# Analyses of CMD X and CMD UUU on Audiogenic Seizure and Dendritic Spine Phenotypes in the Mouse Model of Fragile X Syndrome.

## External_############

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|---|---|
| **Report date:** | XXXX |
| **Study performed during:** | XXXX<br>XXXX |
| **Author(s):** | |
| **Sponsor:** | COMPANY Pharmaceuticals |
| **Performing Laboratory:** | NeuroDetective International network laboratory |
| **Keywords:** | Fragile X syndrome, Fmr-1 KO mice, amyloid-beta precursor protein, amyloid beta, audiogenic seizure |

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# 1 Authentication

I, the undersigned, hereby declare that this work was performed under my direction and the study was conducted according to the procedures herein described and this report represents a true and accurate record of the results obtained. I, the undersigned declare, that the following report constitutes a true and faithful account of the procedures adopted, and the results obtained in the performance of this study. This study was formally exempt from compliance of Good Laboratory Practice regulations. However, the principles and procedures of the Good Laboratory Practice were followed to the maximal possible extend.

| | | |
|---|---|---|
| PI<br>Study Author | Date | Signature |

Hitherto, we accept this final report in its present form.

| | | |
|---|---|---|
| Dr. AAA<br>Study Coordinator | Date | Signature |
| Dr. BBB<br>Director, In vivo Pharmacology | Date | Signature |

# 2 Personnel Involved

| | |
|---|---|
| **Study Director** | |
| **Project performed by:** | |
| | |
| **Responsible for animal care:** | |

# 3 Summary

The efficacy of COMPANY compounds CMD UUU (batch #########) and CMD X (batch #TTT) was tested in *in vitro* and *in vivo* assays. The *in vitro* assays included a dose response assessment of AβPP expression and dendritic spine length and density. CMD UUU significantly reduced dendritic expression of AβPP at concentrations 0.25 μM, 0.5 μM and 1.0 μM. CMD X significantly reduced AβPP expression at 0.0625 μM, 0.25 μM, 0.5 μM, 1.0 μM and 2.5 μM. MPEP was not effective in this assay contrary to our previous results that demonstrated a 40% decrease in dendritic AβPP levels with MPEP treatment. The lack of effect with MPEP could be due to the difference in solvents used to dissolve/suspend the drug. Both CMD UUU and CMD X significantly decreased dendritic spine length by two-fold with 15 and 75 min treatment at 0.25 μM. The 0.25 μM dose was chosen as the lowest common effective dose so that the two drugs could be compared over time. The CMD X was effective with the 5 min treatment, whereas the CMD UUU was not. Neither COMPANY drug significantly affected spine density. Both drugs reduced the percentage of immature spines (filopodia) by 1.8-2.4-fold. The *in vivo* analyses included a dose assessment of audiogenic seizure (AGS) activity. Both compounds were effective at reducing wild running (WR) and AGS and death rates in *Fmr1*$^{KO}$ mice at 3 and 10 mg/kg. Neither compound was effective at 1 mg/kg. MPEP was effective at both 10 and 30 mg/kg. In conclusion, both CMD UUU and CMD X were effective at attenuating *in vitro* and *in vivo* phenotypes associated with Fragile X syndrome in a mouse model of the disorder.

# 4 Introduction

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and the leading known genetic cause of autism (Hagerman, 2002). Fragile X mental retardation protein (FMRP) is absent or expressed at substantially reduced levels in FXS. There is great interest in determining how the loss of FMRP causes FXS phenotypes at the cellular, molecular and system level. FMRP is a messenger RNA (mRNA) binding protein that represses the translation of a subset of dendritic mRNAs whose products affect synaptic plasticity and function. Metabotropic glutamate receptor (mGluR) signaling causes pulsatile translation of post-synaptic mRNAs by temporarily blocking FMRP. *Fmr1*$^{KO}$ mice, which lack FMRP expression, exhibit many of the physical and behavioral characteristics of humans with FXS including lower seizure thresholds, abnormal dendritic spine morphology, macroorchidism, abnormal anxiety-related responses and cognitive impairment. Thus, they are an ideal model system to study the effects of therapeutic interventions on FXS phenotypes. Inhibition of $mGluR_5$ has been proposed as a therapeutic treatment for FXS (Bear, 2004).

FMRP binds to and controls the postsynaptic translation of amyloid beta precursor protein (AβPP) mRNA. Activation of group 1 mGluR signaling with DHPG leads to the release of the translational repressor FMRP from *App* mRNA accompanied by increased AβPP synthesis, which can be blocked by the $mGluR_5$ antagonist MPEP. AβPP is cleaved to beta-amyloid (Aβ) and soluble AβPP (sAβPP). Altered levels of AβPP and Aβ are observed in brain tissue from mice and humans with FXS. These proteins are also dysregulated in Alzheimer's disease (AD), Down Syndrome (DS) and autism suggesting that a common molecular mechanism is involved in disease pathology. AβPP and Aβ play important roles in synapse formation and apoptosis during development and their dysregulation likely contributes to the seizure, behavioral, electrophysiology and dendritic spine phenotypes characteristic of FXS. Consistent with this hypothesis, AGS, anxiety, the ratio of mature versus immature dendritic spines, neocortical hyperexcitability and metabotropic glutamate receptor (mGluR)-mediated long term depression (LTD) (mGluR-LTD) are partially or completely reverted to normal in *Fmr1*$^{KO}$ mice after removal of one *App* allele. Thus, $mGluR_5$ blockade is a potential treatment for reducing the translation of mRNAs, such as *App* mRNA, that are normally synthesized in response to $mGluR_5$ activation and down regulated by FMRP. Reducing AβPP synthesis will decrease the generation of pathogenic catabolites of AβPP leading to normalization of FXS phenotypes. The *Fmr1*$^{KO}$ mouse model with APP/Aβ as biomarkers provide an ideal system to test the efficacy of novel $mGluR_5$ antagonists.

