4, 21, 36, 49), and also consistent with findings from human premutation carriers and FXTAS patients (22, 25). Therefore, the presence of both significantly elevated Fmr1 mRNA and
intermediate levels of FMRP models the human condition (22, 25) and distinguishes preCGG
hippocampal neurons and astrocytes from the FXS mouse model. Although prior studies have
emphasized a toxic gain-of-function effect of elevated messenger RNA associated with the
premutation, the current results from mouse preCGG hippocampal neurons also support a role
of reduced FMRP in alterations of brain activity recently reported by Hessl and coworkers using
functional MRI of amygdala of premutation carriers (50).

The principal mechanisms contributing to the defects in basal electrical activity exhibited
by preCGG neurons appear to be associated with a gain of function in type I mGluRs and/or a
loss of function in GABA_A receptor signaling. This conclusion is supported by data indicating
that: (1) type I mGluR receptor agonist DHPG, but neither NMDA and AMPA receptor agonists,
increased CB firing patterns in WT neurons with increased spike rate and mean burst duration
similar to those observed in preCGG hippocampal neurons, (2) selective mGluR1/5 antagonists
(CPCCOEt and MPEP) abrogated CB activity in preCGG neurons, (3) preCGG astrocytes have
impaired Glu uptake, (4) WT cultures exposed to the astrocyte Glu transport competitive
antagonist TBOA produced CB firing patterns indistinguishable from those of preCGG neurons,
(5) GABA_A receptor block with picrotoxin phenocopied CB firing behavior observed in preCGG
neurons, and (6) the allosteric GABA_A receptor enhancer allopregnanolone essentially restored
WT electrical spiking patterns.

These functional deficits are directly pertinent to the altered patterns of neuronal
complexity reported earlier using the same in vitro preCGG model (36), and possibly contribute
to migration defects in the neocortex of embryonic preCGG mice (35). Abnormal electrical firing
patterns of neural networks could perhaps promote the seizures and developmental problems,
including autism spectrum disorders and attention deficit hyperactivity disorder (ADHD), seen in
a subgroup of young premutation boys (51) (52). Neuronal activity is essential for normal
neuronal migration (53), dendritic growth (54), and synaptic plasticity (55), processes mediated
by spatially and temporally orchestrated intracellular Ca^{2+} signals. Cultured hippocampal
neurons display spontaneous synchronous Ca$^{2+}$ oscillations (40) that are spaced at regular temporal intervals (this study). However, preCGG neurons exhibit synchronized clusters of Ca$^{2+}$ oscillations that appear to coincide with intense CB firing. Low to moderate intracellular Ca$^{2+}$ signals promote dendritic growth, whereas more intense Ca$^{2+}$ signals have been proposed to cause dendritic retraction (56, 57). Therefore, the abnormal CB electrical activity and abnormal patterns of spontaneous Ca$^{2+}$ oscillations observed in preCGG hippocampal neurons are likely to contribute, at least in part, to impaired dendritic growth and synaptic architecture, including increased puncta volumes in presynaptic synapsin and postsynaptic phalloidin (36).

A balance between excitatory and inhibitory synaptic transmission tightly controls spontaneous neuronal activity in hippocampal neurons. In simulations of neural networks, manipulation of excitatory and inhibitory inputs produced distinct neuronal burst-firing behaviors, including clustered bursts (45), similar to those exhibited by preCGG neurons. CB firing activity is pronounced in preCGG neurons by 21 DIV at which time VGAT and VGLUT, markers of excitatory and inhibitory neurons, are expressed at significantly lower levels than in WT neurons. The ratio of VGAT /VGLUT does not differ between preCGG and WT at either 7 or 21 DIV, suggesting that a shift in the relative composition of glutamatergic and GABAergic neurons in the preCGG cultures is not responsible for CB firing activity. PreCGG neurons express 20.2% lower VGLUT1 relative to that on WT at 21 DIV, but such a modest reduction of VGLUT1 protein, the dominant isoform expressed in adult hippocampal neurons (58, 59), is unlikely to have an influence on basal glutamate transmission (60), especially when considering the pronounced developmental upregulation of VGLUT1 expression observed between 7 and 21 DIV with both WT and preCGG neuronal cultures. The developmental upregulation of VGLUT1 observed in our in vitro study is consistent with the previous findings in rat cerebral cortex and human dorsolateral prefrontal cortex (61, 62).

