Title: Evaluating the Neurophysiologic and Clinical Effects of Single-Dose Acamprosate, Lovastatin, Minocycline and Placebo in Fragile X Syndrome

Principal Investigator: Craig Erickson, M.D.
Associate Professor of Psychiatry
Children's Hospital Medical Center
3333 Burnet Avenue
Cincinnati, OH 45229-3039
Office 513-636-4611
craig.erickson@cchmc.org

Co-Investigators Ernest Pedapati, M.D.
Logan K. Wink, M.D.
Ismail Badran, M.D.
Kelli Dominick, M.D., PhD
Rebecca Shaffer, PsyD
Amie Duncan, PhD

1.0 Introduction

Fragile X Syndrome (FXS) is a family of genetic conditions caused by mutations in the *Fmr1* gene. FXS is the most prevalent cause of inherited intellectual disability (Crawford, Acuna, & Sherman, 2001). Approximately 1:4,000 males and 1:8,000 females have FXS (Crawford et al., 2001). This impairment ranges from learning disabilities to severe intellectual disability. Between 15% and 60% of individuals with FXS have autism (Budimirovic & Kaufmann, 2011). Other symptoms also can include characteristic physical (e.g. elongated face, prominent ears, and enlarged testes) and behavioral (e.g. stereotypic movements and social anxiety) features. Sensory hypersensitivities are an especially common, clinically distressing feature of FXS. The aim of this project is to determine whether single-dose administration of acamprosate, minocycline and lovastatin, which lead to recovery of neuronal function in *Fmr1* knockout (KO) mice, reduce (i.e., normalize) sensory evoke-related potentials (ERPs) in FXS patients. The proposed study will contribute to an understanding of neural hyperexcitability as a substrate of sensory hypersensitivities in FXS.

2.0 Specific Aims

Prior research has documented that pharmacological strategies targeting different neural mechanisms can reduce neocortical hyperexcitability in *Fmr1* KO mouse models. Unlike nearly all areas of clinical psychopharmacology, marked to full recovery of function has been shown within hours of drug administration in animal models. We propose to leverage that observation to investigate single-dose drug effects on event-related potential (ERP) biomarkers and behavior in humans with FXS.

Primary Aim
Our aim is to clinically validate the acute pharmacologic recovery of auditory processing deficits seen in *Fmr1* KO mouse models, and to assess the relative impact and consistency of effect across patients of drugs targeting three different mechanisms on this recovery. If ERP normalization is observed in the proposed pharmacologic study, it will not only provide new knowledge about underlying mechanisms, but potentially speed drug development by guiding selection of mechanisms to target, choice of drugs to investigate, and outcomes to assess in next-step early phase clinical trials.

Secondary Aim
Neurophysiologic assessments will mirror the assessments in “Mechanisms and brain circuits underlying fragile X syndrome” IRB # 2015-8425 and include spectral analysis, N1 amplitude and habituation ERP, chirps, and talk/listen paradigms. Behavioral measures were chosen based on prior validation in FXS populations and an ability to allow brief assessments with limited learning effects. The primary clinical measure will be the Clinical Global Impressions Improvement (CGI-I) scale (Guy, 1976). The CGI-I requires the clinician to assess how much the patient's illness has changed relative to pre-dose, from 1 (very much improved) to 7 (very much worse). The recently validated Aberrant Behavior Checklist (ABC) using the 6-factor FXS model (Sansone et al., 2012) will be used as a secondary outcome of behavioral status.

To assess cognitive outcomes as a secondary aim, we selected the following tests: Woodcock Johnson (WJ)-III Spatial Relations (visual spatial thinking/5 min (McGrew & Woodcock, 2001); Grooved Pegboard Test (sensory motor integration/15 min); RBANS List Learning (immediate memory/5 min (Randolph, 1998); and Test of Attentional Performance for Children (KiTAP) (attention and inhibition/30 min (Knox et al., 2012). The WJ-III Auditory Attention test will assess

auditory processing-speech sound discrimination, which aligns well with our focus on auditory cortical function. This test was validated in ages 5-81 and requires 5 min to complete (McGrew & Woodcock, 2001).

3.0 Background:

Fmr1 gene full mutation leads to a cascade of brain changes, which offer multiple potential pharmacologic targets to treat functional deficits associated with FXS (Wijetunge, Chattarji, Wyllie, & Kind, 2013). This protocol is designed to investigate clinical homologues in sensory processing abnormalities that can provide more sensitive and mechanism-related assessments of pharmacologic effects. We plan to evaluate single-dose drug effects on a core symptom of FXS, auditory hypersensitivity, using robust ERP measures of auditory response that translate from mouse models to humans and back. Specifically, our study will evaluate the effects of a single-dose approach to test drug effects on putative ERP biomarkers and behavior of persons with FXS. In 2009, Berry-Kravis et al. (Berry-Kravis et al., 2009) reported that a single oral dose of fenobam, an mGluR5 antagonist (Porter et al., 2005), was well tolerated and resulted in response (defined as 20% improvement) in auditory prepulse inhibition (PPI). These PPI effects were demonstrated just 60 minutes after oral dosing (Berry-Kravis et al., 2009). This study lays a foundation for using acute pharmacologic studies to probe putative etiologic pathways of FXS. We will extend this pharmacologic strategy to test the neurophysiologic effects of multiple drugs targeting different mechanisms in the same patients.

Among the most promising targets for pharmacologic intervention in FXS are treatments targeting mGluR5 and GABA systems and the ERK and MMP-9 pathways (Wijetunge et al., 2013). *Fmr1* mutation results in upregulation of mGluR5 receptors and “downstream” effects of increased mGluR5 signaling in extracellular signal related kinase (ERK) and matrix metalloproteinase-9 (MMP-9) pathways that lead to abnormal dendritic spines and auditory circuit hyperexcitability [Preliminary data from our collaborators at UTSW (Huber & Gibson) and UC Riverside (Abdulrazak & Ethell)]. Fortunately, safe and well-tolerated drugs with actions relevant to these mechanisms already exist in agents FDA-approved for other conditions: acamprosate inhibits mGluR5 (and enhances GABA function (Harris et al., 2002)); lovastatin inhibits ERK (Osterweil et al., 2013), and; minocycline inhibits MMP-9 (Bilousova et al., 2009). Limited, but growing evidence suggests these agents can counter auditory hyperexcitability associated with the *Fmr1* mutation. Acute administration of acamprosate results in a significant reduction in UP state duration (a measure of cortical hyperexcitability) in somatosensory cortex slices of *Fmr1* KO mice (Huber & Gibson preliminary data). Acamprosate has also been shown to reduce cortical hyperexcitability in ERP and magnetoencephalopathy studies in the substance dependence literature (Boeijinga et al., 2004) (Pietrzak & Czarnecka, 2005). Lovastatin, an ERK inhibitor, corrects audiogenic seizures in the *Fmr1* KO mouse model (Osterweil et al., 2013). A related ERK inhibitor, U0126, blocks hyperexcitability in somatosensory cortex as shown in reduced UP states in *Fmr1* KO mice (UTSW collaborators Huber & Gibson preliminary data). Our UC Riverside collaborators (Abdulrazak & Ethell) recently found that chronic minocycline treatment partially attenuates the number and severity of audiogenic seizures in *Fmr1* KO mice (Dansie et al., 2013). A study of mice and rats found that minocycline was protective against partial seizure induction *in vivo* (Wang, Englot, Garcia, Lawton, & Young, 2012). Particularly relevant to our proposed pharmacologic study, a single oral dose of minocycline exerted acute inhibitory effects on cortical excitability (motor cortex response to transcranial magnetic stimulation) in normal humans just 4 hours after dosing (Lang, Rothkegel, Terney, Antal, & Paulus, 2013). Chronic treatment with minocycline has already been shown to significantly reduce auditory N1 ERP in a double-blind, placebo-controlled crossover treatment trial in children with FXS (Schneider et al., 2013).

This pharmacologic study is designed to leverage clinical and preclinical preliminary data showing robust acute drug effects on neurophysiological parameters. If our strategy of single-dose rapid testing of drug effects on ERP biomarkers is effective, the approach would inform preclinical work on the mechanisms most relevant to auditory hyperexcitability, speed the identification of effective pharmacologic treatments, develop an urgently needed biomarker strategy for predicting and evaluating drug effects, and ultimately, could allow for rapid personalization of treatment in FXS.