The objectives of this proposal are to treat *Fmr1*$^{KO}$ mice with novel COMPANY compounds and determine if the drugs:

Phase 1. rescue dendritic spine abnormalities

Phase 2. attenuate AGS

In aggregate these studies will compare the efficacy of novel COMPANY compound(s) with an $mGluR_5$ control antagonist (MPEP) and determine if one or more of these novel compounds are a potential therapy for the treatment of FXS.

# 5 Experimental Procedure

## 5.1 Test Item

CMD UUU (batch #000)

CMD X (batch #TTT)

MPEP [Tocris catalog #1212, Batch #10, date on tube: DATE]

## 5.2 Location of Study

## 5.3 Animals and Management

### *5.3.1 Animals*

The *Fmr1*$^{KO}$ mice were originally developed by Frank Kooy and backcrossed >11 times to FVB mice (Dutch-Belgium Consortium, 1994). We received *Fmr1*$^{KO}$ mice in the C57BL/6 background from Dr. Bill Greenough (University of Illinois at Urbana-Champaign). Pregnant female mice (age 3 months old, no weight data available) were used in the in vitro experiments. Male and female mice were tested for AGS at age postnatal day 21 [weight range: 5.00-12.25 g].

### *5.3.2 Acclimatisation and housing conditions*

Mice were housed in static microisolator cage on a 6 a.m.-6 p.m. light cycle with ad libitum access to food (Purina 5015 mouse diet) and water. The cages contained seeds and a nestlet as the only sources of environmental enrichment. All animal husbandry and euthanasia procedures were performed in accordance with NIH and an approved animal care protocol through the Research Animal Resources Center. *Fmr1* genotypes were determined by PCR analysis of DNA extracted from tail biopsies.

## 5.4 Surgery

Phase I (*in vitro*):

For the neuronal cell preparations, embryos were harvested by abdominal surgery from timed pregnant females (embryonic day 18). Isoflurane was used as an anaesthetic and death was the endpoint for the mother.

## 5.5 Treatment

### *5.5.1 Treatment Groups*

Phase I (in vitro):

The treatment groups for the in vitro studies are listed in Table 1 (AβPP levels in dendritic spines) and Table 2 (dendritic spine length, density and filopodia). Table 3 lists the treatment groups to determine the amount of drug in the cell culture media with/without filtration.

**Table 1: Conditions to Determine APP Levels in Dendritic Spines**

| Group | Treatment Group | Dose Level (μM) |
|---|---|---|
| 1 | Control | 0 |
| 2 | MPEP | 2.5 |
| 3 | CMD UUU | 0.0625 |
| 4 | CMD UUU | 0.125 |
| 5 | CMD UUU | 0.25 |
| 6 | CMD UUU | 0.5 |
| 7 | CMD UUU | 1.0 |
| 8 | CMD UUU | 2.5 |
| 9 | CMD X | 0.0625 |
| 10 | CMD X | 0.125 |
| 11 | CMD X | 0.25 |
| 12 | CMD X | 0.5 |
| 13 | CMD X | 1.0 |
| 14 | CMD X | 2.5 |

**Table 2: Conditions to Determine Dendritic Spine Length, Density and Filopodia**

| Group | Treatment Group | Dose Level (μM) | # spines analyzed |
|---|---|---|---|
| 1 | Control<br>5 min | 0 | 399 |
| 2 | Control<br>15 min | 0 | 390 |
| 3 | Control<br>75 min | 0 | 383 |
| 4 | CMD UUU<br>5 min | 0.25 | 530 |
| 5 | CMD UUU<br>15 min | 0.25 | 567 |
| 6 | CMD UUU<br>75 min | 0.25 | 490 |
| 7 | CMD X<br>5 min | 0.25 | 475 |
| 8 | CMD X<br>15 min | 0.25 | 592 |
| 9 | CMD X<br>75 min | 0.25 | 468 |
| 10 | MPEP<br>5 min | 2.5 | 435 |
| 11 | MPEP<br>15 min | 2.5 | 424 |
| 12 | MPEP<br>75 min | 2.5 | 333 |

