Bryostatin-1 restores hippocampal synapses and spatial learning and memory in adult fragile X mice

Miao-Kun Sun, Jarin Hongpaisan, Chol Seung Lim, and Daniel L. Alkon

Blanchette Rockefeller Neurosciences Institute, 8 Medical Center Drive
Morgantown, WV 26505
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Correspondence and requests for materials should be addressed to:
Miao-Kun Sun, Blanchette Rockefeller Neuroscience Institute, 8 Medical Center Dr., Morgantown, WV 26505, USA; Phone: 304-293-1701; Fax: 304-293-7536; E-mail: masun@brni.org.

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A list of nonstandard abbreviations: BDNF, brain-derived neurotrophic factor; FMR1, fragile mental retardation gene 1; FMRP, fragile mental retardation protein; FXS, fragile X syndrome; GSK, glycogen synthase kinase; HRP, horseradish peroxidase; mGluR, metabotropic glutamate receptor; PKC, protein kinase C; PSD, postsynaptic density

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Abstract

Fragile X syndrome (FXS) is caused by transcriptional silencing in neurons of the FMR1 gene product, fragile X mental retardation protein (FMRP), a repressor of dendritic mRNA translation. The lack of FMRP leads to dysregulation of synaptically driven protein synthesis and impairments of intellect, cognition, and behavior, a disorder which currently has no effective therapeutics. Fragile X mice were treated with chronic bryostatin-1, a relatively selective PKCε activator with pharmacological profiles of a rapid mGluR desensitization, synaptogenesis, and synaptic maturation/repairing. Differences in the major FXS phenotypes, synapses, and cognitive functions were evaluated and compared among the age-matched groups. Chronic treatment with bryostatin-1 rescues adult fragile X mice from the disorder phenotypes, including normalization of most FXS abnormalities in hippocampal brain-derived neurotrophic factor (BDNF) expression and secretion, PSD-95 levels, GSK-3β phosphorylation, transformation of immature dendritic spines to mature synapses, densities of the presynaptic and postsynaptic membranes, and spatial learning and memory. Our results show that synaptic and cognitive function of adult FXS mice can be normalized through pharmacological treatment and that bryostatin-1-like agents may represent a novel class of drugs to treat fragile X mental retardation even after postpartum brain development has largely completed.
Introduction

Fragile X syndrome (FXS) is the most common monogenically inherited form of intellectual disability (Kaufmann et al., 1999; Santoro et al., 2012). The FXS includes general deficits in cognition (Van der Molen and Van der Molen, 2005; Yang et al., 2013), abnormal in memory (Koekkoek et al., 2005), and anxiety and autistic-like behavior (Sabaratnam et al., 2003; Garber et al., 2008).

In nearly all the cases, FXS is caused by an expansion of an untranslated CGG repeat in the 5’ untranslated region of the X-linked gene fragile mental retardation 1 (FMR1; Verkerk et al., 1991; Ludwig et al., 2014). The expansion leads to DNA methylation of FMR1 and transcriptional silencing, thus loss of the fragile X mental retardation protein (FMRP). FMRP is a selective RNA binding protein (Schaeffer et al., 2013), regulating the translation of dendritic mRNAs (Ashley et al., 1993; Gonçalves et al., 2013; Darnell and Klann, 2013).

The lack of FMRP alters signal processing at synapses, such as that involving metabotropic glutamate receptor (mGluR) signaling (Weiler et al., 2007; Lüscher and Huber, 2010; Bhattacharya and Klann, 2012). The leading ‘mGluR theory of FXS’ proposes that overactive mGluR signaling, normally balanced by FMRP, underlies much of the brain changes of FXS (Bhogal and Joegens, 2010; McLennan et al., 2011; Santoro et al., 2012; Hajós, 2014). Indeed, a mouse model that combines FMR1 inactivation with a 50% reduction in mGluR5 expression rescues several anatomic and behavioral consequences of FXS. mGluR5 antagonism has also been shown effective in several FXS phenotypes (Connor et al., 2011; Veloz et al., 2012; Hajós, 2014).