4.0. Concise Summary of Project:

4.1 Study Design

This pharmacologic study employs a double –blinded 4-drug single dose, 4-period, 4-sequence balanced-crossover design. 36 patients age 15 to 55 years with full mutation fragile X syndrome characterized by greater than 200 CGG repeats in the FMR1 gene by Southern Blot and PCR genetic testing will be eligible to participate if they previously successfully completed baseline testing (behavioral, medical, neurophysiological tests) for our study "Mechanisms and brain circuits underlying fragile x syndrome (IRB # 2015-8425)". In this project, subjects will be randomly assigned to 4 different sequences for receiving acamprosate, lovastatin, minocycline and placebo, with a 2-week washout between administrations. Patients will be stratified based on gender and then randomized in a 1:1 ratio to one of the 4 possible drug sequences (e.g.,1: ADBC; 2: BACD; 3: CBDA; and 4: DCAB). The study is expected to enroll 36 subjects who randomize, once they are randomized, subjects will not be replaced, Subjects and legally authorized representatives will be informed during consent that they will be blinded to treatment status throughout the study. Agents of this study will be dosed as a single administration to each patient at the following doses: acamprosate=1,332 mg, lovastatin=40 mg, minocycline extended release 270 mg (bioequivalent to 200 mg immediate release minocycline). The timing of evaluations in relation to the single-dose of each agent/placebo is guided primarily by the time to maximum plasma concentration (Tmax) of the drugs but is also supported by considerable preclinical and clinical literature. The primary outcome ERP will be conducted 4 hours post-dosing, with post-dose clinical and cognitive evaluations being done immediately thereafter. Pharmacokinetic data regarding ½-lives of the drugs support a 2 week washout period between drug exposures.

The power analysis supports our plan to enroll subjects until at least 36 subjects have fully completed the drug dosing protocol. The study will last approximately 10-11 weeks and there will be a total of 5 visits in the study (and 5 follow-up phone calls with a study coordinator).

4.2 Blindness and Breaking the Blind

The study blind will be maintained throughout the duration of the clinical trial. Only the CCHMC Investigational Pharmacy will be aware of study drug assignment. The blind will be broken only at the discretion of a participating study physician or Medical Monitor in cases necessary to assure patient safety. Such significant safety events would include, but are not limited to, any circumstance where an adverse effect documented as possibly, probably, or definitely related to study treatment and moderate or more severe in intensity results in the need for additional medical intervention (hospitalization, emergency/urgent care room visit, additional outpatient prescribing/other management etc.).

5.0 Study Procedures:

5.1 Baseline Visit Procedures

All FXS age 15-55, who successfully complete testing (behavioral, medical, neurophysiological tests) for our study "Mechanisms and brain circuits underlying fragile x syndrome (IRB # 2015-8425)" will be eligible to participate in this pharmacologic study. At the day of recruitment, consenting participants will complete a urine drug screen (if possible) and females of childbearing age (as determined by report regarding age of menarche or menopause) will be required to take a urine or serum pregnancy test before study procedures. A positive test will exclude them from participating in the study. Safety labs including comprehensive metabolic panel (CMP), antinucleotide antibody (ANA), and complete blood count with differential (CBC with differential) will be collected at baseline. All subjects will have the option to provide a blood sample for Extracellular Signal Related Kinase (ERK) Activation Molecular Biomarker Assay and Amyloid Precursor Protein (APP) Molecular Biomarker Assay (described in further detail below). Baseline cognitive and clinical measures (described in further detail below) will be assessed. Each patient will have a medical examination performed by a study physician who will review medical history, and assess height, weight, vitals (Blood pressure, heart rate, respiratory rate, and temperature), muscle tone, Tanner staging, head circumference, history of recurrent otitis media, dysmorphologies and other comorbid conditions at baseline.

Subjects who successfully complete baseline testing will be randomized to study treatment and assigned to 4 different sequences for receiving acamprosate, lovastatin, minocycline and placebo. Subjects will return within 7 days to receive the first dose of the first of 3 drugs or placebo.

5.2 Drug Administration Visit Procedures

The day of each drug administration day, a medical evaluation/physical exam, vital signs, event related potentials (ERP) neurophysiology measures, RBANS, KiTap and CGI-I will be completed as "baseline" assessments for the day. Approximately two hours later, study drug (acamprosate 1,332 mg, lovastatin 40 mg, or minocycline ER 270 mg) or matching placebo will be administered orally to the subject with FXS. Optional pharmacokinetic blood samples will be drawn 1 to 2 hours post dose. Approximately, four hours post-dose event related potentials (ERP) will be performed, followed immediately thereafter by cognitive, clinical, and side effect/safety (including vital sign) assessments. Optional pharmacokinetic blood sampling will be after the ERP evaluation approximately 4 to 6 hours following dosing. Optional Molecular assays (ERK/APP) will be drawn at the same time as Pk draw. At the end of single-dose drug administration days, Side Effect/Safety (including vital sign) assessments will be repeated. These visits will take approximately 8 hours to complete. These four drug administration days will occur once every 2 weeks over the course of 8 weeks. A detailed description of each procedure being performed on drug administration days is provided below in Table 2.

5.3 Follow Up Phone Call Procedures

We will bolster safety by adding weekly Side Effect/Safety assessments by phone midway between planned drug administration visits and the day following drug administration and finally 1 month after final study drug administration. Additional visits will be provided at any time that signs of concern arise.

Table 2. Schedule of Assessments

Visit	1 Base line	2 Pre-Dose	2 Post-Dose	Call	Call	3 Pre-Dose	3 Post-Dose	Call	Call	4 Pre-Dose	4 Post-Dose	Call	Call	5 Pre-Dose	5 Post-Dose	Call	Call	Safety follow-up Call
Day	Day -7to 0	Day 1	Day 1	Day 2	Day 8	Week 2	Week 2	Week 2+1	Week 3	Week 4	Week 4	Week 4+1	Week 5	Week 6	Week 6	Week 6+1	Week 7	Week 10
Informed Consent	X																	
Physical Exam	X	X				X				X				X				
Urine drug screen	X																	
Medical History	X																	
Vital Signs	X	X	X			X	X			X	X			X	X			
Urine or serum pregnancy test	X																	
Safety Labs	X																	
Biomarker Molecular Assays (optional)	X		X				X				X				X			
Pk Assays (optional)			X				X				X				X			
Neurophysiology Measures		X	X			X	X			X	X			X	X			
CGI		X	X			X	X			X	X			X	X			
ABC	X	X				X				X				X				X
WJ Subtests and Pegboard Test	X		X				X				X				X			
RBANS and KiTap		X	X			X	X			X	X			X	X			
Side effects/ Safety		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X*
Randomization	X																	

*The Safety Follow-up visit at week 10 will include phone call with physician regarding participants overall health status and a general inquiry about AE's. Day 0 and Day 1 may occur on the same day. Safety labs include comprehensive metabolic panel, ANA, and CBC.

5.4 Neurophysiology Measures

For acquisition of electrophysiological) data, we will use up to 128 lead channels placed according to the standard 10-20 electroencephalography array (which allows for a brief 5-10 minute setup) referenced to the CMS-DRL ground. We will use an EGI NetAmp400 (EGI, Eugene, OR) with hydrocel nets. Participants will view a standard silent movie during acquisition for all paradigms except talk-listen, as is common practice to facilitate participation with developmentally disabled subjects. Auditory stimuli will be delivered with Etymotic or Seinheiser earphones via a programmable sound module (Presentation Software). data will be acquired continuously, amplified (12,500x), and digitized (1000 Hz). Eye movements will be recorded (EOG) for offline data correction. For all neurophysiology studies, data will be average referenced and artifacts related to muscular, cardiac and ocular activity will be corrected using the ICA toolbox in Matlab. Source estimates of ERP components and power spectra in frequency bands of interest are computed for time epochs of interest implemented on the canonical mesh using multiple sparse priors under group constraints.

Spectral analysis: Spectra analysis will be completed on data acquired with eyes open for 5 minutes. Data will be segmented into 1-sec epochs, detrended, and transformed into time-frequency space in Matlab with a fast Fourier transform (FFT) algorithm, yielding 1Hz frequency steps.

Auditory habituation ERP: ERPs will be recorded during passive listening (150 sets of four 75 ms broadband noise bursts) separated by an ISI of 500 ms, with inter-set interval of 4000 ms, for ~14 minutes of recording. Data will be filtered from .5-55 Hz (6 and 12 db/octave rolloff, respectively, zero-phase). For each 4 stimulus train, trials will be blocked into 3000ms epochs, from 500 ms prior to stimulus onset to 500 ms after stimulus offset, baseline corrected using the prestimulus interval, and averaged across trials. Spatial PCA in BESA 5.3 (MEGIS Software, Grafelfing, Germany) will be used to define spatial topographies for ERP components in the grand average of all subjects. The PCA component capturing the N1 waveform will be identified with topography defined as the largest negative deflection between 50-200 ms post-stimulus.