VGAT expression remains unchanged as WT hippocampal neurons mature in vitro, an observation consistent with developmental studies in human brain (62). By contrast, VGAT
levels decrease nearly 27% from 7 to 21 DIV in preCGG neurons. Reduced expression of VGAT could result in insufficient reuptake of GABA to presynaptic vesicles, negatively influencing the strength of GABAergic synaptic transmission (43). Although impaired GABAergic synaptic function in preCGG neurons in combination with impaired Glu buffering by preCGG astrocytes could contribute to their CB firing pattern and clustered Ca^{2+} oscillations, we cannot discount the contributions of defects in mGluR1/5 and/or GABA_{A} signaling pathways to preCGG impairments, especially given our results with pharmacological modulation of their receptors. Ca^{2+} signaling dysregulation in FMRP-null models of FXS have been recently described (63, 64). Defects in activity-dependent Ca^{2+} influx and Ca^{2+} store release have been demonstrated in the null Drosophila FMR1 brain mushroom body and are dependent on developmental stage (63). High frequency stimulation of hippocampal CA3 neurons in the Fmr1 knock-out mouse model significantly enhanced Ca^{2+} influx after the first few stimuli (64). These effects have been ascribed to the loss of FMRP expression. However, in our preCGG mouse model there is substantial FMRP expression (~50% of WT levels) in both preCGG hippocampal astrocytes and neurons. Although we cannot rule out that intermediate levels of FMRP in preCGG neurons could contribute to CB activity and altered Ca^{2+} oscillatory behavior, this phenotype is more likely a consequence of a gain-of-function imparted by the ~8-fold increase in expanded CGG-repeat Fmr1 mRNA or a combination of both intermediate FMRP levels and elevated FMR1 mRNA levels (15, 26, 49). As a further point regarding the influence of FMRP on the present observations, It has been recently reported that the variation of FMRP among individuals with normal FMR1 alleles is greater than four-fold in the general population (38, 65), in the absence of any clinical features of FXS.

GABA receptor signaling is critical to the regulation of neuronal firing activity. Application of GABA to cultured neurons reduced neuronal network activity (66), whereas inhibition of GABA_{A} receptors by bicuculline, a competitive antagonist of GABA_{A} receptors, elicited a highly synchronized, periodic burst-firing pattern (66, 67), similar to those we elicited in WT neurons
exposed to picrotoxin. Altered mGluR5 receptor signaling has been demonstrated in Fmr1 KO FXS mouse model, where the mGluR5-dependent long-term synaptic depression is enhanced during synaptic transmission (68). This enhancement was later proved to be due to excessive protein synthesis downstream of mGluR5 activation with the loss of FMRP expression, the latter normally acting as a negative regulator of mRNA translation (69). Here we show that block of mGluR1/5 resolves CB firing behavior. However, mGluR1/5 signaling impairments that contribute to CB firing behavior in preCGG neurons occurs in the presence of appreciable but reduced levels FMRP, and thus may be etiologically distinct from FXS, although may respond to medications now used for targeted treatment trials in FXS (70).

A finding of potential clinical importance for novel therapeutic prophylaxis of premutation-related disorders or FXTAS intervention is our observation that the neurosteroid allopregnanolone can mitigate CB firing patterns in a concentration-dependent and reversible manner. Collectively these data further support the concept that impaired mGluR1/5 and GABA<sub>A</sub> receptor signaling pathways contribute to physiological impairments in preCGG neurons and identify novel intervention strategies for targeted treatment of premutation developmental disorders and FXTAS.