## Table 3: Culture Media Samples +/- Filtration

| Sample Label | Treatment | Dose | Comments |
|---|---|---|---|
| 1A | no treatment | **0 uM** | not filtered |
| 2A | no treatment | **0 uM** | not filtered |
| 3A | vehicle: 0.025% DMSO | **0 uM** | not filtered |
| 4A | vehicle: 0.025% DMSO | **0 uM** | not filtered |
| 5A | 2.5 uM MPEP | **2.5 uM** | not filtered |
| 6A | 2.5 uM MPEP | **2.5 uM** | not filtered |
| 7A | 0.0625 uM CMD UUU | **0.0625 uM** | not filtered |
| 8A | 0.025 uM CMD UUU | **0.0625 uM** | not filtered |
| 9A | 0.125 uM CMD UUU | **0.125 uM** | not filtered |
| 10A | 0.125 uM CMD UUU | **0.125 uM** | not filtered |
| 11A | 0.25 uM CMD UUU | **0.25 uM** | not filtered |
| 12A | 0.25 uM CMD UUU | **0.25 uM** | not filtered |
| 13A | 0.5 uM CMD UUU | **0.5 uM** | not filtered |
| 14A | 0.5 uM CMD UUU | **0.5 uM** | not filtered |
| 15A | 1.0 uM CMD UUU | **1.0 uM** | not filtered |
| 16A | 1.0 uM CMD UUU | **1.0 uM** | not filtered |
| 17A | 2.5 uM CMD UUU | **2.5 uM** | not filtered |
| 18A | 2.5 uM CMD UUU | **2.5 uM** | not filtered |
| 19A | 0.0625 uM CMD X | **0.0625 uM** | not filtered |
| 20A | 0.025 uM CMD X | **0.0625 uM** | not filtered |
| 21A | 0.125 uM CMD X | **0.125 uM** | not filtered |
| 22A | 0.125 uM CMD X | **0.125 uM** | not filtered |
| 23A | 0.25 uM CMD X | **0.25 uM** | not filtered |
| 24A | 0.25 uM CMD X | **0.25 uM** | not filtered |
| 25A | 0.5 uM CMD X | **0.5 uM** | not filtered |
| 26A | 0.5 uM CMD X | **0.5 uM** | not filtered |
| 27A | 1.0 uM CMD X | **1.0 uM** | not filtered |
| 28A | 1.0 uM CMD X | **1.0 uM** | not filtered |
| 29A | 2.5 uM CMD X | **2.5 uM** | not filtered |
| 30A | 2.5 uM CMD X | **2.5 uM** | not filtered |
| 1B | no treatment | **0 uM** | filtered |
| 2B | no treatment | **0 uM** | filtered |
| 3B | vehicle: 0.025% DMSO | **0 uM** | filtered |
| 4B | vehicle: 0.025% DMSO | **0 uM** | filtered |
| 5B | 2.5 uM MPEP | **2.5 uM** | filtered |
| 6B | 2.5 uM MPEP | **2.5 uM** | filtered |
| 7B | 0.0625 uM CMD UUU | **0.0625 uM** | filtered |
| 8B | 0.025 uM CMD UUU | **0.0625 uM** | filtered |
| 9B | 0.125 uM CMD UUU | **0.125 uM** | filtered |
| 10B | 0.125 uM CMD UUU | **0.125 uM** | filtered |
| 11B | 0.25 uM CMD UUU | **0.25 uM** | filtered |
| 12B | 0.25 uM CMD UUU | **0.25 uM** | filtered |
| 13B | 0.5 uM CMD UUU | **0.5 uM** | filtered |
| 14B | 0.5 uM CMD UUU | **0.5 uM** | filtered |

| | | | |
|---|---|---|---|
| 15B | 1.0 uM CMD UUU | **1.0 uM** | filtered |
| 16B | 1.0 uM CMD UUU | **1.0 uM** | filtered |
| 17B | 2.5 uM CMD UUU | **2.5 uM** | filtered |
| 18B | 2.5 uM CMD UUU | **2.5 uM** | filtered |
| 19B | 0.0625 uM CMD X | **0.0625 uM** | filtered |
| 20B | 0.025 uM CMD X | **0.0625 uM** | filtered |
| 21B | 0.125 uM CMD X | **0.125 uM** | filtered |
| 22B | 0.125 uM CMD X | **0.125 uM** | filtered |
| 23B | 0.25 uM CMD X | **0.25 uM** | filtered |
| 24B | 0.25 uM CMD X | **0.25 uM** | filtered |
| 25B | 0.5 uM CMD X | **0.5 uM** | filtered |
| 26B | 0.5 uM CMD X | **0.5 uM** | filtered |
| 27B | 1.0 uM CMD X | **1.0 uM** | filtered |
| 28B | 1.0 uM CMD X | **1.0 uM** | filtered |
| 29B | 2.5 uM CMD X | **2.5 uM** | filtered |
| 30B | 2.5 uM CMD X | **2.5 uM** | filtered |

Phase 2 (in vivo): The treatment groups for the AGS are listed in Table 4 of Appendix 3 and include: vehicle: 1% HPMC/1% Tween-80; 1, 3 and 10 mg/kg CMD UUU; 1, 3 and 10 mg/kg CMD X and 10 and 30 mg/kg MPEP.

### *5.5.2 Selection of Dose Levels*

Dose levels of CMD UUU and CMD X were determined following evaluation of existing relevant data and suggested by the study sponsor. Dose levels took into account the established concentration of MPEP known to reduce dendritic AβPP levels, dendritic spine length and AGS. See Table 1 (Treatment Conditions for assessment of dendritic AβPP levels), Table 2 (Treatment Conditions for assessment of dendritic spine length, density and percent filopodia), and Table 3 (Treatment Conditions to test culture media for drug levels).

### *5.5.3 Route and Means of Administration*

Phase 1 (*in vitro*):

The cells were dosed *in vitro* at a constant dose volume of 1 ml dosing solution per well.

Phase 2 (*in vivo*):

Mice were dosed for the AGS studies by I.P. injection 30 min prior to seizure testing with a dosing volume of 20 mL/kg.

### *5.5.4 Treatment Regime*

Phase I (*in vitro*):

*Fmr1*$^{KO}$ primary neurons were prepared from embryos and cultured for 15 days. They were treated with the indicated doses of $mGluR_5$ inhibitor in 1 mL of NeuralBasal culture media containing B27 supplement for the indicated times.