Chirp modulated sweep: Subjects will passively listen to auditory stimuli consisting of a 1000 Hz tone amplitude modulated (AM) by a chirp sinusoid that linearly increases in frequency from 0-100 Hz over a period of 2 sec. Two hundred of these stimuli will be separated by a 1.5-2 sec inter-trial interval, for ~12.5 minutes of testing. Data will be filtered from .5-120 Hz (6 and 12 db/octave rolloff, respectively, zero-phase). Trials will be blocked into 3500 ms epochs, from 500 ms before to 1000 ms after stimulus offset. Representative topographies for the steady-state response will be computed using spatial PCA in BESA 5.3 (MEGIS Software, Grafelfing, Germany) on the grand average of all subjects to determine the sensors used for the final analysis. Data will be averaged over the representative sensors and Morlet wavelets and/or Gabor transform will be utilized to compute time-frequency plots, single trial power and phase locking across trials.

Talk/Listen Paradigm: In the Talk condition, participants pronounce short (<300ms), sharp vocalizations of the phoneme “ah” in a self-paced manner about every 1-2s, for 180s. The speech is recorded and transmitted back to participants through headphones in real time (zero delay). In the Listen condition, participants listen to the recording from the Talk condition played back. Participants are coached to produce “ah” vocalizations >75dB and < 85dB by monitoring intensity with a dB meter. Sound intensity is kept the same in Talk and Listen conditions for each participant by ensuring that a 1000Hz tone (generated by a Quest QC calibrator) produces equivalent dB intensities when delivered through earphones during the Talk and Listen

conditions. Data will be filtered 1- 50Hz and then epoched from -800 to 800 ms with respect to the onset of each “ah” and baseline corrected using data from the -800 to -500ms epoch preceding vocalization. ERP averages are generated using a robust averaging approach included in SPM8. Inspection of the grand average ERP waveform in preliminary studies and previous work with this paradigm indicates three components: N1 peak at ~100ms after speech onset, P2 peak at ~200ms after speech onset, and a slow negative component before speech onset from -300 to 0 ms. Mean amplitudes of these components will be extracted at the anterior locus from which each of these three components are robust (collapsed across 26 electrodes surrounding FCz): N1: 80-120ms; P2: 170-210 ms; pre-speech: -300-0 ms. Correlations will be computed between amplitude of the pre-speech component and N1 ERP suppression (difference between N1 amplitude in the Talk vs. Listen conditions).

5.5 Blood Sampling

All subjects will have the option to provide a blood sample as noted in Table 2 to complete both molecular pharmacodynamic and pharmacokinetic studies. Pharmacokinetic data will be limited to a spot pK draw following neurophysiology evaluation approximately 4 to 6 hours following dosing. Molecular assays will be drawn at the same time as pK draw and at Baseline.

5.5.1 Extracellular Signal Related Kinase (ERK) Activation Molecular Biomarker Assay

ERK is a central cellular signaling kinase who dysregulation is implicated in the pathophysiology of FXS and in autism spectrum disorder. Our group has previously utilized ERK activation as a pharmacodynamics marker in FXS drug study (Erickson et al. 2011). For ERK biomarker assays, blood will be drawn at Baseline and 1-2 and 4-6 hours post dose at all drug dosing days. Assays will be analyzed in Dr. Craig Erickson’s Molecular Translational Biomarker Lab at CCHMC. All blood biomarker assays will be performed blinded to study assignment. Blood will be collected and lymphocytes will be isolated, washed, counted, and re-suspended. Samples will be frozen until ERK total and phosphorylated ERK levels will be determined in duplicate using commercially available ELISA kits (Abcam) per manufacture instructions. *All ERK measurements will be attempted, but are not required for study participation.*

5.5.2 Amyloid Precursor Protein (APP) Molecular Biomarker Assay

APP and its derivatives are involved in neuronal growth, maturation, and senescence. APP dysregulation has been implicated in the pathophysiology of FXS and autism of unknown cause. Our group has published on the use of plasma APP derivative analysis in FXS clinical trials (Erickson et al. 2014). For APP biomarker assays assessing plasma levels of sAPP (total), sAPPα, Aβ40, and Aβ42, blood will be drawn at Baseline and 1-2 and 4-6 hours post dose at all drug dosing days. Assays will be analyzed in Dr. Craig Erickson’s Molecular Translational Biomarker Lab at CCHMC. All blood biomarker assays will be performed blinded to study assignment. Plasma will be isolated and platelets will be removed prior to platelet-free plasma storage at -80ºC. Aliquots will be thawed and pre-coated ELISA plates will be used to determine the amount of sAPPα (IBL), sAPP (IBL), and Aβ peptides (Wako Chemical Industries, Japan) in duplicate per manufacturer instructions. *All APP measurements will be attempted, but are not required for study participation.*

5.5.3 Pharmacokinetic (PK) sampling

Pharmacokinetic evaluation blood samples will be drawn 1 to 2 and 4 to 6 hours post dose (max two PK draws per drug dosing day) on each of four drug dosing days. *All PK measurements will be optional and are not required for study participation.*

Acamprosate, minocycline, and lovastatin concentrations will be determined with a low volume, validated liquid chromatography tandem mass spectrometry (LC-MS/MS) assay provided by the iC42 Clinical Research and Development Laboratory (Uwe Christians, MD, PhD, director), University of Colorado, Aurora, Colorado, USA.

Concentration data will be analyzed by compartmental and non-compartmental pharmacokinetic analysis with the software package WinNonlin (Version 4.0.1, Pharsight Corporation, Palo Alto, CA) using a weighed least-squares algorithm. Population PK analysis will be conducted using NONMEM version 7.2.0 (ICON, Ellicott City, MD) on a 64-bit Linux Operation System with an Intel Fortran Compiler (v 12.0). PDx-Pop (version 5, ICON, Ellicott City, MD) will be used as the graphical user interface for running NONMEM and for processing NONMEM output. Visualization of NONMEM output was implemented by Xpose 4 package in R (v 2.15.0.). First order conditional estimation with interaction (FOCE-I) will be employed throughout to simultaneously estimate the typical population PK parameters, random effect of inter-individual variability and residual errors. Model structure selection will be based on goodness-of-fit criteria, including convergence with at least 3 significant digits, diagnostic plots, physiological plausibility of the parameter estimates and Akaike Information Criterion (AIC). Inter-individual variability (IIV) will be modeled using an exponential model which assumes a normally distributed inter-individual variable with a mean of zero and a variance of ω2.

Parameter estimates generated will include Cmax, total body clearance, distribution and elimination half-lives, volume of distribution and the area under the curve (AUC).

6.0 Study Outcome Measures

6.1 Clinical Assessments

- CGI-I: The Clinical Global Impressions Improvement (CGI-I) will be the primary clinical measure completed both before and after the subject receives study medication. The CGI-I requires the clinician to assess how much the patient's illness has changed relative to pre-dose, from 1 (very much improved) to 7 (very much worse).

- ABC: The recently validated Aberrant Behavior Checklist (ABC) using the 6-factor FXS model will be used as a secondary outcome of clinical status. This test will be completed only after the subject receives study medication.

6.2 Cognitive Assessments

To assess cognitive outcomes as a secondary aim, we selected tests with minimal practice effects, good reliability (weighted kappa scores ≥0.7), and a lack of floor effects in persons with FXS with a mental age of 3+ years (Berry-Kravis, Sumis, Kim, Lara, & Wuu, 2008). See Table 1 below for descriptions of each of the tests.

Table 1. Cognitive and neuropsychological assessment measures

Measure	*Domain*	*Task Description*	*Estimated Time*

WJ-III Spatial Relations (McGrew & Woodcock, 2001)	Visual Spatial Thinking	Identify parts needed to form a complete shape. Responses can be oral or motoric (pointing).	5 minutes
RBANS: List Learning (Randolph, 1998)	Immediate Memory	A 10-item list of unrelated words is presented orally to the examinee who is then required to immediately recall words presented.	5 minutes
KiTAP: Test of Attentional Performance for Children (Knox et al., 2012)	Attention and Inhibition	Computerized task comprised of 8 subtests where an examinee is required to push a key when a target stimulus is presented on the screen.	30 minutes
Grooved Pegboard Test	Sensory Motor Integration	Pegs, with a key along one side, must be rotated to match the holes in the pegboard before they can be inserted.	15 minutes
WJ-III Auditory Attention (McGrew & Woodcock, 2001)	Auditory Processing-Speech Sound Discrimination	Identify orally presented words amid increasingly intense background noise. Examinee points to a picture of the word presented.	5 minutes

7.0 Side Effect/Safety Assessments

Safety assessments will include comprehensive metabolic panel (CMP), ANA, complete blood count with differential (CBC with differential), urine drug screen, urine or serum pregnancy test in female participants of childbearing age, physical exam, medical history, concomitant medication review, vital signs at baseline.