MATERIALS AND METHODS

Materials

Anti-VGAT and anti-VGLUT1 antibodies were purchase from Synaptic Systems GmbH (Goettingen, GERMANY). Mouse anti-β-actin antibody was from Cell Signaling Techonology (Danvers, MA, USA). Chicken-anti-FMRP antibody was generated in P. Hagerman’s laboratory (38). NewBlot Nitro Stripping Buffer, Odyssey Blocking Buffer and IRDye-labeled secondary antibody were from LI-COR Biotechnology (Lincoln, NE, USA). Fluo-4 and pluronic F-127 were from Invitrogen (Carlsbad, CA, USA). L-trans-pyrrolidine-2,4-dicarboxylic acid (L-trans-PDC), DL-threo-β-benzylxoyaspartic acid (TBOA), 2-methyl-6-(phenylethynyl)pyridine hydrochloride
MPEP), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt),
dihydroxyphenylglycine (DHPG), N-methyl-D-aspartic acid (NMDA) and α-2-amino-3-(5-methyl-
3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) were from Tocris Bioscience (Ellisville, MO, USA).
Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) was synthesized by M.A. Rogawski and
characterized as >99% pure.

**PreCGG mouse model**

All preCGG KI and WT mice in the C57 B6 background were housed under standard
vivarium conditions. PreCGG hemizygous male mice (150-190 CGG expansion repeat; average
170) were obtained by breeding homozygous preCGG females with preCGG hemizygous males
from founders derived from Erasmus University (20). Male WT and preCGG pups delivered on
the same day were used for paired cultures. The *FMR1* genotype was verified by PCR (36). All
animal use protocols were approved by the University of California, Davis IACUC.

**Genotyping**

DNA was extracted from mouse-tail and genotyping of the *FMR1* expansion size were
performed using the forward and reverse primers previously described (36).

**Hippocampal neuron-astrocyte co-culture**

Hippocampal astrocytes-neuron mixed cultures were obtained from postnatal day 0-1
(P0-1) WT or preCGG KI male pups. Mice were decapitated, their brains removed, and
hippocampus dissected in a sterile hood. Hippocampal neurons were dissociated and plated
onto poly-L-lysine coated 6-well or clear-bottom, black well, 96-well imaging plate (BD, Franklin
Lakes, NJ, USA) at densities of 2×10^6/well or 1×10^5/well, respectively, as previously described
(36). For microelectrode array (MEA) experiments, 60µl of cell suspension at a density of 2×10^6
cells/ml were added as a drop to the center of MEA to cover the 64 electrode probes. After 2 h
incubation, a volume of 1.5ml of serum-free neurobasal supplemented medium containing NS21
supplement, 0.5mM L-glutamine, and HEPES was added to each MEA. The medium was
changed twice a week by replacing half volume of culture medium with serum-free neurobasal
supplemented medium. The cells were maintained in an atmosphere at 37 °C with 5% CO₂ and 95% humidity.

**Hippocampal astrocytes culture**

Enriched hippocampal astrocytes were obtained from postnatal day 0-1 (P0-1) WT or preCGG KI male pups. The dissection and dissociation of the cells is the same as the neuronal preparation. After digestion and centrifugation as described for isolation of neurons, cells were suspended in DMEM medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 0.10mg/ml streptomycin, pH 7.4. Cells were plated onto poly-L-ornithine coated T-75 culture flask at a density of 2-3 x 10⁶ cells/flask and maintained in an incubator at 37 °C with 5% CO₂ and 95% humidity. The culture medium was changed twice a week and the cells were used between 2-4 weeks and no more than 3 passages.

**Western blot**

The sample preparation for western blot was performed as described previously (71). Equal amounts (20 µg) of samples were loaded onto a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane by electroblotting. The membranes were blocked with Odyssey blocking buffer (for VGLUT1 and VGAT antibodies) or 5% non-skimmed milk in PBS buffer+0.1% Tween-20 (for FMRP antibody) for 1.5-2 h at room temperature. After blocking, membranes were incubated overnight at 4°C in primary antibody dilution (anti-VGAT, 1:5,000; anti-VGLUT1, 1:10,000; anti-FMRP, 1:20,000 and anti-β-actin, 1:20,000). The blots were washed and incubated with the IRDye (800CW or 700CW)-labeled secondary antibody (1:10,000) for 1 h at room temperature. After washing with 0.1% Tween in PBS for 5 times, the membrane was scanned with the LI-COR Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA). The densitometry was performed using LI-COR Odyssey Infrared Imaging System application software 2.1. Membranes were stripped with NewBlot Nitro Stripping Buffer and rebotted for analysis of additional proteins.
Quantitative measurements of Fmr1 mRNA levels

Total RNA from primary hippocampal cultures was isolated by standard method (Trizol, Ambion Inc., Austin, TX, USA). Precise estimates of Fmr1 mRNA levels in total RNA were obtained by real time PCR. Details of the method and its application to the study of Fmr1 mRNAs are described as previously (22). The reference gene was β-glucoronidase (GUS). The analysis was repeated for 3 different RNA concentrations, in duplicate, and incorporated standards for each determination to compensate for any changes in reaction efficiency.

