R01 FX ENTRAIN: Perturbation of Neurodynamics Underlying Sensory Hyperarousal and Statistical Learning in Youth with FXS

PRINCIPAL INVESTIGATOR:

Ernest V. Pedapati, MD, MS, FAAP
Associate Professor, Division of Psychiatry and Neurology
Cincinnati Children's Hospital Medical Center, Division of Psychiatry
3333 Burnet Avenue
Cincinnati, OH 45229-3039
Office 513.636.6553
Fax 513.517.0860
ernest.pedapati@cchmc.org

CO-INVESTIGATORS:

Craig Erickson, MD	*Professor, Psychiatry, CCHMC/University of Cincinnati (UC)*
Lauren Ethridge, PhD	*Asst. Professor, Pediatrics/Psychology, OUHSC*
Makoto Miyakoshi, PhD	*Project Scientist, CCHMC*
Stephanie Jones, PhD	*Professor, Brown University*
Laura Batterink, PhD	*Asst. Professor, Western University*
Steve Wu, MD	*Associate Professor, Neurology, CCHMC/UC*
Elizabeth Smith, PhD	*Asst. Professor, Psychologist, CCHMC*
Meredith Will, PhD	*Asst. Professor, Psychologist, CCHMC*

VERSION NUMBER/DATE:

Version 1.0 // 4.03.2023

REVISION HISTORY

Revision #	Version Date	Summary of Changes	Consent Change?

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1.0 Study Summary

Study Title	R01 FX ENTRAIN: Perturbation of Neurodynamics Underlying Sensory Hyperarousal and Statistical Learning in Youth with FXS
Study Design	The proposed project includes a case-control descriptive study (Aims 1 & 2) and an acute, crossover clinical trial (Aim 3).
Primary Objectives	Aim 1: Translational neurodynamics in resting and sensation. Aim 2: Cognitive studies (Statistical Learning). Aim 3**:** Sensory and cognitive perturbation studies.
Research Intervention	Closed-loop alpha auditory entrainment
Study Population	The study population includes subjects 5 to 15 years of age with Fragile X Syndrome (FXS) with full FMR1 mutations, as well as two age-and-sex matched cohorts of control subjects consisting of typically developing controls (TDC) and subjects with autism spectrum disorder (ASD).
Sample Size	180 human subjects will be recruited in total: 120 subjects total for case-control portion (60 FXS, 60 ASD, 60 TDC) and 60 subjects total for clinical trial (20 FXS, 20 ASD, 20 TDC).
Study Specific Abbreviations/ Definitions	FXS, Fragile X Syndrome KO, knockout TDC, typically developing control AAE, alpha auditory entrainment EEG, electroencephalography ITC, inter-trial coherence ASD, autism spectrum disorder FMRP, Fragile X mental retardation protein CFC, cross-frequency power-power coupling ERP, event-related potentials TCD, thalamocortical dysrhythmia SL, statistical learning PAF, peak alpha frequency PPF, peak power frequency WT, wild type BCI, brain-computer interface WLI, word learning index BLE, behavior learning effect AM, amplitude modulated LSL, lab streaming layer (software component) GUID, global unique identifier NDAR, National Database for Autism Research NIMH, National Institute of Mental Health ERSP, event-related spectral power CNS, central nervous system

2.0 Objectives

Our **central hypothesis** is that in Fragile X Syndrome (FXS), reduced alpha (~10 Hz) pacing and increased gamma (>30 Hz) noise impairs, via altered timing, the detection of sensory patterns and the ability to mount precise neural responses, thereby impeding cognitive functioning and contributing to increased sensory hypersensitivity. Our **approach** involves three scientific aims, which, if addressed, would ascertain underlying mechanisms that may alleviate sensory and cognitive impairments.