At dosing visits, Side Effect/Safety Assessments will include: a general inquire about AE's, a detailed inquiry of common side effects of the 3 study drugs, and vital signs. In an effort to ensure subject safety, we plan to schedule all study drug dosing visits at the CTRC. Phone calls to the patient/caregiver assessing for adverse effects will be completed 1 day following each dose. The Safety Follow-up visit at week 10 will include phone call with physician regarding participants overall health status and a general inquiry about AE's.

Additional study safety oversight will be provided by Dr. Sergio Delgado, Outpatient Clinical Director and Professor with the division of child and adolescent psychiatry at Cincinnati Children's Hospital will serve as independent study medical monitors for the study. The monitors will review recruitment and adverse events every 6 months and report assessment to PI.

8.0 Research Methods

8.1 Criteria for Inclusion of Subjects:

- Subjects ages 15-55, with FXS who completed the study entitled "Mechanisms and brain circuits underlying fragile X syndrome (IRB # 2015-8425). FXS is

defined as full FMR1 mutations (>200 CGG repeats) confirmed by genetic testing.

- General good health as determined by physical exam, medical history and laboratory work up.

8.2 Criteria for Exclusion of Subjects:

- Subjects with a history of intolerance to acamprosate, lovastatin, or minocycline will be excluded.
- Subjects will also be excluded if they have taken any investigational drug within 3 months, have a history of substance abuse or dependence within 6 months, or significant psychiatric or CNS neurological disease unrelated to FXS.
- Uncontrolled seizures impact EEG data as do anticonvulsants, barbiturates, lithium and benzodiazepines and are exclusions (within 5 half-lives). Those taking other psychiatric medications must be on stable doses for 4 weeks before any testing.
- For female subjects of child bearing potential, a positive urine pregnancy test.
- Potential subjects with a creatinine clearance < 50 mL/min will be excluded.
- Identified medical issues, inability to tolerate study procedures or study drug per the discretion of the Principal Investigator.

8.3 Vulnerable Populations:

Inclusion of Children in Research:

One special class of subjects that will be involved in this study is children. FXS is a genetic disorder that is a cause of intellectual disability and neuropsychiatric disorders. A central aim of this study is to examine the effects to investigate single-dose drug effects on event-related potential (ERP) biomarkers and behavior in humans with FXS. This can only be done by studying affected individuals across the age span. We have chosen ages 15-55 to represent a broad developmental spectrum. The upper age of 55 is chosen because of variable and common age-related changes in behavior, sensory function and brain function beyond that age. The age 15 is chosen for the low end of the age range because of the level of cooperation required to complete procedures.

Persons with Cognitive Impairment or other limited decision making capacity

To be inclusive and most generalizable in our study findings, and because some elements of our protocol can be completed with minimal cognitive requirements such as ERP monitoring of cortical responses to tones, we will recruit any study eligible patient with Fragile X Syndrome regardless of level of cognitive disability into as many study components as they can complete. We will recruit all FXS patients who we believe can complete study procedures and use IQ scores to describe study participants in research reports.

Inclusion of Women and Minorities:

No person shall be excluded from participation in this research study on the basis of gender alone. However, FXS occurs at a higher rate in males than in females. Therefore, it is anticipated that the largest number of proband participants will be male. Every effort will be

made to recruit and include minorities among the participants in this research study. In regard to gender and minority representation, our research cohort will reflect consecutive referrals to our clinic meeting the inclusion-exclusion criteria of the study. Recruitment is expected to be 30% minority because of the population our clinics serve.

9.0 Sources of Research Material:

In addition to sociodemographic information, we may collect sensitive clinical information including information about medical history, drug use, psychiatric symptoms and developmental history during individual testing sessions and via questionnaires completed during pre-screening or testing sessions. This information can be collected as part of structured and semi-structured interviews, self-report and caregiver questionnaires, and clinical tests of behavioral functioning.

Neuropsychological and electrophysiological data may be acquired during individual testing sessions. Medical records may be obtained after obtaining a signed permission for release of records.

All information collected is for research purposes only. If some assessments were recently done in the clinic by our faculty before recruitment, then we will use the existing clinical data to reduce patient burden; but we expect this overlap to be rare. All material will be protected by limited access and stored under a unique ID number.

10.0 Recruitment Methods and Consenting Process:

10.1 Recruitment

Recruitment for the study will be conducted by the Cincinnati Fragile X Research and Treatment Center Subjects ages 15-55, will be recruited from participants who have completed the study entitled “Mechanisms and brain circuits underlying fragile X syndrome (IRB #2015-8425).

10.2 Informed Consent Process

Screening and consent procedures for the study will be completed prior to enrollment. Participants (if they have decisional capacity) or legally authorized representatives together with participants initially will receive written materials and be given an oral explanation of the study. Informed consent procedures will be conducted by research staff or investigators. In addition, members of the protocol team will be available to address any questions for participants or families.

Each participant must give informed consent or assent to participate when possible. Given the developmental disability associated with FXS, particularly in males, some subjects themselves will not be able to provide assent or consent. Consent also will be provided by the guardian of minors and from adult subjects who are not their own legal guardian.

Participants (and if they have one, their legally authorized representative) will be informed during their initial consent, prior to the initiation of the pharmacologic study, and on several additional occasions throughout their experience in the study that they may elect to not to participate at any time.

Consent and assent will be collected using the procedures outlined above. This will take place after initial screening but before any formal assessments and experimental procedures begin.

Both written and oral information will be provided to participants and legally authorized representatives. Refusal to participate in the study will not limit their ability to seek services at Cincinnati Children's Hospital Medical Center. One signed and witnessed copy of the consent and assent forms will be stored in secure storage and the participant and legally authorized representative will each be given a copy of the signed forms, as is appropriate. Participants will receive payment for taking part in this study.

Some individuals may be interested in study participation but live far away from CCHMC. In these circumstances, the consent forms will be mailed to potential participants after they have initiated contact with our research program. Our research team will arrange for a conversation with the potential participant by phone after the documents have been received. During the phone conversation, our research team member will review the consent form (i.e., study purpose, voluntary participation, study procedures, risks and benefits, and confidentiality) and answer any questions. Then, the participant will sign the documents and mail the documents back to the researcher. Once the researcher at our Center receives them, he/she will sign the consent forms and return copies of the documents to the participant for their records and place the originals in a secure area.

If a subject turns 18 years-old while enrolled in the study, the parent(s)/legal guardian(s) will be asked to provide appropriate documentation indicating that they are actively seeking legal guardianship. Copies of documentation will be filed in the subject's study chart. If parent(s)/legal guardian(s) are not pursuing legal guardianship when the subject turns 18 years-old, then the subject will need to demonstrate the capacity to provide informed consent and voluntarily sign the informed consent document in order to continue participation in the study.

11.0. Potential Risks:

11.1 Study Drugs

All participants will receive single doses of 3 FDA-approved drugs (acamprosate, lovastatin, minocycline) that have proven benign safety profiles during chronic treatment. **Minocycline and acamprosate were well-tolerated in studies of humans with FXS** (Erickson et al., 2013; Leigh et al., 2013). Lovastatin, not tested in humans with FXS, is one of the most widely prescribed medicines worldwide. At the doses proposed for this study the incidence of adverse effects is low. Furthermore the administration of a single dose will limit the risk for side effects that might emerge with longer term treatment. The study PI (Dr. Erickson) has experience in the use of pharmacotherapy in persons with FXS. All participants will have a careful medical examination and detailed monitoring of side effects in the Fragile X clinic.