One of the major goals of FXS research is to develop effective therapies. Despite extensive efforts, therapeutic options for FXS remain limited. Whilst preclinical studies with mGluR5
antagonism appear promising, therapeutic values of mGluR inhibitors for FXS are still not clear. One problem with inhibition of the mGluR signaling, for instance, is that the inhibition exaggerates spine immaturity in the fragile X mice (Cruz-Martín et al., 2010), an effect opposite to the intended therapeutic outcomes. Here, we show that bryostatin-1 (Nelson et al., 2009; DeChristopher et al., 2010), a highly potent and relatively specific PKCε activator (of also PKCα), with pharmacological profiles of rapid mGluR desensitization, synaptogenesis, and synaptic maturation/repairing (Hongpaisan and Alkon, 2007, 2011; Sun et al., 2008), rescues synaptic and memory functions and other phenotypic features in adult fragile X mice. Bryostatin-1 has a much lower ED$_{50}$ for inducing PKCε translocation than its ED$_{50}$s for PKCα or PKCδ (Szallasi et al., 1994). The structural features of the bryostatins have been well defined by leading experts in function-oriented synthesis (Wender et al., 1988, 2011). Bryostatin-1-like agents may represent a novel class of drugs for treating FXS.
Methods and Materials

Animals and drug treatment

Two types of mice (male, The Jackson Laboratories, ME, USA; 9-10/group) were used: FVB.129P2-Pde6b\(^+\) Tyr\(^{c-ch}\) Fmr1\(^{tm1Cgr}\)/J (formerly as FVB.129P2-Fmr1\(^{tm1Cgr}\)/J; or fragile X mice) and FVB.129P2-Pde6b\(^+\) Tyr\(^{c-ch}\)/AntJ (as the controls). These mice do not suffer from blindness. They were housed in a temperature-controlled (20-24°C) room for at least a week, allowed free access to food and water, and kept on a 12-hour light/dark cycle.

All mice were randomly assigned to different groups. We began to treat the fragile X mice when they reached an age of 2 months with bryostatin-1 (20 \(\mu\)g/m\(^2\), tail i.v., 2 doses/week for 13 weeks). The dose was chosen, based on our preliminary dose-response studies that smaller doses were not effective against disorders-induced synaptic and cognitive impairments. Non-treated groups received the same volume of vehicle at the same frequency. Synaptic and memory functions and other phenotypic features were evaluated 10 days after the last dose.

Total RNA isolation and Reverse transcription-polymerase chain reaction

Bilateral hippocampi were dissected (n=5) for total RNAs isolation, using an RNeasy mini kit (Qiagen). RNA purity was confirmed by spectrophotometry (\(A_{260}/A_{280}>1.8\)) and RNA integrity was visualized by agarose gel electrophoresis. For Reverse transcription (RT) reaction, 500 ng of total RNA was reverse-transcribed, using oligo(dT) primer and Superscript III (Invitrogen) at 50°C for 1 hour. Real- time PCR was performed for 40 cycles with SYBR Green I PCR master mixture and processed on LightCycler 480 II (Roche) machine. The following primers (Origene) were used: mouse BDNF, 5’-GGCTGACACTTTTGAGCACGTC-3’ and 5’-CTCCAAAGGCACTTTGACTGCT-3’ and mouse GAPDH, 5’-
TGAACGGGAAGCTCACTGGCAT-3’ and 5’-TCAGATGCCTGCTTCACCACCT-3’ as a control. Threshold cycle (Ct) for BDNF was normalized on the house keeping GAPDH (dCt) and every experiment sample was referred to its control (ddCt). Relative change values were expressed as $2^{-\text{ddCt}}$.

**Western Blot analysis**

Bilateral hippocampi ($n=5$) were homogenized in cold lysis buffer [10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 1% SDS, and 1x complete protease inhibitor cocktail (Thermo Scientific)]. After protein concentration measurement with the BCA method, the same amount of proteins (25 µg/lane) was then separated, using 18% (for BDNF) or 8% [for FMRP, PSD-95, GSK-3β, phospho-GSK-3β (Ser9) and α-tubulin] SDS-PAGE gel and transferred onto nitrocellulose membranes (Invitrogen). The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline plus 0.1% Tween 20 (TBS-T) for 1 h and then incubated with rabbit anti-BDNF (Santa Cruz Biotech., 1:500), rabbit FMRP (Cell Signaling, 1:1,000), mouse anti-PSD-95 (Santa Cruz Biotech., 1:1,000), rabbit GSK-3β (Cell Signaling, 1:1,000), rabbit phospho-GSK-3β (Ser9) (Cell Signaling, 1:1,000) for in TBS-T for overnight at room temperature. After 3 times of washing with TBS-T, the membranes were incubated with Horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Lab, 1:5,000) in TBS-T for 1 h at room temperature and immunoreactive bands were visualized by SuperSignal West Pico chemiluminescent substrate. To control for loading differences across lanes, membranes were stripped and reprobed using mouse anti-α-tubulin (Sigma, 1:5,000) and HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Lab, 1:5,000). Levels of
immunoreactivity were assessed by densitometric analysis of films, using the ImageJ 1.44a system, and normalized to α-tubulin levels.