Specific Aim 1: Translational neurodynamics in resting and sensory studies. We hypothesize that fine-temporal alpha and gamma neurodynamics will uncover the basis of participant- and subgroup-level variation that drives FXS group effects. **1A**: We will acquire resting-state electroencephalography (EEG) and sensory auditory chirp paradigms in 40 youth (5 to 15 years old) with FXS and age- and sex-matched typically developing controls (TDC) and autism spectrum disorder controls (ASD). Neurodynamics (peak power frequency, spectral events) and biophysical modeling of each paradigm will be compared between groups. Neurodynamics will be assessed individually and in combination as predictors of psychological measures, medication effects, co-occurring neuropsychiatric diagnoses, and computerized cognitive testing. **1B:** We aim to identify neurodynamic features that distinguish $Fmr1^{-/-}$ KO from wild type (using preexisting resting-state EEG and sensory auditory chirp murine data). **1C (Exploratory)**: We aim to identify neurodynamic features that are conserved between $Fmr1^{-/-}$ KOs and participant human subgroups based on genotype. We hypothesize that full mutation, non-mosaic males with FXS will share similar neurodynamics as the $Fmr1^{-/-}$ KO (knockout) mouse. Shared neurodynamics between $Fmr1^{-/-}$ KO and a human subgroup will have a high translational impact.

Specific Aim 2: Cognitive studies. We hypothesize statistical learning (SL) will be impaired in FXS and the degree of SL impairment will be related to cognitive and language abilities in FXS. **2A:** Passive SL Task will compare structured (word) entrainment via EEG intertrial coherence (ITC) between FXS, TDC, and ASD. **2B:** Psychological measures (i.e., cognitive testing, expressive language sampling, and language ability) will be examined to identify key subgroups and predictors of neural response. As the Passive SL Task can capture an objective proxy of a cognitive process across a wide variety of functional levels, it will have a high impact on understanding heterogeneity in FXS.

Specific Aim 3: Sensory and cognitive perturbation studies. We hypothesize that "bottom-up" alpha auditory entrainment of the corticothalamic drive will normalize the ability to mount precise neural responses to stimuli (increased ITC) and diminish asynchronous gamma "noise." **3A:** Acute perturbation with closed-loop (to individualize stimulus) alpha auditory entrainment (AAE) will be conducted in FXS and sex- and age-matched TDC and ASD controls (n=20 per group). AAE will be performed in two visits as a double-blind, sham-controlled crossover study such that each participant will receive AAE and sham in random order. We will assess if closed loop AAE leads to normalizing phase synchronization of the sensory auditory chirp and the Passive SL Task. **3B (Exploratory)**: We will determine if AAE can enhance word learning as measured by reaction time in the Active SL Task.

3.0 Background

Fragile X Syndrome (FXS), a monogenetic neurodevelopmental disorder, manifests varying levels of intellectual disability, autistic features, and sensory hypersensitivity[1]. To date, despite over 60 publications of phenotypic rescue in animal models, pharmacological and behavioral treatments have yielded little clinical success in improving cognitive symptoms in FXS in human trials[2, 3]. Central to this discrepancy is a poor understanding of the constituent neurodynamics of averaged group effects and individual variability in human brain activity as related to higher-level cognitive symptomatology and clinical phenotype[4, 5]. This could, in part, account for the major discordance between mouse and human drug treatment effects. Additional factors including development, environment, and medications can further meaningfully contribute to heterogeneity. In large, published cohorts, we and others have identified reproducible, group-level abnormalities in resting-state EEG and sensory auditory chirp responses linked with intellectual disability, neuropsychiatric symptoms, and sensory

hyperarousal[6-14]. This data demonstrates that individuals with FXS do not mount precise neural responses to the sensory auditory chirp and, instead, show increases in "noise" in the form of asynchronous gamma activity[10]. Furthermore, a marked reduction in alpha power suggests altered thalamocortical function, reducing the ability to detect signal from noise in the environment and representing potential tractable targets for "bottom-up" entrainment. However, to translate these findings to effective treatments for individuals with FXS, two significant knowledge gaps must be addressed: 1) how does individual variability in neural activity in FXS contribute to core, impairing sensory and cognitive symptoms; and 2) could individually targeted, external entrainment of this neural activity improve sensory and cognitive function?