Product sheets are provided for NeuroBasal media (Gibco Life Technologies catalog #21103) and B27 supplement (Gibco Life Technologies catalog #17504 in Appendix 2.

Phase 2 *(in vivo)*:

Drugs of interest (vehicle, MPEP, CMD UUU, CMD X) or their vehicle (1% HPMC/1% Tween-80) were administered 30 min prior to AGS testing.

## 5.6 Experimental Methods

### *5.6.1 Assessment of dendritic AβPP levels.*

Neuronal Cell Culture, Confocal Microscopy and Image Analysis: Pregnant females (embryonic day 18) were anesthetized with isoflurane prior to decapitation and transfer of the uterine sac to ice-cold Hank's buffered saline solution (HBSS) (Cellgro catalog #21-021-CV; see Appendix 2 for formulation). Cortices were removed, washed with ice-cold HBSS, lysed with 0.5 mg/mL trypsin for 25 min at 37$^0$C, washed with HBSS, suspended in NeuroBasal medium (supplemented with 2% B27 supplement, penicillin/streptomycin, 0.5 mM glutamine), triturated 70X with a 10 mL pipet and passed through a 70 mm cell strainer. Cells were counted by trypan blue dye exclusion and plated at 1.3 X $10^5$ cells/mL on poly(D)-lysine coated glass coverslips in 12 well tissue culture dishes and cultured for 15 days at 37$^0$C/5% $CO_2$.

Neuronal cells were treated with the indicated concentrations of $mGluR_5$ inhibitor, fixed and stained with anti-AβPP antibody. For fixation, treated cells were washed with Dulbecco's phosphate buffered saline (DPBS) (Cellgro catalog #21-031-CV; see Appendix 2 for formulation), fixed in 4% paraformaldehyde (PHA) for 10 min at room temp and permeabilized with methanol (-20$^0$C) for 15 min. Fixed, permeabilized cells were stained with anti-22C11 against the amino-terminus of AβPP (Chemicon #mAB348, Temecula, CA) (1:2000, overnight) and visualized with goat anti-mouse rhodamine-conjugated secondary antibody (Invitrogen, Carlsbad, CA) (1:500 for 20 min in the dark). Washes and antibody dilutions were in DPBS containing 2% FBS. Coverslips were fixed to slides with 12 μL ProLong Gold Antifade (Invitrogen, Carlsbad, CA) and dried overnight.

Images were acquired with a Nikon C1 laser scanning confocal microscope (Nikon Eclipse E600 upright microscope) using the 543 Diode (1mw Mellet Griot) laser, the Nikon Plan Apo 60X/1.40 oil objective with Zeiss Immersol$^{TM}$ 518F oil at ambient temperature, and Nikon EZ-C1, v3.91 software (Nikon Corp, Tokyo, Japan). AβPP levels in the puncta of 4-7 dendrites per sample were quantitated with IMAGE J software using the Analyze Particles function (Rasband, UUU.S., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006).

### *5.6.2 Assessment of dendritic spine length and density.*

Primary mouse neurons were prepared from embryonic mice dissected brains from timed pregnant *Fmr1*$^{KO}$ female mice as previously described (Westmark, 2007). Cells were cultured for 15 days on poly(D)-lysine coated glass coverslips inside of 12-well tissue culture dishes, treated with the indicated concentration of $mGluR_5$ inhibitor for the indicated times, fixed with 4% paraformaledehyde and stained with DiI dye (Gibco Life Technologies, catalog #D282). DiI is a lipophilic, orange-red fluorescent, membrane stain that diffuses laterally to stain the entire cell. For the staining, the wells were aspirated and sprinkled with DiI crystals and a small amount of DBPS was added to the edge of the wells to prevent dehydration of the cells. Cells were stained for 10 min, copiously washed with DPBS to remove all crystals and fixed to slides with ProLong Gold Antifade (Life Technologies Corporation, Carlsbad, CA, USA). Slides were allowed to dry for at least 3 days to allow complete migration of the DiI into dendritic spines. Dendritic spines were imaged on a Zeiss Axioplan 2 Imaging Photomicroscope equipped with a MBF Biosciences automated XYZ stage and MicroFire A/R camera. Images were taken using the 100X objective (Zeiss FLUAR 100X/1.30 oil) and Zeiss Immersol$^{TM}$ 518F oil at ambient temperature. Spine length was quantitated with StereoInvestigator v9 software. Contours were drawn around the protrusions and the feret max (length) and feret min (widest width) of the contours were calculated. A minimum of 2 coverslips were analyzed per neuronal cell prep and images of neurons were taken from multiple areas of those coverslips. Spines (333-592) were quantitated per condition. The feret width was divided by feret max and protrusions having a ratio less than 0.5 were classified as filopodia and those with a ratio greater than or equal to 0.5 were classified as spines.

### *5.6.3 Audiogenic Seizure Testing*

*Fmr1*$^{KO}$ in the C57BL/6 background have peak sensitivity to AGS at postnatal day 21 (P21) (Yan, 2005). Thus, we assessed AGS in *Fmr1*$^{KO}$ in the C57BL/6 background at P21. Mice were treated with vehicle or indicated dose of drug of interest by I.P. injection and 30 min later transferred to a Plexiglas box (13"L X 8"UUU X 7"UUU) and exposed to a high-pitched siren (118 dB) from a personal body alarm (LOUD KEY$^{TM}$). We scored the number of mice exhibiting wild running (WR), tonic seizures (AGS) and death. See Appendix 3: Table 4 (AGS raw data with statistics).