**Enzyme-linked immunosorbent assay (ELISA)**

Bilateral hippocampi were homogenized in ice cold homogenization buffer consisting of 20 mM Tris-Cl (pH 7.4) buffer containing 137 mM NaCl, 1% Triton X-100, 10% glycerol, and 1X protease inhibitor cocktail (Thermo Scientific) and used for measuring BDNF. The homogenates were centrifuged at 14,000 x g for 30 min. From the resulting supernatants, total BDNF amounts were measured with the mouse BDNF ELISA technique (Insight Genomics).

**Confocal microscopy for dendritic spine morphology**

Mice were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused through the heart by gravity with phosphate buffered saline (PBS) at room temperature. They were then perfused with 4% paraformaldehyde in PBS (20 ml for lightly fixed) at room temperature to avoid the negative impact of hypothermia on the number of dendritic spines (Kirov et al., 2004). Right dorsal hippocampi were dissected and sectioned with a vibratome (Leica VT1000S). A series of sections were selected from the right dorsal hippocampi by starting at approximately 1200 μm and selecting 1 section every 400 μm.

The number of distinct shapes of dendritic spines on individual dendritic shafts was studied with 1,1'-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes/Invitrogen, Carlsbad, CA, USA) staining, as previously described (Hongpaisan and Alkon, 2007).
Dendritic spines, in the stratum radiatum, were imaged (>510 nm/568 nm; excitation/emission) with a Zeiss Axio Observer Z1 microscope equipped with 710 confocal scanning system, using 100x Plan-APO Chromat oil immersion objective (1.4 NA). A series of randomized confocal images (1024 pixels x 1024 pixels) were confocally scanned every 0.45 μm. Individual spines identified on a single image were verified on adjacent images of series of confocal images in order to approximate the 3-dimensional structure of the spines with the Image J program (http://rsb.info.nih.gov/ij/). The criteria to differentiate the morphology of dendritic spines were based on the relative ratio between spine head and neck length, as described by Sorra and Harris (2000).

Densities of pre- and postsynaptic structures and BDNF immunohistochemistry

For immunohistochemical studies, hippocampal sections were incubated free-floating overnight at room temperature with primary antibodies (Millipore/Chemicon, Billerica, MA, USA): mouse monoclonal anti–growth-associated protein (GAP)-43/B-50 (1:2,000), polyclonal rabbit anti-neurogranin (1:400), and mouse monoclonal anti-synaptophysin (1:2000). Tissue sections were then incubated with either Alexa Fluor 568 goat anti-rabbit IgG (1:200; Invitrogen/Molecular Probes) for 3 h at room temperature or biotinylated anti-mouse secondary antibody (1:20; Vector Laboratories, Burlingame, CA, USA) for 3 h at room temperature and then streptavidin-conjugated Alexa Fluor 488 (1:100; Invitrogen/Molecular Probes) for 3 h at room temperature. Sections were mounted with VECTASHIELD mounting medium (Vector Laboratories) with DAPI to counterstain nuclei.

Confocal images (Zeiss confocal microscope, 63x objectives) were acquired of hippocampal sections between 0.6 μm thick, in-line scan mode, and with a pinhole of approximately 1.00 Airy
unit. Confocal images with similar levels of DAPI fluorescence intensity were quantified with the ImageJ program.

The densities per 63 x 63 x 0.6 μm$^3$ volume of the CA1 stratum radiatum (where apical dendrites of CA1 pyramidal neurons are located) of postsynaptic membranes (neurogranin grains), presynaptic membranes (GAP-43 grains), presynaptic axonal terminals (the presynaptic vesicle membrane protein synaptophysin grains), and presynaptic vesicle concentration (synaptophysin fluorescence intensity) were measured. The microscope lateral resolution was 20 nm in XY and 100 nm in Z, and its point-spread function (PSF) was automatically collected by the microscope software. By collecting confocal images at 0.6 μm thickness, the overlap of synaptic membranes or axonal boutons was minimized.