Significance of Aims

Constituent Neurodynamics of Alpha and Gamma Alterations (Aim 1):

Despite being a single-gene disorder, FXS patients exhibit market variability in EEG, molecular, and behavioral phenotypes compared to the Fmr1-/-KO mouse model[6, 15-18]. This high variability is observed within and across males with full mutation (mosaic and non-mosaic) and females who, due to an X-linked disorder, exhibit obligate mosaicism[7, 15, 19, 20]. Though a link between Fmr1-/-KO and males with full mutation FXS can be predicted based on suspected Fmr1 expression[21], the degree to which they share conserved physiology is yet unknown. Identifying parallel neurophysiological markers between mice and a particular human subset would represent a significant breakthrough for precision medicine in FXS. EEG is a highly feasible method to postulate whole-brain hypotheses in FXS, for which there is consensus on group-level effects in humans[8-10, 14] and Fmr1-/-KO mice[6, 22, 23]. Therefore, Aim 1 studies will investigate the neurodynamic basis and biophysical modeling of alpha and gamma alterations in resting-state and sensory auditory chirp EEG17 to parse heterogeneity.

Statistical Learning as a High-value Target in FXS (Aim 2): Though resting-state and sensory studies can provide insight into FXS physiology, there is an urgent need to develop cognitive paradigms that can be studied across the FXS phenotype and be broadly applicable to cognitive development. Children with FXS, for example, do not keep pace with the typical rate of cognitive development, and their skills plateau prematurely[24]. Statistical learning (SL) is the process by which humans extract patterns in sensory input, through passive exposure and without effort or conscious intention to learn. SL is present from birth[25] and operates continuously across the lifespan to extract and flexibly update statistical regularities from the environment[26]. While SL operates across many different domains, it is thought to play a significant role in language learning, most notably in speech segmentation[27]. Pauses or other acoustic cues do not reliably demarcate words in continuous speech. Becoming sensitive to the statistical regularities between neighboring syllables may allow learners to discover word boundaries in speech[28, 29]. Given most individuals with FXS show significant, lifelong language impairments, including delay of first words, deficits in SL would be expected in FXS[27]. Confirming this hypothesis and determining whether intervention is effective at improving SL would have far-reaching clinical implications, as improving communication abilities is an almost ubiquitous goal for families of individuals with FXS[30].

Since SL is performed passively and can be measured through EEG independently of behavior, it can be captured in children with FXS regardless of cognitive functioning ability[31, 32]. During SL, complex patterns are passively extracted through environmental exposure. Interestingly, the neural acquisition of patterned data heuristically resembles the responses to sensory auditory chirp when monitored by EEG and can similarly be quantified using inter-trial coherence (ITC)[31]. In this regard, SL is an ideal bridge between passive EEG sensory

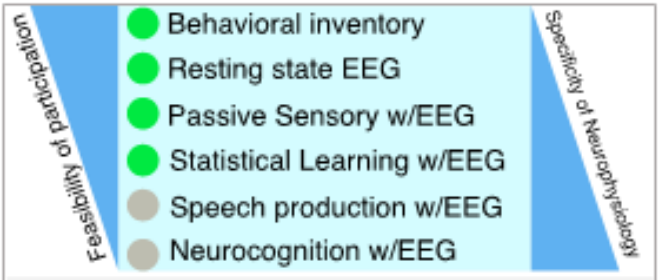


Fig 4: Addressing a gap in FXS research. Higher complexity tasks reduce feasibility. Statistical learning can be studied passively with EEG. Elements collected during this study are designated in green.

paradigm like the sensory auditory chirp and more complex but less feasible cognitive or language EEG paradigms (**Fig. 4**).