11.1.1 Acamprosate

Common side effects (more than 1 in 100 patients):

- headache, abdominal pain, back pain, infection, flu syndrome, chest pain, chills, suicide attempt
- palpitations (feelings of having rapid, fluttering or pounding heartbeats), fainting
- vomiting, upset stomach, constipation, increased appetite
- swelling of the legs and feet, weight gain
- muscle pain, joint pain
- sleepiness, decreased interest in sex, memory loss, thinking abnormal, shaking,

vasodilatation, high blood pressure
- stuffy nose, cough increased, shortness of breath, sore throat, bronchitis (lung infection)
- rash
- abnormal vision, change in sense of taste
- difficulty in getting or keeping an erection

Less common side effects (between 1 in 1000 patients and 1 in 100 patients):
- fever, intentional overdose, bodily discomfort, allergic reaction, abscess, neck pain, hernia (part of an internal organ or tissue that bulges through a weak area of muscle), intentional self-injury
- low blood pressure, fast or irregular heart rate, bleeding, angina pectoris (chest discomfort due to poor blood flow through the blood vessels in the heart), migraine, varicose vein, heart attack, phlebitis (vein inflammation), a drop in blood pressure when getting up from a seated position or lying down
- abnormal liver function tests, stomach flu, gastritis (inflammation of the stomach lining), difficulty with swallowing , burping, bleeding of the stomach or intestines, pancreatitis (inflammation of the pancreas), rectal bleeding, liver cirrhosis (scarring of the liver), inflammation of the esophagus, vomiting blood, nausea and vomiting, hepatitis (inflammation of the liver)
- anemia (low red blood cell count), bruising, eosinophilia (higher than normal level of a type of white blood cells, which could be a sign of an allergic-type drug reaction), lymphocytosis (an increase in a type of white blood cells, which could be a sign of an allergic-type drug reaction), thrombocytopenia (abnormally low amount of platelets, which can increase risk of bleeding)
- weight loss, high blood sugar and diabetes, (increase in chemicals made by the liver (a sign of liver injury), , hyperuricemia (high level of uric acid), gout (type of arthritis caused by too much uric acid in blood), thirst, low vitamin levels, bilirubinemia (presence of excess bilirubin in the blood, which can be sign of liver dysfunction)
- leg cramps
- convulsion, confusion, increased interest in sex, dizziness, withdrawal syndrome, general lack of interest, suicidal thoughts, neuralgia (nerve pain), hostility, agitation (a state of marked excitement or restlessness), unrealistic thoughts, abnormal dreams, hallucinations, hypesthesia (a reduced sense of touch or sensation)
- asthma, nosebleed, pneumonia;
- acne, eczema (skin irritation condition), hair loss, maculopapular rash (flat discolored area of the skin covered with small bumps), dry skin, hives, exfoliative dermatitis (severe inflammation of the entire skin surface, vesiculobullous rash (a rash like a blister),
- ringing in the ears, lazy eye, deafness;
- metrorrhagia (bleeding from the uterus between menstrual periods), frequent urination, urinary tract infection, sexual dysfunction, loss of bladder control, vaginitis (inflammation of the vagina)

Rare side effects (less than 1 in 1000 patients):
- ascites (build-up of fluid inside the abdomen), face swelling, photosensitivity reaction (sensitivity to sunlight), abdomen enlarged, sudden death
- heart failure, closing of an artery of the intestines, cardiomyopathy (weakening of the heart muscle), deep thrombophlebitis (a blood clot blocking one or more of your veins), shock (dangerously low blood pressure)
- melena (black stools from bleeding), stomach ulcer, inflammation of the gallbladder, inflammation of the colon, duodenal ulcer (an ulcer in the first part of the small intestine),

mouth ulceration, live cancer
- goiter (enlargement of the thyroid gland), hypothyroidism (underactive thyroid)
- leukopenia (low white blood cell count, which could increase the risk of infection), swollen or enlarged lymph nodes, monocytosis (increase in number of particular type of white blood cells, which could be a sign of an allergic-type drug reaction).
- alkaline phosphatase increased (a possible sign of liver, bone or gall bladder injury), creatinine increased (a possible sign of kidney injury), low sodium, lactic dehydrogenase increased (a possible sign of tissue injury)
- rheumatoid arthritis, muscle injury
- alcohol craving, psychosis, excessive abnormal movements, twitching, depersonalization (periods of feeling disconnected or detached from one's body and thoughts), increased salivation, paranoid reaction, torticollis (a muscle spasm of the neck in which the head is tipped to one side)), brain disturbance resulting in confusion, manic reaction (elevated, expansive, or irritable mood)
- laryngismus (spasm of the throat, which can cause difficulty breathing), blood clot of the vessels in the lung
- psoriasis (a skin condition that causes skin redness and irritation)
- eye inflammation, double vision, sensitivity of eyes to light
- kidney stone, abnormal ejaculation, blood in urine, an abnormally heavy and prolonged menstrual period, a need to get up at night to urinate, excessive urination, a sudden urge to urinate

11.1.2 Lovastatin

Common side effects (more than 1 in 100 patients):
- weakness
- abdominal pain, constipation, diarrhea, upset stomach, flatulence, nausea
- muscle cramps, muscle pain
- dizziness, headache
- rash
- blurred vision

Less common side effects (between 1 in 1000 patients and 1 in 100 patients):
- chest pain
- acid regurgitation (“heartburn”), dry mouth, vomiting
- leg pain, shoulder pain, joint pain
- insomnia (difficulty falling or staying asleep), paresthesia (numbness and tingling)
- hair loss, itching
- eye irritation

Rare side effects (less than 1 in 1000 patients):
Myopathy/Rhabdomyolysis
Lovastatin, like other “statin” cholesterol-lowering medicines, can cause myopathy (muscle injury) which shows as muscle pain, tenderness or weakness with an increase of creatine kinase (“CK” a protein leaked from the muscles when injured) to ten times the normal limit. Myopathy sometimes takes the form of rhabdomyolysis (high levels of muscle proteins leaked to the bloodstream) with or without acute kidney failure, and rare fatalities have occurred. The risk of myopathy is increased by high levels of “statin” drug inhibitory activity in plasma.

As with other "statin" cholesterol-lowering medicines, the risk of myopathy/rhabdomyolysis is dose related. In a clinical study (EXCEL) in which patients were carefully monitored and some interacting drugs were excluded, there was one case of myopathy among 4933 patients randomized to lovastatin 20-40 mg daily for 48 weeks, and 4 among 1649 patients randomized to 80 mg daily.

There have been rare reports of immune-mediated necrotizing myopathy (IMNM), an autoimmune myopathy, associated with statin use. IMNM is characterized by: proximal muscle weakness and elevated serum creatine kinase, which persist despite discontinuation of statin treatment; muscle biopsy showing necrotizing myopathy without significant inflammation; improvement with immunosuppressive agents.

All patients starting therapy with lovastatin, or whose dose of lovastatin is being increased, should be advised of the risk of myopathy and told to report promptly any unexplained muscle pain, tenderness or weakness particularly if accompanied by malaise or fever or if muscle signs and symptoms persist after discontinuing lovastatin. Lovastatin therapy should be discontinued immediately if myopathy is diagnosed or suspected. In most cases, muscle symptoms and CK increases resolved when treatment was promptly discontinued. Periodic CK determinations may be considered in patients starting therapy with lovastatin or whose dose is being increased, but there is no assurance that such monitoring will prevent myopathy.

Many of the patients who have developed rhabdomyolysis (high levels of muscle proteins leaked to the bloodstream) on therapy with lovastatin have had complicated medical histories, including kidney disease usually as a consequence of long-standing diabetes. Such patients should have closer monitoring. Lovastatin therapy should be discontinued if markedly elevated CPK levels occur or myopathy is diagnosed or suspected. Lovastatin therapy should also be temporarily withheld in any patient experiencing an acute or serious condition predisposing to the development of kidney failure secondary to rhabdomyolysis, e.g., sepsis (infection in the bloodstream); hypotension (low blood pressure); major surgery; trauma; severe metabolic, endocrine, or electrolyte disorders; or uncontrolled epilepsy.

11.1.3 Minocycline ER

Common side effects (more than 1 in 100 patients):

- headache
- fatigue
- dizziness
- itching
- bodily discomfort
- mood change
- sleepiness
- hives
- tinnitus (ringing in the ears)
- joint pain
- dizziness
- dry mouth
- muscle pain

Less Common or Rare (occurrence rates not further specified in PI), but potentially serious side effects

Teratogenic Effects

Minocycline can cause fetal harm when taken by women during pregnancy. Minocycline should not be used during pregnancy or by individuals of either gender who are attempting to conceive a child.

The use of minocycline during tooth development (last half of pregnancy, infancy and childhood to the age of 8 years) may cause permanent discoloration of the teeth (yellow-gray-brown). This adverse reaction is more common during long-term use of the drug but has been observed following repeated short-term courses. Enamel hypoplasia (a defect of the teeth in which the enamel is hard but thin and deficient in amount, which can cause abnormally shaped teeth) has also been reported. Minocycline, therefore, should not be used during tooth development.

All tetracyclines (the class of drug that minocycline is in) form a stable calcium complex in any bone-forming tissue. A decrease in fibula (a bone in the leg) growth rate has been observed in premature human infants given oral tetracycline in doses of 25 mg/kg every 6 hours. This reaction was shown to be reversible when the drug was discontinued.

Results of animal studies indicate that tetracyclines (like minocycline) cross the placenta, are found in fetal tissues, and can cause retardation of skeletal development on the developing fetus. Evidence of embryotoxicity has been noted in animals treated early in pregnancy.