## 5.7 Fluid Collection

Phase I (*in vitro*):

Culture media was removed from primary neurons treated with XXX (0.0625, 0.125, 0.25, 0.5, 1.0 and 2.5 μM), TTT (0.0625, 0.125, 0.25, 0.5, 1.0 and 2.5 μM) and MPEP (2.5 μM) after a 60 min treatment period. For the collection of non-filtered samples, the media was transferred to Eppendorf tubes. For the collection of filtered samples, the media was transferred to Centricon-10 filter units (Centricon, catalog #4205) having a 10,000 molecular weight cut-off and spun at 3,850 x g. Samples were frozen at -80$^0$C.

Phase 2 (*in vivo*):

After AGS testing, mice are anesthetized with isoflurane and blood is removed from the abdominal aortic artery with a 23g needle and mixed with 20 µL of 10 mg/mL sodium heparin to prevent coagulation. The mice were then decapitated and the brains were dissected, but in half, and frozen in dry ice. After all blood samples were collected, tubes were spun at 5,000rpm for 10 min. The upper plasma layer was removed and frozen on dry ice. See Appendix 4: Table 5 (List of Plasma and Brain Samples Shipped).

## 5.8 Observation of Symptoms

Phase I (*in vitro*):

N/A.

Phase 2 (*in vivo*):

Mice were monitored after administration of the compounds.

### *5.8.1 Method of Sacrifice*

Phase I (*in vitro*):

Timed pregnant mice (embryonic day 18) were sacrificed by exposure to isoflurane followed by decapitation.

Phase 2 (*in vivo*):

After AGS testing, mice are anesthetized with isoflurane, the blood was removed from the abdominal aortic artery and the mice were decapitated prior to dissection of the brains.

## 5.9 Statistical Analysis

Phase I (*in vitro*):

Student T-test

Phase 2 (*in vivo*):

Chi Square analyses

## 5.10 Archives

The data archives are located _____

# 6 Results

## 6.1 General symptoms observations

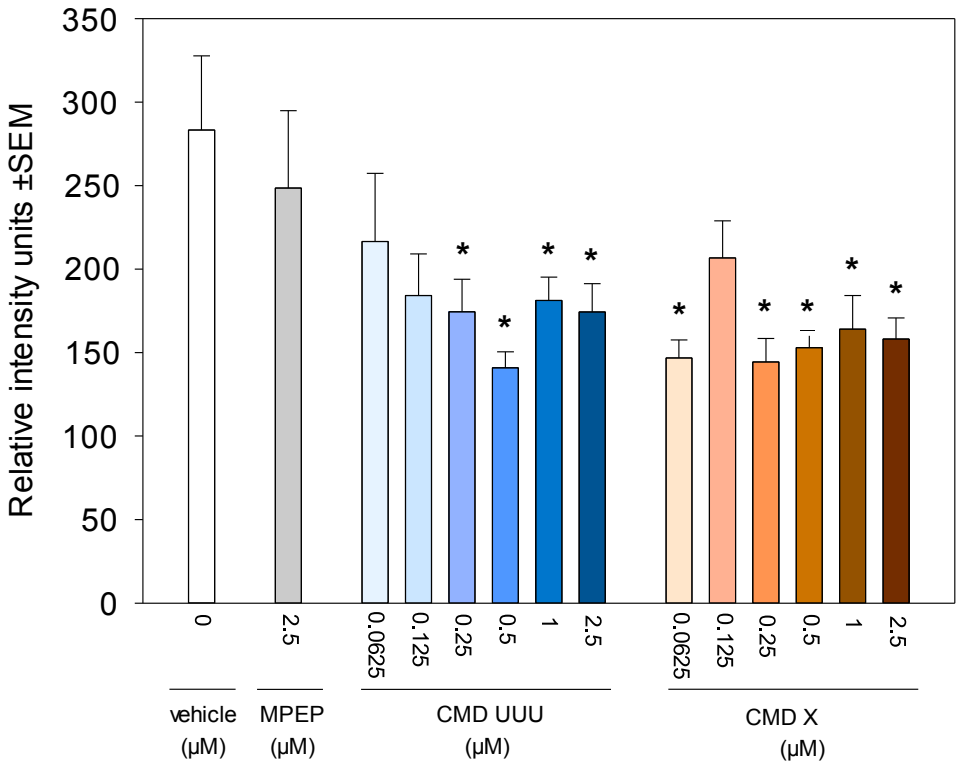
Primary neurons exhibited excellent plating, growth and staining with both anti-AβPP and DiI. Example photographs are given in Appendix 1.

Mice were monitored after administration of the compounds. They took a few minutes to recover from the injections and then they exhibited normal home cage activity. 30 min post-injection, mice were exposed to the 110 dB siren in a sound-attenuating chamber and monitored for WR, AGS and death.

## 6.2 Assessment of dendritic AβPP levels.

CMD UUU significantly reduced dendritic expression of AβPP at concentrations 0.25 μM – 1.0 μM. CMD X significantly reduced AβPP expression at all concentrations tested except 0.125 μM. It is not clear why 0.125 μM did not work. MPEP was not effective in this assay contrary to our previous results that demonstrated a 40% decrease in dendritic AβPP levels with MPEP treatment.