Ca^{2+} oscillation determination

The WT and premutation hippocampal neurons cultured in the same clear bottom, 96-well imaging plate were used to determine the spontaneous Ca^{2+} oscillations as described previously (72).

MEA recording

All MEA recordings were conducted at 37 °C in culture medium without perfusion with the MED64 system (Alpha MED Scientific Inc., Ibaraki, Osaka, Japan). The MED probe contains 64 electrodes in an 8 × 8 grid with inter-electrode spacing of 150µm. MEA probes were loaded in MED-C03 chamber and the raw data were acquired using Mobius software (Alpha MED Scientific Inc., Ibaraki, Osaka, Japan). Signals from the amplifier were digitized at a rate of 20kHz and using highpass filter (cutoff frequency of 100 Hz). Supplemental Figure S1A shows single spike waveforms from preCGG hippocampal neurons at 21 DIV. Mobius software was used to detect spontaneous events that exceeded a threshold of -25 µV (Supplemental Fig. S1B), the typical peak-to-peak noise level of MEA electrodes was ±5–8 µV. The timestamps for each channel were then saved and exported to an excel sheet. For raster plot as well as spike rate and burst analysis, data were imported into the NeuroExplorer software (version 4.0, NEX Technologies, Littleton, MA) and analyzed using the raster or burst-firing analysis function. For activity to be defined as a burst, spikes had to occur within 350 ms of each other. In addition, a burst consisted of a minimum of four spikes with minimum burst duration of 100ms. The
minimum separation between bursts was set at 350 ms. Using this measurement, the burst duration is measured from the start of a burst until the gap to the next spike is more than 350 msec, so one repetitive burst may include multiple high frequency bursts (Supplemental Fig. S1C).

**Data analysis**

Graphing and statistical analysis were performed using GraphPad Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA). Statistical significance between different groups was calculated using Student’s t test or by an ANOVA and, where appropriate, a Dunnett’s Multiple Comparison Test. The p values below 0.05% were considered significant. Kinetic parameters (K_m and V_max) for glutamate uptake were determined by nonlinear regression analysis of the saturation curves using the Michaelis-Menten equation.
FUNDING
This project was supported by National Institutes of Health [grant numbers RC1 AG036022 to P.J.H. and I.N.P., UL1 DE019583 to P.J.H., RL1 AG032119 to P.J.H. and I.N.P., RL1 AG032115 to R.J.H, R01 ES011269 to I.N.P., R21 NS072094 to M.A.R.], Congressionally Directed Medical Research Programs [grant number W81XWH-09-1-0746 to M.A.R.], and an unrestricted research grant from the J.B. Johnson Foundation to I.N.P.

ACKNOWLEDGEMENTS
The authors wish to express their gratitude to the families who have supported research in developmental disorders. Drs. Rob Willemse (Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands) and Rob Berman (Department of Neurological Surgery, UC Davis) are gratefully acknowledged for providing the preCGG mice.

CONFLICTS OF INTEREST
Randi Hagerman has received funding from Novartis, Roche, Seaside therapeutics and Forest to carry out targeted treatment trial in those with FXS or autism. She is also on the FXS Treatment Advisory Board of Novartis. The other authors have no conflicts of interest to declare.
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FIGURE LEGENDS

Fig. 1 | Premutation cultures express higher levels of Fmr1 mRNAs with decreased FMRP proteins compared with WT paired cultures. (A) Representative western blot in paired cultures of WT and preCGG hippocampal astrocytes as well as neurons. The band with molecular weight around 72kDa is FMRP; (B) Quantification of FMRP expression level relative to β-actin in paired WT and preCGG cultures of hippocampal astrocytes, and 14 and 21 DIV neuronal cultures. Data were pooled from two independent cultures; (C) Fmr1 mRNA comparison between WT and preCGG paired cultures of hippocampal astrocytes and 14 as well as 21 DIV neurons. Data were pooled from two independent culture days each performed in duplicates.