Pseudomembranous Colitis

Note, the risk of pseudomembranous colitis typically occurs only with repeated dosing of antibiotics over days or weeks (just a single dose of minocycline will be taken in this study, thus the risk for pseudomembranous colitis is exceedingly low). A description of pseudomembranous colitis, as usually only seen with repeated dosing follows.

Clostridium difficile associated diarrhea (CDAD) has been reported with nearly all antibacterial agents, including minocycline, and may range in severity from mild diarrhea to fatal colitis. Treatment with antibacterial agents alters the normal flora of the colon leading to overgrowth of C. difficile. C. difficile produces toxins A and B which contribute to the development of CDAD. Hypertoxin producing strains of C. difficile cause increased morbidity and mortality, as these infections can be refractory to antimicrobial therapy and may require colectomy. CDAD must be considered in all patients who present with diarrhea following antibiotic use. Careful medical history is necessary since CDAD has been reported to occur over two months after the administration of antibacterial agents. If CDAD is suspected or confirmed, ongoing antibiotic use not directed against C. difficile may need to be discontinued. Appropriate fluid and electrolyte management, protein supplementation, antibiotic treatment of C. difficile, and surgical evaluation should be instituted as clinically indicated.

Hepatotoxicity

Post-marketing cases of serious liver injury, including irreversible drug-induced hepatitis and fulminant hepatic failure (sometimes fatal) have been reported with minocycline use in the treatment of acne.

Metabolic Effects

The anti-anabolic action of the tetracyclines (like minocycline) may cause an increase in blood urea nitrogen (BUN). A BUN test measures the amount of nitrogen in blood that comes from the waste product urea. Urea is made when protein is broken down in the body. While an increase in BUN is not a problem in those with normal kidney function, in patients with significantly impaired kidney function, higher serum levels of minocycline-class drugs may lead to azotemia (high levels of nitrogen-containing waste products in the blood stream),

hyperphosphatemia (high levels of phosphate), and acidosis (which can disturb normal functioning of the body).

Central Nervous System Effects
Central nervous system side effects including light-headedness, dizziness or vertigo have been reported with minocycline therapy. Patients who experience these symptoms should be cautioned about driving vehicles or using hazardous machinery while on minocycline therapy. These symptoms may disappear during therapy and usually rapidly disappear when the drug is discontinued.

Benign Intracranial Hypertension
Pseudotumor cerebri (benign intracranial hypertension/pressure) in adults and adolescents has been associated with the use of tetracyclines like minocycline. Minocycline has been reported to cause or precipitate pseudotumor cerebri, the hallmark of which is papilledema. Clinical manifestations include headache and blurred vision. Bulging fontanels have been associated with the use of tetracyclines in infants. Although signs and symptoms of pseudotumor cerebri resolve after discontinuation of treatment, the possibility for permanent problems such as visual loss that may be permanent or severe exists. Patients should be questioned for visual disturbances prior to initiation of treatment with tetracyclines. If visual disturbance occurs during treatment, patients should be checked for papilledema.

Autoimmune Syndromes
Tetracyclines like minocycline have been associated with the development of autoimmune syndromes (a condition that occurs when the immune system mistakenly attacks and destroys healthy body tissue). The long-term use of minocycline in the treatment of acne has been associated with drug-induced lupus-like syndrome (condition that mimics the symptoms of systemic lupus erythematosus, or SLE, but is not actually lupus). Lupus-like syndrome can cause rash (especially of the face), skin sensitivity to sunlight, fatigue, joint pain and arthritis, and overall feeling of sickness. Long-term use of minocycline can also cause autoimmune hepatitis (autoimmune reaction to the liver) and vasculitis (autoimmune reaction to the blood vessels). Rare cases of serum sickness have presented shortly after minocycline use, including symptoms of fever, rash, arthralgia, and malaise.

Photosensitivity
Photosensitivity manifested by an exaggerated sunburn reaction has been observed in some individuals taking tetracyclines. This has been reported rarely with minocycline.

Serious Skin/Hypersensitivity (allergic) Reaction
Cases of anaphylaxis (severe, whole-body allergic reaction), serious skin reactions, and drug rash with eosinophilia (type of white blood cell) and systemic (whole-body) symptoms (DRESS) syndrome have been reported post marketing with minocycline use in patients with acne. DRESS syndrome consists of skin reaction (which is sometimes severe), eosinophilia, and one or more of the following: organ complications such as inflammation of the liver, lungs, kidneys or heart. Fever and swelling of the lymph nodes may be present. In some cases, death has been reported.

Tissue Hyperpigmentation
Note, this side effect is exceedingly unlikely to occur because it tends to occur only with prolong taking of minocycline over days, weeks, or months (and just a single dose of minocycline will be taken in this study). Tetracycline-class antibiotics like minocycline are known to cause hyperpigmentation (darkening pigment). Minocycline may induce

hyperpigmentation in many organs, including nails, bone, skin, eyes, thyroid, visceral tissue, oral cavity (teeth, linings of the mouth, bone), sclerae ("whites" of the eye) and heart valves. Skin and oral pigmentation has been reported to occur independently of time or amount of drug administration, whereas other tissue pigmentation has been reported to occur upon prolonged administration. Skin pigmentation includes diffuse pigmentation as well as over sites of scars or injury.

Superinfection
As with other antibiotic preparations, use of minocycline may result in overgrowth of non-susceptible organisms, including fungi.

11.2 Cognitive Testing

There are minimal risks anticipated to individuals participating in observational or clinical behavioral assessments. In the rare case that an individual becomes overly fatigued or distressed during assessment sessions, the session will be discontinued and rescheduled. Evaluation procedures, when appropriate, are designed in a way that is compatible with the attention span of a person with an intellectual disability, greater activity level, and need for access to his or her parents. In addition, specialized procedures for children with FXS (e.g., reducing language demands, providing praise for task attention and attempts, using work-reward routines) will be implemented. Three clinical psychology investigators with extensive experience working clinically with developmentally disabled patients are investigators in the study.

11.3 Neurophysiology Procedures

There are minimal risks associated with participation in the neurophysiology part of the study. The auditory stimuli presented are relatively benign, and have been well tolerated in our pilot studies. We are sensitive to monitoring participant distress associated with sensory paradigms, which we have found more commonly in participants younger than 12 years of age. The most common risk associated with these tasks is boredom. In rare circumstances the electrode gel used to place the recording electrodes to the scalp can cause minor irritation to the skin, which resolves rapidly with no permanent effects.

11.4 Pregnancy and Protection against Risk

Females of childbearing age (as determined by report regarding age of menarche or menopause) enrolling in the pharmacologic study will be required to take a urine pregnancy test before study procedures. A positive test will exclude them from participating in this portion of the study. The PI or his designee will discuss with the subject in private the results of any drug screen and/or pregnancy test and if positive, that they will no longer be able to participate in the study. Consent forms will make clear that this information will be shared with parents of minors. A subject who tests positive will be referred to appropriate medical care.

11.5 Risk of Discrimination

There is a theoretical risk for discrimination towards individuals who are at risk for a medical disorder or have a medical disorder in their family. Potential discrimination may include barriers to insurability, employability, or other unidentified adverse effects. Extensive efforts are made to protect all research subjects from prejudice, discrimination, or uses of this information that will adversely affect them.

11.6 Venipuncture Procedures

The risks of venipuncture are modest and include mild discomfort, infection, bleeding, and fainting. Standard methods and precautions will be used to protect the puncture site from bleeding and infection. The Research Coordinator or Research Assistant will be familiar with the subjects and will accompany the subject and their parents to the blood drawing setting. Parents are encouraged to remain with the subject at all times. To minimize the subject's anxiety and phobic reactions, we utilize Child Life personnel when needed and available. We also suggest that the parents reassure the subject concerning their safety. At the discretion of the nurse or the investigator, to help reduce pain at the site of the venipuncture, we will offer the use of a topical anesthetic.

12.0 Procedures for Protecting Against and Minimizing Potential Risks

Effective screening will be used to eliminate subjects who are at greatest risk because of concurrent medical conditions. The subjects will be evaluated and cared for in an advanced well-staffed pediatric neuropsychiatric research environment. Thus, the direct observation by nursing staff and research psychiatrists will allow for careful monitoring of potential adverse effects including drug side effects. If adverse reactions become excessive, the subject will be treated and removed from the study. There will be repeated monitoring of behavior and vital signs that will allow the treatment team to assess the status of the subject and alter or terminate the study if this is warranted. All of the research data is kept in locked files to ensure confidentiality. The other procedures to ensure confidentiality follow the regulations and policies of the Medical Center.

All SAEs will be reviewed in detail by the PIs and Independent Safety Monitor. If the stopping rule is met, the study will close to enrollment for interim analysis of safety and efficacy data and the Safety Monitor would provide a recommendation to the PI regarding continuation of the trial.