**Fig. 1. Effects of vehicle, MPEP, CMD-UUU, and CMD-X on APP staining intensity in cultured primary neurones from *fmr-1* KO mice**



## 6.3 Assessment of dendritic spine length and density.

Both CMD UUU and CMD X significantly decreased dendritic spine length by two-fold with 15 and 75 min treatment at 0.25 mM. The 0.25 mM dose was chosen as the lowest common effective dose so that the two drugs could be compared over time. The CMD X was effective with 5 min treatment, whereas the CMD UUU was not.

MPEP gave spurious results, and in conjunction with the AβPP confocal data, suggests that MPEP was not active when dissolved in DMSO. Neither CMD UUU nor CMD X drug significantly affected spine density. Both drugs reduced the percentage of immature spines (filopodia)

**Fig. 2. Effects of vehicle, Compound UUU, Compound X and MPEP on spine length in cultured primary neurones from *fmr-1* KO mice**

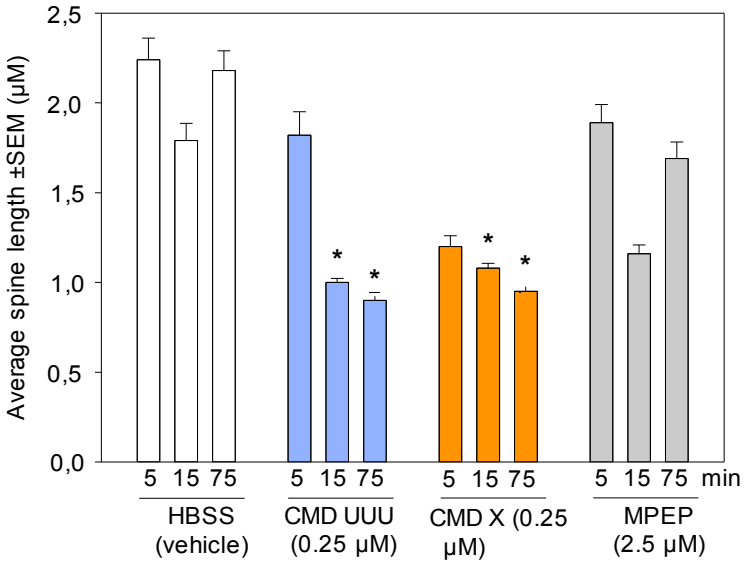



**Fig. 3. Effects of vehicle, XXX, UUU and MPEP on spine density in cultured primary neurones from *fmr-1* KO mice**

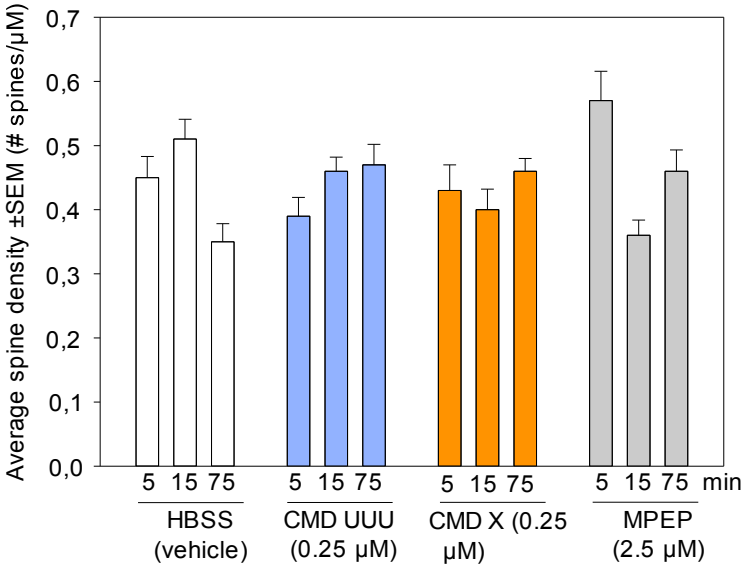
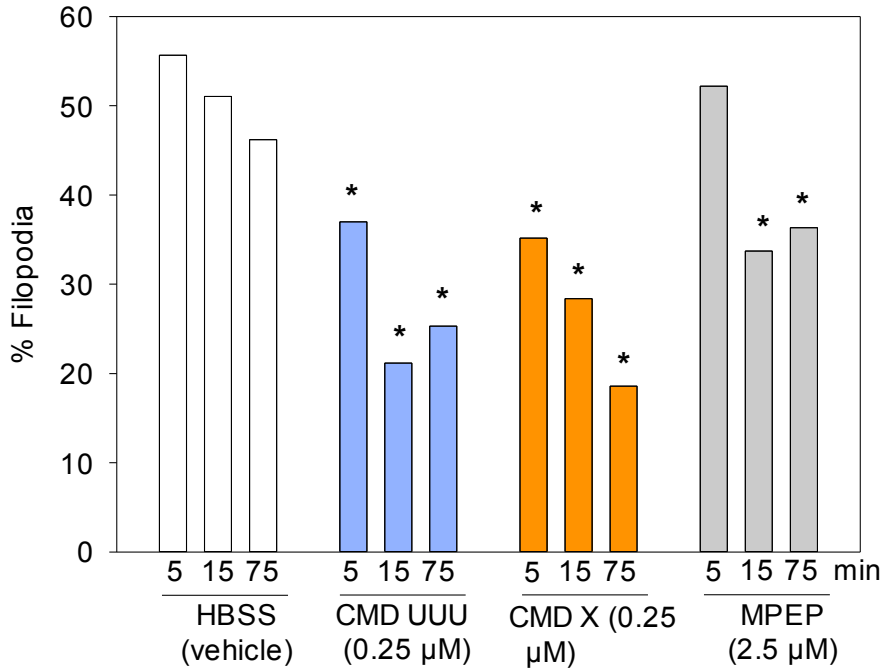


**Fig. 4. Effects of vehicle, XXX, UUU and MPEP on percentage of filopodia in cultured primary neurones from *fmr-1* KO mice**




## 6.4 Audiogenic seizure testing

WR and AGS but not mortality were significantly attenuated with both 3 and 10 mg/kg CMD UUU and CMD X. However, mortality rates were found to be 0%. Neither drug was effective at 1 mg/kg. MPEP significantly attenuated WR and AGS but not mortality at 10 mg/kg.