Fig. 2 | Hippocampal neurons with CGG expansion predominantly display a pattern of spontaneous field potential activity having clustered burst (CB) firing. (A) Representative traces of firing for WT and preCGG hippocampal neurons at 7 DIV and 21 DIV in a 10 seconds epoch; (B) Representative raster plots for the neuronal firing of WT and preCGG hippocampal neurons at 7 DIV and 21 DIV; (C)&(D) Quantification of the spike frequency and mean burst duration for WT and preCGG hippocampal neurons at 7 DIV and 21 DIV. Summary data were from 26 MEAs for WT and 37 MEAs for preCGG hippocampal neurons.

Fig. 3 | Hippocampal neurons with CGG expansion predominantly display clustered Ca^{2+} oscillations. Representative intracellular Ca^{2+} oscillations in WT (A) and preCGG (B) hippocampal neurons measured at 14 DIV; (C) Quantification of the percentage of wells displaying clustered Ca^{2+} oscillations. These data were summarized from 5 independent cultures with a total number of wells of 71 and 93 for the WT and preCGG hippocampal neurons, respectively.

Fig. 4 | 21 DIV hippocampal neurons with CGG expansion display decreased expression levels of VGAT and VGLUT1. (A) Representative western blots for VGAT and VGLUT1. (B) Quantification of the expression levels for VGAT and VGLUT1. These data were normalized to the expression levels of VGAT or VGLUT1 on 7 DIV WT neurons. (C) Quantification of the ratio of VGAT over VGLUT1 expression level. (D) Quantification of the developmental change of VGAT and VGLUT1. These data were normalized to the expression levels of respective 7 DIV neurons for each genotype. N=4 from 3 separate
culture days.

Fig. 5 | Glutamate uptake in pure hippocampal astrocyte cultures. (A) Non-selective glutamate transporter inhibitors, DL-TBOA (1 mM) and l-trans-PDC (1 mM), blocked glutamate uptake in both WT and preCGG hippocampal astrocytes. These data were repeated in two cultures, in triplicate, with similar results. (B) Kinetic analysis for glutamate uptake in WT and preCGG hippocampal astrocytes. The experiments were repeated in 4 independent cultures performed in triplicate with similar results. Uptake as a function of glutamate concentration was fitted by the Michaelis-Menten equation. The \( V_{\text{max}} \) values for glutamate uptake do not show a statistically significant difference between WT and preCGG hippocampal astrocytes. However, the \( K_m \) value was shifted from 20.0±2.8µM for WT to 28.4±2.2µM for preCGG (n=4, \( p<0.01 \)).

Fig. 6 | Glutamate and TBOA induce clustered burst-firing pattern in WT hippocampal neurons. (A)&(D) Representative trace for firing activity before and after WT hippocampal neurons exposed to glutamate (100 µM) or TBOA (100 µM), respectively. (B)&(E) Representative raster plots for the neuronal firing before and after WT hippocampal neurons exposed to glutamate or TBOA, respectively. (C) &(F) Quantification of the spike frequency (left Y axis) and mean burst duration (right Y axis) for WT hippocampal neurons before and after glutamate or TBOA exposure respective. Data were repeated twice in duplicate with similar results.

Fig. 7 | DHPG produces clustered burst-firing pattern in WT hippocampal neurons. (A) Representative trace for firing activity before and after WT hippocampal neurons exposed to 10 µM DHPG. (B) Representative raster plots for the neuronal firing before and after DHPG exposure. (C) Quantification of the spike frequency (left Y axis) and mean burst duration (right Y axis) for WT hippocampal neurons before and after DHPG exposure. Data were repeated twice in duplicate with similar results.