13.0. Potential Benefits:

Subjects may or may not benefit directly from study participation. The possibility of contributing to the discovery of treatments for FXS may benefit all affected individuals and their families. The following knowledge can be gained from this research: a more detailed understanding of auditory processing abnormalities in FXS, a better understanding of the pathophysiology of FXS, new perspectives on pharmacologic mechanisms in FXS, and insight into possible biomarkers that can be used to track drug effects in FXS.

The potential gain in knowledge can change our clinical understanding and treatment of FXS and related disorders. This could lead to more effective treatments and possibly earlier identification and personalized treatment of at-risk children. Given the severity of impairments associated with FXS, this could dramatically impact the lives of affected individuals and their families. Given the potential for gain, the relatively modest risks are reasonable for the study participants, most of whom stand to receive modest personal gains by participating in the research.

13.1 Risk vs Benefit Ratio

The subjects will be exposed to the risks of blood sampling and the potential side effects of acamprosate, lovastatin, or minocycline. For the patients, the benefits potentially offsetting this will be a more intensive and thorough psychiatric and medical evaluation, a documented objective treatment trial, and the possibility of more accurate prescription of treatment designed to meet the individual subject's needs. Since some of the subjects will have had previous drug trials with poor response or intolerable or dangerous side effects, the opportunity for a more thorough evaluation and clinical trial may be beneficial. Thus, with the risk of drug treatment minimized, the more intensive evaluation and treatment may compensate for the negative risks. The overall benefit to family members and society is considerable.

14.0. Subject Safety and Data Monitoring:

Dr. Sergio Delgado, Outpatient Clinical Director with the division of child and adolescent psychiatry at Cincinnati Children's Hospital Medical Center (CCHMC), will serve an independent study monitor for the study. The PI and Co-investigators at CCHMC will be primarily responsible for monitoring data quality and adverse events. A physician will monitor adverse effects at each visit. In addition, he or she will review vital signs and laboratory data, as they become available. All of these values are reviewed continuously by a physician. The monitor will review recruitment and adverse events every 6 months and report their assessment to the PI. The independent monitor will also review any SAEs and significant unanticipated events as they occur.

All problems (i.e. adverse events, unanticipated events, etc.) will be characterized by the following:

- Severity = Mild, Moderate or Severe
- Relatedness = Definite, Probable, Possible, Unlikely or Unrelated
- Expectedness = Expected or Not Expected

A stopping rule for excessive toxicity is in place in the event of ≥ 2 SAEs considered possibly, probably or definitely related to treatment on study. All SAEs will be reviewed in detail by the PIs and Independent Safety Monitor. If the stopping rule is met, the study will close to enrollment for interim analysis of safety and efficacy data and the Safety Monitor would provide a recommendation to the PI regarding continuation of the trial.

Events will be reported consistent with Institutional IRB reporting policies. An aggregate listing of all safety events will be reported to the IRB in summary form at the time of continuing review. These reports will also be provided to any applicable regulatory agencies per applicable regulatory agency requirements. Data (such as lab values, vital signs, and outcome measure data) will be entered from source documents to Case Report Forms by the study coordinator. The study coordinator will review case report form entries for accuracy by comparison with the source documents. Research records and source documents will be maintained in a research chart and stored in the investigator's locked file cabinet. Information will be kept in a secure database that is password protected. Records will be kept secure, and individually identifiable information will not be included in any reports or data sets.

All subjects will be assured that data will be kept confidential. Data will be identified only by a unique identification number and not by the subject's name. Paper data will be stored in a

locked office in a locked cabinet to which only staff affiliated with the project will have access. Any discrepancies in the maintenance of confidentiality or other irregularities involving the data will be reported to the PI. Any such events will be documented and reviewed by the PI and reported to the IRB within a timeframe set out by guidelines set forth by the CCHMC IRB.

14.1 Adverse Events

Any adverse events--whether due to assessment, medication or other aspects of the study--will be reported to the PI. Adverse events will be documented and reviewed by the PI within 24 hours of receipt. Serious/unanticipated events will be reported to the IRB within 48 hours by phone, email, or fax. All adverse events will be compiled, and reported in summary form to the IRB, on an annual basis and at the conclusion of the study.

15.0 Procedures to Maintain Confidentiality:

Unique numeric identifiers that do not include any PHI will be assigned. All data will be maintained either in locked storage facilities with limited access or in secure, electronic facilities. Data transmitted electronically will be protected by encryption over a very secure network that limits access only to CCHMC faculty and staff who are reviewed and specifically cleared for access to the password protected, secure network. The data will be stored in protected hard drives in a server system that is protected by two firewalls, one at the hospital level and a second at the Departmental level. De-identified data will be shared with the national database for ASD research (NDAR) as may be required as part of NIH funding via this mechanism, using a Global Unique Identifier (GUID) and the Data Dictionary technology developed by NIH. We have successfully used these methods during the past 5 years to share behavioral phenotyping and genetic information (a more detailed description of data sharing procedures is below).

15.1 Data Management

Data will be collected on paper source documents, CRFs, and/or in the electronic medical record and will be entered by study staff in a password protected, encrypted REDCap[64] database. The REDCap database will be built upon a developmental disability-focus clinical trial REDCap database. REDCap data collection projects rely on a thorough study-specific data dictionary defined in an iterative self-documenting process by the research team. REDCap provides a secure, web-based application that is flexible and provides 1) an intuitive interface for users to enter data and have real time validation rules (with automated data type and range checks) at the time of entry; 2) HIPAA-compliant and 21 CFR Part 11-ready audit trails for tracking page views, data manipulation and export procedures; 3) record locking and electronic signature functions; 4) fine grained control of user rights to view and manipulate data; 5) a report builder for reporting, monitoring and querying patient records; 6) automated export procedures for seamless data downloads.

16.0 Biostatistics:

The sample size analysis was based on a primary outcome of N1 amplitude. This is the best established ERP abnormality and the only validated ERP drug outcome measure in FXS patients. The estimate of effect size was drawn from a double-blind trial of minocycline vs. placebo in FXS on N1 amplitudes (Schneider et al., 2013). Assuming that omnibus drug effect size of d~0.68, and no carryover or period effects, then 36 patients (9 in each of 4 drug sequences) provides power of 0.80 with α of 0.05 (two-tailed) ("Power Analysis and Sample

Size (PASS)," 2008). Patients will be recruited from the STU 052013-043 population until 36 patients have completed the pharmacologic study.

A 4-drug, 4-period, 4-sequence balanced-crossover design (Williams, 1989) will be used for the proposed pharmacologic study. The primary ERP outcome will be N1 amplitude. Secondary outcomes will be N1 habituation, auditory evoked gamma power in the AM modulated chirps, and N1 amplitude reduction during the talk/listen task, and clinical and cognitive outcomes.

The analysis will be a linear mixed model analysis of repeated measures. A separate mixed model analysis will be conducted on each outcome. If deemed necessary, the outcomes will be appropriately transformed to obtain a more normal distribution. The fixed effects of drug, drug sequence, and drug period will be examined. Potential carryover effects of drug will be estimated in the mixed model and, if significant, the drug effects will be adjusted. Patients will be nested within drug sequence and accounted for as a random effect in the mixed model. The intercept will be included as a random effect (if it improves model fit). Sex, age, and IQ will be considered as covariates in the respective mixed models, as will baseline clinical and cognitive data for analyses of those parameters. Restricted maximum likelihood estimation, Type 3 tests of fixed effects, and generalized least-squares will be used, with the Kenward-Roger correction (Kenward & Roger, 1997) applied to the appropriate and best fitting covariance structure. The robust covariance matrix estimator will also be considered. The six pairwise differences of least squares means among the drug effects will be estimated, and these comparisons will be carried out using the Tukey multiple comparison procedure. Hedges' $\hat{g}$ will be calculated and interpreted as the effect size estimator for the pairwise contrasts between the drug effects. Findings will be examined in relation to *Fmr1* gene expansion and methylation. Baseline and changes in neurophysiologic measures will be examined for relationships to cognitive and clinical outcomes as an exploratory evaluation of these variables as potential biomarkers of drug effects in FXS. The mixed model procedures of PROC MIXED in SAS software (SAS Institute, Inc., Cary, NC) will be used to conduct the mixed model analysis making use of all available data from all participants to provide a robust mechanism for handling data that are assumed missing at random (Little, 1995) (Mallinckrodt et al., 2003) (Rubin, 1976) (Wolfinger & Chang, 1995). The level of significance for all tests will be set at $\alpha = .05$ (two-tailed) and, to address multiple testing, p-values will be adjusted using the False Discovery Rate (Benjamini & Hochberg, 1995).