**Electron microscopy**

Electron microscopy was used to evaluate synaptic details. Mice at an age of 2 months were treated with bryostatin-1 (25 μg/kg, i.p., twice/week) for 5 weeks. Under anesthesia (pentobarbital, 80 mg/kg, i.p.), mice were perfused through the left cardiac ventricle with phosphate buffered saline (PBS) by gravity and then fixed with 2.5% glutaradehyde and 3% paraformadehyde in PBS at room temperature. Right dorsal hippocampi were dissected and sectioned with a vibratome at 400 μm thickness. The hippocampal sections were re-sectioned to 100 μm thickness and washed three times with cold PBS and post-fixed in cold 1% OsO4 for 1 hour and then rinsed with cold distilled water. Hippocampal slices were dehydrated in a cold, graded ethanol series. Sections were followed by resin embedding at room temperature. Ultrathin sections (70 nm) were stained with uranyl acetate and lead acetate and viewed at Zeiss Libra 120+electron microscope. Random sampling to determine synaptic density was achieved by
orienting the hippocampal CA1 area under low-power magnification. The random area that immediately appeared after switching to a higher magnification (8,000X magnification and a pixel resolution of 266.859 pixels/micron) was imaged with a CCD camera (UltraScan, Gatan, Pleasanton, CA).

During double blind quantification, electron micrographs (64 μm² CA1 area at 8,000 X) were digitally zoomed up to 16,000 X magnification by the Preview program in an Mac Pro computer with an Mac OS X operating system and a 30” monitor. Spines were defined as structures that do not have mitochondria and form synapses with axon boutons that contain presynaptic vesicles.

**Spatial learning and memory and visible platform test**

A modified water maze task (2 trials/day for 8 days), a difficult task for revealing mild impairments, was used to evaluate spatial learning and memory.

The training of water maze task began on the 10th day after the last dose of bryostatin-1, a time gap to separate potential acute effects of the agent from the chronic therapeutic impacts. The maze pool had a diameter of 114 cm and height of 60 cm and was filled with 40 cm H₂O (22±1°C), mixed with 200 ml of non-toxic white Tempera (BesTemp, Certified Color Corp, Orange, CA). Mice were trained to find a hidden platform, centered in one of the quadrants and submerged about 2 cm below the water surface. At the start of all trials, mice were placed individually in the water facing the maze wall, using different starting positions each trial, and allowed to swim until they find the platform, where they remained for 20 s. A mouse that failed to find the platform within 1.5 min was guided there by the investigator, with 90 s scored. The swim path was recorded with a video-tracking system. After the training trials, a probe trial was given, 24 hours after the last training trial, with the platform removed to assess memory retention.
for its location by the distance the mouse moved in the quadrants. The video-tracking system tracked the animal’s movements in each quadrant during a period of one minute.

A visible platform test was used to evaluate sensorimotor ability of the mouse. The platform was placed at a new location and was marked with a pole that protruded 9 inches above the water surface. The escape latency and the route of mouse’s swimming across the pool to the visible platform were recorded with the video-tracking system.

Statistical analysis was performed using the analysis of variance (ANOVA), followed by Newman-Keuls multiple comparisons test. $P<0.05$ was considered statistically significant. All procedures were conducted according to National Institutes of Health Animal Care and Use Committee guidelines and approved by the Ethical Committee of the Institute.
Results

Bryostatin-1 increases BDNF levels, PSD-95, and GSK-3β phosphorylation

FMRP only highly expressed in the wild type, but not in the fragile X mice (Fig. 1A). Bryostatin-1 had no significant impact on its expression (Fig. 1A).

The quantitative RT-PCR study revealed that the hippocampal BDNF mRNA levels did not differ significantly between the fragile X mice and the wild type. Bryostatin-1 increased the BDNF mRNA level by about 2.1-fold in the wild type ($F_{1,58} =19.64$, $p<0.001$) and by about 2-fold in the fragile X mice ($F_{1,58} =17.23$, $p<0.001$; Fig. 1B).

The BDNF protein levels in hippocampal extracts were measured with Western Blot analysis. Bryostatin-1 increased production of the 14 kDa BDNF form by about 45% in the wild type ($F_{1,28} =4.727$, $p<0.05$) and 44% ($F_{1,28} =4.59$, $p<.05$) in the fragile X mice, respectively (Fig. 1C). Using sandwich ELISA method, no significant differences were found between the untreated groups, but bryostatin-1 enhanced BDNF production by about 2.43-fold ($F_{3,156} =8.682$, $p<0.001$; Fig. 1D). Using immunohistochemistry and confocal microscopy, we also observed significant group differences in BDNF expression (Fig. 1E; $F_{3,114} =3.633$, $p<0.01$). Bryostatin-1 restored BDNF levels in the hippocampal CA1 stratum radiatum in the Fragile X mice (Fig. 1F).