**Fig. 5. Effects of vehicle, CMD UUU, CMD X , and MPEP on audiogenic seizures (AGS) in *fmr-1* KO mice**

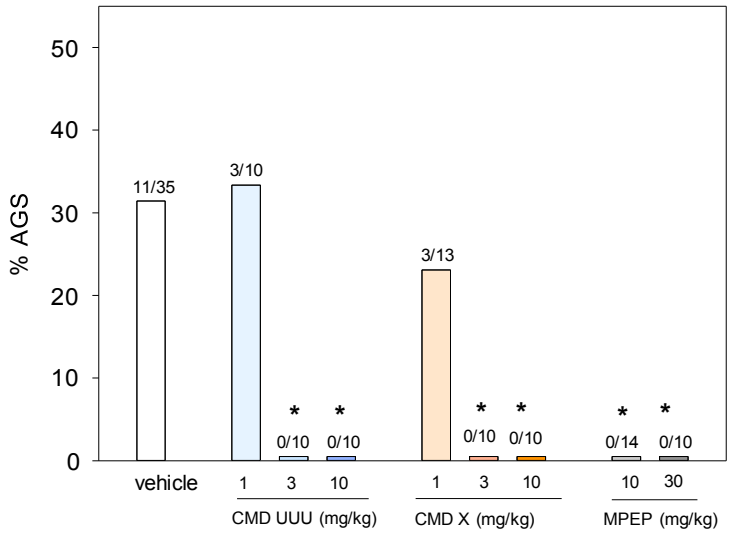


**Fig. 6. Effects of vehicle, XXX, UUU, and MPEP on wild running (WR) in *fmr-1* KO mice**

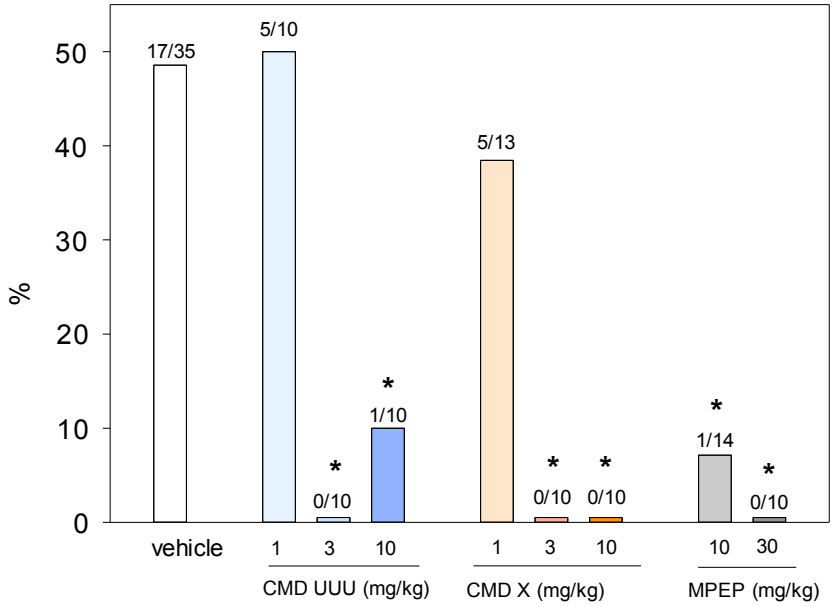
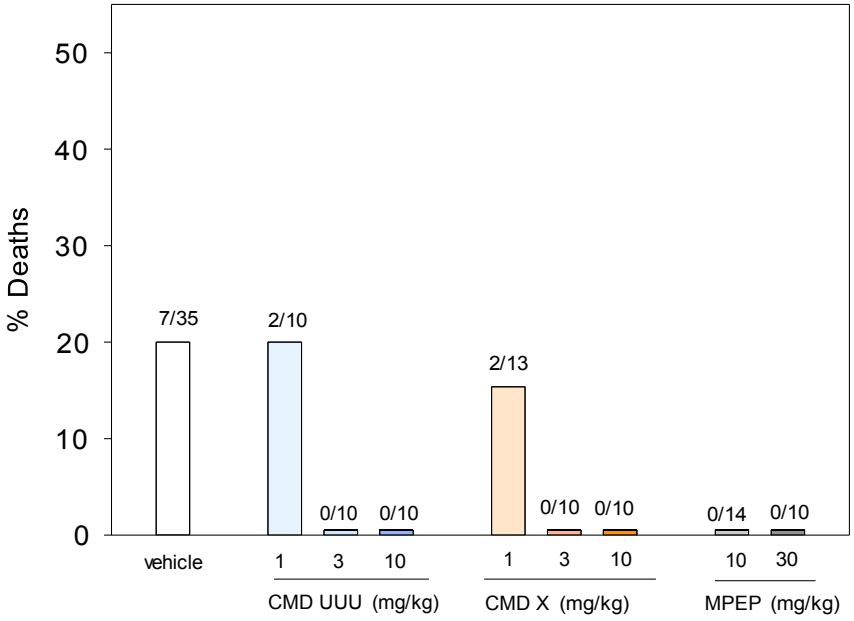



**Fig. 7. Effects of vehicle, XXX, UUU, and MPEP on deaths in *fmr-1* KO mice**



## 6.3 Analysis of body fluids (brain) concentrations

*In vitro* (filtered and non-filtered culture media) and in vivo (brain and plasma) samples were collected and shipped to COMPANY for further analyses (Appendix 4:Table 5).