Fig. 8 | Picrotoxin together with DHPG induces clustered burst-firing pattern in WT hippocampal neurons. (A) Representative firing traces before and after WT hippocampal neurons were exposed to Picrotoxin (100 µM) or a combination of picrotoxin (100 µM) and DHPG (10 µM), respectively. (B) Representative raster plots of the neuronal firing before and after picrotoxin or picrotoxin and DHPG exposure. (C) Quantification of the spike frequency (left Y axis) and mean burst duration (right Y axis) before and after exposure to picrotoxin or
picrotoxin and DHPG exposure. These data were repeated twice in two independent cultures with similar results.

Fig. 9 | MPEP and CPCCOEt reversed the clustered burst-firing pattern in preCGG hippocampal neurons in a concentration-dependent manner. (A) &(C) Representative raster plots for the neuronal firing before and after preCGG hippocampal neurons were exposed to MPEP or CPCCOEt, respectively. (B) &(D) Quantification of the spike frequency (left Y axis) and mean burst duration (right Y axis) for preCGG hippocampal neurons before and during exposure to different concentrations of MPEP and CPCCOEt, respectively. These data were repeated twice in duplicate with similar results.

Fig. 10 | Allopregnanolone reversibly reduced the spike frequency and burst duration of the preCGG hippocampal neurons. (A) Representative raster plots for the neuronal firing of preCGG hippocampal neurons before and during exposure to as well as washout of allopregnanolone. (B) Quantification of the spike frequency (left Y axis) and mean burst duration (right Y axis) for preCGG hippocampal neurons before and during exposure to as well as after washout of allopregnanolone, respectively. Data were repeated two times with independent cultures.
ABBREVIATIONS

CB, clustered burst
CPCCOEt, ethyl 7-hydroxyimino-1,7a-dihydrocyclopropa[b]chromene-1a-carboxylate
DIV, days in vitro
DHPG, (S)-3,5-dihydroxyphenylglycine
FLIPR, fluorescent laser plate reader
FMR1, fragile X mental retardation 1 gene
FMRP, fragile X mental retardation protein
FXS, Fragile X syndrome
FXTAS, fragile X-associated tremor/ataxia syndrome
Glu, glutamate
KI, knock-in
L-trans-PDC, L-trans-pyrrolidine-2,4-dicarboxylic acid
MEA, microelectrode array
mGluR, metabotropic glutamate receptor
MPEP, 2-methyl-6-(phenylethynyl)pyridine hydrochloride
FXPOI, Fragile X-associated primary ovarian insufficiency
TBOA, DL-threo-β-benzyloxyaspartic acid
VGAT, vesicular GABA transporter
VGLUT, vesicular glutamate transporter
WT, wild type
Fig. 1

(A) Western blot analysis showing FMRP and β-actin levels in astrocytes and neurons. The blots were probed with WT and preCGG antisera. The molecular weights of the bands are indicated in kilodaltons.  

(B) Bar graph showing the FMRP level (as a percentage of WT) in astrocytes and neurons. The significant differences (p<0.01) are indicated.  

(C) Bar graph showing the Fmr1 mRNA level (as a percentage of WT) in astrocytes and neurons. The significant differences (p<0.01) are indicated.
Fig 3

(A) WT

(B) preCGG

(C) % well with cluster oscillations

WT  preCGG

p<0.01
Fig. 4

A

VGLUT1
VGAT1
β-actin

WT preCGG WT preCGG
7 DIV 21 DIV

B

VGLUT1/β-actin

WT preCGG

7 DIV 21 DIV

C

VGAT/VGLUT1

WT preCGG

7 DIV 21 DIV

D

VGAT/β-actin (%DIV)

WT preCGG WT preCGG

7 DIV 21 DIV

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Fig. 5

A

L-glutamate uptake/30 min (nanomole/mg protein)

WT
preCGG

Ctrl
TBOA
PDC

Ctrl
TBOA
PDC

B

V (nmol/mg/min)

L-glutamate (μM)

WT
preCGG

0 25 50 75 100
Fig. 8

A

B

C

Spike frequency

Mean burst duration (s)

Basal PTX P+D

p<0.01

p<0.01

p<0.01

p<0.01

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Fig. 10

A

B

Mean burst duration (s)

Spike frequency

B

AlloP (uM)

AlloP (uM)

0.1 mM

0.1 mM

Wash

Wash

B

AlloP (uM)

AlloP (uM)

0.1 mM

0.1 mM

Wash

Wash

100 S