We determined expression of PSD-95, a postsynaptic marker protein, to explore whether bryostatin-1 can rescue functional synapses. PSD-95 expression decreased in the hippocampus of the fragile X mice by 34% ($F_{1,28} =4.289$, $p<0.05$, vs. the wild type; Fig. 1G). Bryostatin-1 enhanced PSD-95 expression by about 51% ($F_{1,28} =4.935$, $p<0.05$) and rescued the PSD-95 levels in the fragile X mice ($F_{1,28} =7.793$, $p<0.01$; Fig. 1G).

GSK-3β is highly active in FXS and is one of the core phenotypes of FXS. There were no group differences in the total GSK-3β levels. However, levels of phosphorylation of GSK-3β on
Ser9 were lower by about 33% in the hippocampus of the fragile X mice ($F_{1,28} = 9.884, p<0.01$; Fig. 1H), indicating that GSK-3β was more active in the hippocampus of the fragile X mice. Bryostatin-1 increased phosphorylation of GSK-3β (Ser9) by about 35% ($F_{1,28} = 10.43, p<0.01$) in the wild type and totally rescued phosphorylation of GSK-3β (Ser9) in the hippocampus of the fragile X mice ($F_{1,28} = 11.96, p<0.01$).

**Bryostatin-1 prevents the loss of mature dendritic spines and pre- and postsynaptic membranes and presynaptic vesicles in the fragile X mice**

After water maze learning, dendritic spines on individual apical dendritic shafts of the CA1 pyramidal neurons (stratum radiatum) were stained with DiI and imaged with confocal microscopy by serial scanning, 3D reconstruction, and a double-blind analysis (unknown subject and treatment, Fig. 2A). There was a significant overall group difference for mushroom-shaped spine density ($F_{3,94} = 2.959, p<0.05$; Fig. 2B) and all shape dendritic spine (mushroom, thin, and stubby spines together; $F_{3,94} = 4.245, p<0.01$; Fig. 2C). Both mushroom-shaped spine ($p<0.05$) and all shape dendritic spine ($p<0.01$) densities were significantly lower in the fragile X mice (Fig. 2B,C). A significant overall group difference was found for the density of immature dendritic spines ($F_{3,94} = 8.347, p<0.001$; Fig. 2A,D). We found a significant ($p<0.001$) increase in immature dendritic spine density in the fragile X mice, vs. the wild-type (Fig. 2D).

Bryostatin-1 significantly reduced the density of immature dendritic spines ($p<0.01$; Fig. 2D), but increased the density of mushroom and all dendritic spines ($p<0.01$; Fig. 2B,C), suggesting that bryostatin-1 enhanced the transformation of immature dendritic spines to the mature dendritic spines.
Immunohistochemistry and confocal microscopy were used to study the densities of presynaptic membranes (GAP-43 grains) and postsynaptic membranes (neurogranin grains) in the CA1 stratum radiatum (Fig. 2E). An ANOVA analysis revealed a significant overall group difference for presynaptic membranes ($F_{3,139} = 4.753$, $p<0.01$; Fig. 2F) and postsynaptic membranes ($F_{3,167} = 7.713$, $p<0.001$; Fig. 2G). Compared with the wild-type, the fragile X mice had lower density of presynaptic membranes (Fig. 2F; $p<0.01$) and postsynaptic membranes (Fig. 2G; $p<0.001$) that were reversed by bryostatin-1 ($p<0.05$).

The presynaptic vesicle membrane protein synaptophysin was used as a marker for presynaptic axon boutons (Graham and Redman, 1994). At the resolution of the confocal microscope, one synaptophysin granule equals one presynaptic axonal bouton/terminal (Fig. 2H). No significant group differences were found for the number of synaptophysin-labeled endings (Fig. 2I), suggesting that the number of the axon boutons was not affected by the loss of FMRP or bryostatin-1.

The concentration of presynaptic vesicles was studied by measuring fluorescence intensity of the presynaptic vesicle membrane synaptophysin. There was a significant overall group difference for synaptophysin intensity ($F_{3,122} = 3.431$, $p<0.01$; Fig. 2J). The concentration of presynaptic vesicles decreased in the fragile X mice, vs. the wild-type ($p<0.01$). In the fragile X mice treated with bryostatin-1, the presynaptic vesicle density was restored ($p<0.01$) and reached a level higher ($p<0.01$) than that in the wild-type.