# 7 Discussion and Conclusion

$mGluR_5$ inhibitors are under intense investigation as a promising therapeutic for the treatment of FXS. MPEP is a potent and highly selective noncompetitive antagonist of $mGluR_5$ (Wallberg, 2006; Porter, 2005) that reduces AGS, anxiety phenotypes and dendritic spine protrusion morphology in *Fmr1*$^{KO}$ (Yan, 2005; de Vrij, 2008). We have demonstrated that $mGluR_5$ blockade with MPEP inhibits translation of AβPP (Westmark, 2007) and that several FXS phenotypes can be rescued by genetic manipulation to reduce AβPP/Aβ (Westmark, 2011). Thus, AβPP and Aβ are promising biomarkers for FXS and *Fmr1*$^{KO}$ mice are the best available animal model for the assessment of novel $mGluR_5$ antagonists. The purpose of this project was to compare the efficacy to two novel COMPANY compounds with MPEP both *in vitro* and *in vivo*.

*In vitro*, both Clinet CMD UUU and CMD X reduced dendritic AβPP levels by 50% after 60 min treatment as expected from previous experiments with MPEP. We have demonstrated 2-10 μM MPEP is effective in the past. In this report, we did not observe good efficacy with 2.5 μM MPEP. We have not tested lower concentrations of MPEP. This lack of effect with MPEP is discussed below. The Client's drug were effective
at 0.25 μM, and perhaps lower with the CMD X (0.0625 μM), although the aberrant results at 0.125 μM CMD X occlude a definitive conclusion.

CMD UUU and CMD X both reduced dendritic spine length in *Fmr1*$^{KO}$ neurons. The control cells exhibit an average spine length of 2.24 μm as expected. From previous work, *Fmr1*$^{KO}$ neurons have longer spines than WT neurons (WT neurons have an average spine length of 1 μm) and the *Fmr1*$^{KO}$ neurons resemble WT neurons after 15 min and 4 hr treatments with 2.5 μM MPEP (Westmark, 2011). In this proposal, 0.25 μM CMD UUU was effective at 15 and 75 min and 0.25 μM CMD X was effective at all times tested (5, 15 and 75 min). In this study, 2.5 μM MPEP was effective at reducing spine length at 15 min, but not 5 or 75 min. There were no significant differences in spine density with the treatments.

*In vivo*, both CMD UUU and CMD X attenuated WR and AGS at doses of 3 and 10 mg/kg, but neither was effective at 1 mg/kg. The lowest dose of MPEP tested was 10 mg/kg, which significantly reduced both WR and AGS.

It is disturbing that the MPEP was not effective in the *in vitro* studies, but was active in the *in vivo* studies. Perhaps the issue was the difference in solvents used to dissolve/suspend the drugs between the *in vitro* and *in vivo* work. The CMD UUU and CMD X compounds are not aqueous soluble. For the *in vivo* work, these drugs as well as the MPEP were prepared as a fine suspension in 1% HPMC/1% Tween-80 using a IKA-Ultra Turrax mill. For the *in vitro* studies, we did not know how 1% HPMC/1% Tween-80 would affect the neuronal cells. The detergent would be expected to lyse the cells. The CMD UUU and CMD X compounds were not soluble in our normal vehicle, HBSS; thus, both COMPANY compounds and the MPEP were dissolved in a small volume of DMSO and then diluted in HBSS prior to treating the neuronal cells. The final concentration of DMSO on the cells was 0.025% and we saw no evidence of the drugs precipitating out of solution upon dilution of the DMSO stocks with HBSS. We normally

do not dilute MPEP with DMSO, but wanted to treat all of the drugs the same. Perhaps the DMSO inactivated the MPEP?

In conclusion, both CMD UUU and CMD X were effective at attenuating *in vitro* and *in vivo* phenotypes associated with Fragile X syndrome in a mouse model of the disorder. We were not able to discern a difference in efficacy between the drugs in the *in vivo* studies as both were effective at 3 and 10 mg/kg, but not at 1 mg/kg. With the *in vitro* work, CMD X was effective at all times tested for the dendritic spine length analyses whereas CMD UUU was not active at the shortest time tested suggesting that CMD X was more effective. It is difficult to compare the drugs with MPEP as the *in vitro* MPEP experiments failed and the lowest dose of MPEP tested in the AGS was 10 mg/kg, which was active. In the literature, 30 mg/kg MPEP is routinely used to inhibit AGS; thus, our data suggests that a dose response curve will need to be performed with MPEP to determine if new compounds are more or equally effective.

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# 8 Appendices

Appendix 1: Photographs

APP Stained Cells

DiI Labeled Cells

Appendix 2: Product Profiles

NeuroBasal Media

B27 Supplement

HBSS

DPBS

Appendix 3: AGS raw data and statistics

Appendix 4: Sample IDs for shipment