17.0 Subject Cost and Payment:

Subjects will be paid $100 on a ClinCard for their time and transportation at the end of each fully completed visit. Other than cost of travel to and from visits and potential time away from work for caregivers, we anticipate no other costs for subjects associated with study participation.

References:

Benjamini, Y, & Hochberg, Y. (1995). Controlling the flase discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, 57*, 289-300.

Berry-Kravis, E., Hessl, D., Coffey, S., Hervey, C., Schneider, A., Yuhas, J., . . . Hagerman, R. (2009). A pilot open label, single dose trial of fenobam in adults with fragile X syndrome. *J Med Genet, 46*(4), 266-271. doi: 10.1136/jmg.2008.063701

Berry-Kravis, E., Sumis, A., Kim, O. K., Lara, R., & Wuu, J. (2008). Characterization of potential outcome measures for future clinical trials in fragile X syndrome. *J Autism Dev Disord, 38*(9), 1751-1757. doi: 10.1007/s10803-008-0564-8

Bilousova, T. V., Dansie, L., Ngo, M., Aye, J., Charles, J. R., Ethell, D. W., & Ethell, I. M. (2009). Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model. *J Med Genet, 46*(2), 94-102. doi: 10.1136/jmg.2008.061796

Boeijinga, P. H., Parot, P., Soufflet, L., Landron, F., Danel, T., Gendre, I., . . . Luthringer, R. (2004). Pharmacodynamic effects of acamprosate on markers of cerebral function in alcohol-dependent subjects administered as pretreatment and during alcohol abstinence. *Neuropsychobiology, 50*(1), 71-77. doi: 10.1159/000077944

Budimirovic, D. B., & Kaufmann, W. E. (2011). What can we learn about autism from studying fragile X syndrome? *Dev Neurosci, 33*(5), 379-394. doi: 10.1159/000330213

Crawford, D. C., Acuna, J. M., & Sherman, S. L. (2001). FMR1 and the fragile X syndrome: human genome epidemiology review. *Genet Med, 3*(5), 359-371. doi: 10.109700125817-200109000-00006

Dansie, L. E., Phommahaxay, K., Okusanya, A. G., Uwadia, J., Huang, M., Rotschafer, S. E., . . . Ethell, I. M. (2013). Long-lasting effects of minocycline on behavior in young but not adult Fragile X mice. *Neuroscience, 246*, 186-198. doi: 10.1016/j.neuroscience.2013.04.058

Erickson, C. A., Wink, L. K., Ray, B., Early, M. C., Stiegelmeyer, E., Mathieu-Frasier, L., . . . McDougle, C. J. (2013). Impact of acamprosate on behavior and brain-derived neurotrophic factor: an open-label study in youth with fragile X syndrome. *Psychopharmacology (Berl), 228*(1), 75-84. doi: 10.1007/s00213-013-3022-z

Erickson CA, Weng N, Weiler IJ, Greenough WT, Stigler KA, Wink LK, McDougle CJ. Open-label riluzole in fragile X syndrome. Brain Res. 2011 Mar 22;1380:264-70. doi: 10.1016/j.brainres.2010.10.108. Epub 2010 Nov 5. PubMed PMID: 21059347.

Erickson CA, Ray B, Maloney B, Wink LK, Bowers K, Schaefer TL, McDougle CJ, Sokol DK, Lahiri DK. Impact of acamprosate on plasma amyloid-β precursor protein in youth: a pilot analysis in fragile X syndrome-associated and idiopathic autism spectrum disorder suggests a pharmacodynamic protein marker. J Psychiatr Res. 2014 Dec;59:220-8.

Guy, W. (ed). (1976). *ECDEU Assessment for Psychopharmacology, Revised Edition*. Rockville, MD: NIMH Publication.

Harris, B. R., Prendergast, M. A., Gibson, D. A., Rogers, D. T., Blanchard, J. A., Holley, R. C., . . . Littleton, J. M. (2002). Acamprosate inhibits the binding and neurotoxic effects of trans-ACPD, suggesting a novel site of action at metabotropic glutamate receptors. *Alcohol Clin Exp Res, 26*(12), 1779-1793. doi: 10.1097/01.ALC.0000042011.99580.98

Kenward, M. G., & Roger, J. H. (1997). Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics, 53*(3), 983-997.

Knox et al.: Feasibility, reliability, and clinical validity of the Test of Attentional Performance for Children (KiTAP) in Fragile X syndrome (FXS). *Journal of Neurodevelopmental Disorders* 2012 4:2.

Lang, N., Rothkegel, H., Terney, D., Antal, A., & Paulus, W. (2013). Minocycline exerts acute inhibitory effects on cerebral cortex excitability in humans. *Epilepsy Res, 107*(3), 302-305. doi: 10.1016/j.eplepsyres.2013.09.006

Leigh, M. J., Nguyen, D. V., Mu, Y., Winarni, T. I., Schneider, A., Chechi, T., . . . Hagerman, R. J. (2013). A randomized double-blind, placebo-controlled trial of minocycline in children and adolescents with fragile x syndrome. *J Dev Behav Pediatr, 34*(3), 147-155. doi: 10.1097/DBP.0b013e318287cd17

Little, RJA. (1995). Modeling the drop-out mechanism in repeated-measures studies. *Journal of the American Statistical Association, 90*, 1112-1121.

Mallinckrodt, C. H., Sanger, T. M., Dube, S., DeBrota, D. J., Molenberghs, G., Carroll, R. J., . . . Tollefson, G. D. (2003). Assessing and interpreting treatment effects in longitudinal clinical trials with missing data. *Biol Psychiatry, 53*(8), 754-760.

McGrew, K., & Woodcock, RW. (2001). *Woodcock-Johnson III Technical Manual*. Itasca, IL: Riverside Publishing.

Osterweil, E. K., Chuang, S. C., Chubykin, A. A., Sidorov, M., Bianchi, R., Wong, R. K., & Bear, M. F. (2013). Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile X syndrome. *Neuron, 77*(2), 243-250. doi: 10.1016/j.neuron.2012.01.034

Pietrzak, B., & Czarnecka, E. (2005). Effect of the combined administration of ethanol and acamprosate on rabbit EEG. *Pharmacol Rep, 57*(1), 61-69.

Porter, R. H., Jaeschke, G., Spooren, W., Ballard, T. M., Buttelmann, B., Kolczewski, S., . . . Malherbe, P. (2005). Fenobam: a clinically validated nonbenzodiazepine anxiolytic is a potent, selective, and noncompetitive mGlu5 receptor antagonist with inverse agonist activity. *J Pharmacol Exp Ther, 315*(2), 711-721. doi: 10.1124/jpet.105.089839

Power Analysis and Sample Size (PASS). (2008). Kaysville, Utah: NCSS, Inc.

Randolph, C. (1998). *Repeatable battery for assessment of neuropsychological status (RBANS)*. San Antonio, Tx: The Psychological Corporation, Harcourt Brace Company.

Rubin, DB. (1976). Inference and missing data. *Biometrika, 63*, 581-592.

Sansone, S. M., Widaman, K. F., Hall, S. S., Reiss, A. L., Lightbody, A., Kaufmann, W. E., . . . Hessl, D. (2012). Psychometric study of the Aberrant Behavior Checklist in Fragile X Syndrome and implications for targeted treatment. *J Autism Dev Disord, 42*(7), 1377-1392. doi: 10.1007/s10803-011-1370-2

Schneider, A., Leigh, M. J., Adams, P., Nanakul, R., Chechi, T., Olichney, J., . . . Hessl, D. (2013). Electrocortical changes associated with minocycline treatment in fragile X syndrome. *J Psychopharmacol, 27*(10), 956-963. doi: 10.1177/0269881113494105

Wang, D. D., Englot, D. J., Garcia, P. A., Lawton, M. T., & Young, W. L. (2012). Minocycline-and tetracycline-class antibiotics are protective against partial seizures in vivo. *Epilepsy Behav, 24*(3), 314-318. doi: 10.1016/j.yebeh.2012.03.035

Wijetunge, L. S., Chattarji, S., Wyllie, D. J., & Kind, P. C. (2013). Fragile X syndrome: from targets to treatments. *Neuropharmacology, 68*, 83-96. doi: 10.1016/j.neuropharm.2012.11.028

Williams, EJ. (1989). Experimental designs balanced for the estimation of residual effects of treatments. *Australian Journal of Scientific Research, 2*, 149-168.

Wolfinger, RD, & Chang, M. (1995). *Comparing the SAS GLM and MIXED procedures for repeated measures.* Paper presented at the Proceedings of the Twentieth Annual SAS Users Group Conference