Density of synapses in the hippocampal CA1 stratum radiatum was also studied with electron microscopy (a double-blind analysis). There were significant overall group differences for synaptic density ($F_{2,114} = 2.968$, $p<0.05$; Fig. 3A,B) and presynaptic vesicle concentration within axonal boutons ($F_{2,114} = 8.121$, $p<0.001$; Fig. 3C,D). The fragile X mice had lower synaptic densities compared to the wild-type, and these differences were reversed by bryostatin-1 ($p<0.05$).
density (Fig. 3A,B; \( p<0.05 \)) and presynaptic vesicle concentration (Fig. 3C,D; \( p<0.01 \)) vs. the wild-type. Bryostatin-1 reversed the decrease in synaptic density in the fragile X mice (Fig. 3A,B; \( p<0.05 \)). With bryostatin-1, the reduction of presynaptic vesicle number was not only rescued (\( p<0.001; \) the fragile X mice+bryostatin-1 vs. the fragile X mice) but also enhanced (\( p<0.05; \) the fragile X mice+bryostatin-1 vs. the wild type; Fig. 3C,D), confirming the results in Fig. 2J.

As one dendritic spine may form more than one synapse, we further assessed the density of all dendritic spines, using an antibody against the dendritic spine-specific protein spinophilin (Fig. 3E). A significant group difference was found \( (F_{3,149}=15.273, p<0.001) \). Bryostatin-1 increased dendritic spine density in the fragile X mice (\( p<0.001; \) Fig. 3F), suggesting that the bryostatin-1-induced increase in synaptic density is correlated with an increase in the number of dendritic spines rather than an increase in multiple synapses of preexisting dendritic spines.

**Bryostatin-1 restores spatial learning and memory of the fragile X mice but did not alter sensorimotor ability**

There were significant group differences in learning \( (F_{3,623}=5.214, p=0.001; \) Fig. 4A). Bryostatin-1 significantly improved the learning of the fragile X mice (the fragile X mice vs. the fragile X mice with bryostatin-1: \( F_{1,319}=15.556, p<0.001 \)), to the level of the controls (the wild-type vs. the fragile X mice with bryostatin-1: \( F_{1,319}=0.827, p>0.05 \)), indicating that bryostatin-1 repaired the learning of the fragile X mice.

The results in the probe test (Fig. 4B-E) were analyzed using the target quadrant ratio (dividing the target quadrant distance by the average of the non-target quadrant values during the probe test; Fig. 4F). There were significant group differences in the ratio \( (F_{3,38}=3.016, p<0.05) \),
indicating differences in the spatial memory. Detailed analysis reveals that bryostatin-1 significantly improved the memory recall in the fragile X mice vs. the mice without bryostatin-1 ($F_{1,19} = 6.640, p < 0.05$), to the level of the control (the wild-type vs. the fragile X mice+bryostatin-1: $F_{1,15} = 0.028, p > 0.05$).

A visible platform test, determined after the probe test, revealed no significant group differences ($F_{3,38} = 1.042, p > 0.05$; Fig. 4G), indicating that there were no group differences in sensorimotor ability and escape motivation.
Discussion

The results of the present study reveal a novel pharmacological profile of bryostatin-1 (DeChristopher et al., 2010; Nelson et al., 2008) for treating FXS after the FXS phenotypes have already been established in the fragile X mice (Bhattacharya and Klann, 2012). Bryostatin-1 rescues the hippocampus from the FXS phenotypes, including a decrease in PSD-95, a decrease in GSK-3β phosphorylation, a decrease in density of presynaptic and postsynaptic membranes, an increase in immature synapses, a decrease in learning-induced mature synapses, and an impairment of hippocampus-dependent spatial learning and memory. Learned-induced formation of dendritic spines is severely impaired in the fragile X mice (Padmashri et al., 2013), probably involving a loss of some dendritic channels (Routh et al., 2013), but can be rescued with bryostatin-1 treatment.

FMRP is an mRNA-binding translation regulator that mediates activity-dependent control of synaptic structure and function (Niere et al., 2012). Its lack results in an overactivity of the mGluRs, a decreased GABAergic system or delayed developmental switch in GABA polarity (D’Hulst et al., 2006; Olmos-Serrano et al., 2010; He et al., 2014), and an elevated activity of GSK3β (Guo et al., 2012). Potential therapeutics, therefore, include mGluR inhibitors (Veloz et al., 2012), GABAergic enhancers (Olmos-Serrano et al., 2010; D’Hulst and Kooy, 2007; Paluszkiewicz et al., 2011; Heulens et al., 2012), and inhibitors of GSK3β (Guo et al., 2012; Yuskaitis et al., 2010). Since intellectual ability, as well as retardation (Wang et al., 2010), involves multiple players in signal processing, bryostatin-1-like agents, for their multi-targeting actions, may represent a more effective class of therapeutics than agents that target a single factor in this complex pathologic process (Vislay et al., 2013).
First, some PKC isozymes play an essential role in various phases and types of learning and memory (Alkon et al., 2005). PKC activators not only increase activity of PKC isozymes and thereby restore PKC signal activity, including neurotrophic activity (Alkon et al., 2007; Sun et al., 2008), synaptic/structural remodeling, and synaptogenesis, but also reduce the accumulation of neurotoxic amyloid (Alkon et al., 2007; Hongpaisan et al., 2011), which may play some pathological roles in the fragile X mice (Westmark et al., 2011). The effects of bryostatin-1 on disorder-induced learning and memory impairments are evidenced as in the present study in that it fully rescued the spatial learning and memory in the fragile X mice. The results are consistent with an earlier observation that infusion of BDNF restored synaptic function in slices from the fragile X mice (Lauterborn et al., 2007). PKCα,ε enhances BDNF expression and via the mRNA stabilizing protein HuD increases expression of other synaptogenic factors, such as, GAP43, IGF, NTF, and NGF (Nelson et al., 2008).

Second, PKCε and other isoforms are known to phosphorylate mGluR5 at multiple sites, inducing a relatively rapid form of desensitization (Gereau and Heinemann, 1998), an action that directly targets the mGluR-mediated overactivity.

Third, bryostatin-1 effectively increases the hippocampal PSD-95 levels in the fragile X mice. The mGluR5 dysfunction includes an altered Homer scaffold interaction (Ronesi et al., 2012). Consistent with other’s finding, the synaptic scaffold protein PSD-95 was downregulated in the hippocampus, probably owing to an alteration in mRNA stability (Zalfa et al., 2007).

Fourth, bryostatin-1 rescues the GSK-3β level to that of the wild type. GSK-3β is a core component of FXS pathology (Yuskaitis et al., 2010) and is mainly regulated by phosphorylation on an N-terminal serine, Ser-9-GSK3β, for its inhibition. This inhibitory serine-phosphorylation of GSK-3 is impaired in the fragile X mice (Min et al., 2009) but can be rescued by bryostatin-1.
Fifth, bryostatin-1 induces transformation of immature dendritic spines to mature synapses in the hippocampus of the fragile X mice and thus avoids the undesired impact of a pure mGluR inhibition on spine immaturity (Cruz-Martin et al., 2010). Bryostain-1 was administered at a dose at which it promotes learning–induced synaptogenesis and synaptic maturation (Hongpaisan and Alkon 2007, 2011). Mice lacking expression of \textit{FMR1} show an abundance of dense, immature dendritic spines (Scott-Lomassese et al., 2011), as in FXS patients (Grossman et al., 2006). The hyper-abundance of immature-looking lengthened dendritic spine could be the results of a failed/delayed maturation (Cruz-Martin et al., 2010; Harlow et al., 2010) and activity-dependent synaptic elimination (Pfeiffer et al., 2010).

In short, bryostatin-1-like agents (Sun and Alkon, 2005; Nelson et al., 2009; DeChristopher et al., 2010) may have important therapeutic value for the treatment of adult FXS.

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\textbf{Authorship Contributions:}

\textit{Participated in research design:} Sun, Hongpaisan, Lim, and Alkon.

\textit{Conducted experiments:} Sun, Hongpaisan, and Lim.

\textit{Performed data analysis:} Sun, Hongpaisan, and Lim.

\textit{Wrote or contributed to the writing of the manuscript:} Sun, Hongpaisan, Lim, and Alkon.
References


Yang JC, Simon C, Niu YQ, Bogost M, Schneider A, Tassone F, Seritan A, Grigsby J,
Hagerman PJ, Hagerman RJ, and Olichney JM (2013) Phenotypes of hypofrontality in older
female fragile x premutation carriers. Ann Neurol in press.

Yuskaitis CJ, Mines MA, King MK, Sweatt JD, Miller CA, and Jope RS (2010) Lithium
ameliorates altered glycogen synthase kinase-3 and behavior in mouse model of fragile X

Zalfa F, Eleuteri B, Dickson KS, Mercaldo V, De Rubeis S, di Penta A Tabolacci E, Chiurazzi P,
Neri G, Grant SG, and Bagni C (2007) A new function for the fragile X mental retardation
Figure Legends

Figure 1. Bryostatin-1 does not alter FMRP but increases BDNF expression/level, PSD-95, and inhibitory phosphorylation of GSK-3β in the hippocampus. A. Western Blot analysis using anti-FMRP antibody. Quantitative data showing each band-intensity normalized to α-tubulin staining in the WC group. B. RT-qPCR analysis with specific primers against BDNF and GAPDH as the control. C. Western Blot analysis showing BDNF level, normalized to α-tubulin levels for the same Western blot lane. D. BDNF ELISA assay. E. Confocal microscopy of BDNF in hippocampal CA1 stratum radiatum, showing high level of BDNF immunofluorescence in the long profile of dendritic shafts. F. Bryostatin-1 rescued a decrease in BDNF (N=28-49 hippocampal CA1 areas). G. Western Blot analysis showing PSD-95 level. H. Western Blot analysis showing GSK-3β and phospho-GSK-3β (Ser9) levels. All data are presented as means±SEM from 3-4 animals. The same abbreviations are used in this and the figures that follow: WC, wild type with vehicle; WB, wild type with bryostatin-1; TC, fragile X mice with vehicle; TB, fragile X mice with bryostatin-1; n.s., not significant; *, p<0.05; **, p<0.01; ***, p<0.001; #, p<0.01, compared with TC.

Figure 2. Bryostatin-1 prevents the loss of mature dendritic spines and an increase in immature spines and protects the losses of pre- and postsynaptic membranes and presynaptic vesicles in the fragile X mice. A. Confocal microscopy of dendritic spines stained with DiI (1,1‘-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate). B. Quantitatively analysis of the numbers of mushroom-shape dendritic spines. C. All mature dendritic spines (mushroom, thin, and stubby spines together). D. Immature dendritic spines per 100 μm dendritic shaft. E. Confocal microscopy of the immunohistochemistry of the presynaptic membrane marker growth-
associated protein-43 (GAP-43) and the postsynaptic membrane marker neurogranin. Bryostatin-1 prevented decreases in (F) pre- and (G) postsynaptic membranes. H. Confocal microscopy and immunohistochemistry of the presynaptic vesicle membrane protein synaptophysin. I. The number of presynaptic axonal boutons (synaptophysin grains). J. Effects of bryostatin-1 on the presynaptic vesicle concentration (synaptophysin fluorescence intensity) in axonal boutons. Data are shown as means ± SEM. *: p<0.05, **, p<0.01; ***: p<0.001. N=21-34 dendritic shafts or 28-61 hippocampal CA1 areas per experimental conditions from 3-6 animals. For simplicity, illustration of WB was not shown.

**Figure 3.** Bryostatin-1 restores the number of synapses in fragile X mice. (A) Electron microscopy (EM) of the stratum radiatum in the right dorsal hippocampal CA1 area. Dendritic spines are highlighted in yellow. B. EMs at higher magnification show dendritic spines forming synapses (red arrows) with presynaptic axonal boutons that contain presynaptic vesicles (yellow arrows). Bryostatin prevented the loss of synaptic density (A, B) and presynaptic vesicle concentration (C, D) within 8 µm x 8 µm x 0.07 µm of the CA1 stratum radiatum. E. Confocal microscopy of dendritic spines immunostained with the dendritic spine-specific protein spinophilin in hippocampal CA1 stratum radiatum revealed that (F) bryostatin increased the maturation of dendritic spine. Data are shown as means±SEM. ***: p<0.001. N=38-61 hippocampal CA1 areas per experimental conditions from 3 animals. For simplicity, illustration of WB was not shown.

**Figure 4.** Bryostatin-1 restores spatial learning and memory in fragile X mice. A. Water maze learning. Data are shown as means±SEM, using the daily 2 trials as a block. B-F. Results of the
probe tests after the training trials shown as the distance in each quadrant (B-F). The quadrant 4 was the target quadrant. F shows target quadrant ratio. G shows the escape latency during the visible platform test. *: $p<0.05$; NS: $p>0.05$. 
Figure 1
Figure 2
Figure 3
Figure 4

A: Escape latency (s) over days 1 to 8 for different groups (WC, WB, TC, TB).

B: Swimming distance in quadrant 4 for WC group.

C: Swimming distance in quadrant 4 for TC group.

D: Swimming distance in quadrant 4 for WB group.

E: Swimming distance in quadrant 4 for TB group.

F: Target quadrant ratio comparison among WC, WB, TC, and TB groups.

G: Escape latency comparison among WC, WB, TC, and TB groups.