Normalizing EEG through Alpha Auditory Entrainment (AAE) (Aim 3): The observed reproducible alterations in brain activity raise a key question: would normalization of targeted EEG activity lead to improved sensory and SL markers within the disorder? Thus far, it remains an open question whether the EEG alterations observed in FXS represent a physiological mechanism that maintains suboptimal brain states or if they simply reflect compensatory changes to other aberrant neural processes. Though previous reports have identified group-level correlations of EEG and behavioral data, the ability of AAE to "pace" alpha rhythms (10 Hz) into typical range can be directly quantified by online monitoring of real-time EEG. If successful, this could lead to a new approach to addressing the underlying cognitive impairment in FXS and thus alter the trajectory of intellectual development in this disorder[2].

Summary: To this end, we have developed a cutting-edge approach to study heterogeneity in EEG signatures in humans, explore their relevance for sensory and cognitive disturbances, and introduce perturbation studies to test causal inferences. Our approach encompasses three key areas that, in our opinion, are of critical importance to advancing the FXS field. First, we will perform fine-temporal analysis (neurodynamics) of resting-state and sensory EEG to capture individual variability, model biophysical properties, and quantify the constituent factors of known group effects. At this juncture, we also ascertain which subgroup of persons with FXS shares neurodynamic profiles with theFmr1-/-KO mouse model. Second, we will study SL in FXS as a cognitive proxy and identify neural markers of SL with individual and subgroup variability. Third, we propose to move beyond correlational studies to examine causal effect, for the first time, that if individualized alpha entrainment will lead to normalization of neural responses in response to sensory auditory chirp and SL word entrainment.

Preliminary Data Justifying Proposed Approach

The investigative team has collected the largest cohort to date of high-resolution FXS patient resting-state EEGs (n=70; 32F; mean age= 20.5+/-10 years old; range=6 to 46) with age/sex-matched controls (n=71; 30F; mean age=22.2+/-10.7 years old; range=6-48). We recently conducted the first source analysis of resting-state EEG in FXS and found 1) decreased peak alpha frequency, 2) reduced alpha power, 3) centralized distribution (known as anteriorization) of "slow" alpha frequencies (8 to 10 Hz), 4) theta power, not alpha power, is inversely coupled to gamma power in FXS, and 5) increased asynchronous gamma power (see **Fig. 5** for summary)[33].

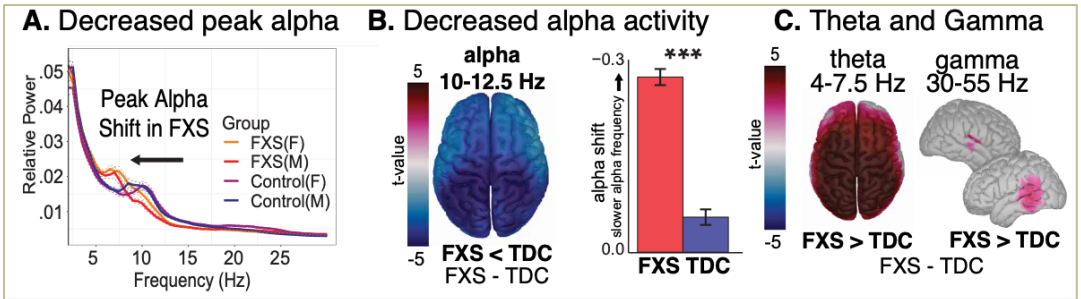


Fig. 5: Alterations in resting EEG (FXS, n=70; TDC, n=71). A) shift of peak alpha frequency towards the theta frequency and a decrease in alpha power. **B)** marked cortical alpha reduction (left) and alpha slowing and "anteriorization" (right) in FXS suggests decreased thalamocortical drive. **C)** increased theta power (left), centralized distribution (known as anteriorization) of "slow" alpha frequencies (shown in **Fig. 2B**), increased asynchronous gamma activity (right). We propose to test the hypothesis that auditory alpha entrainment can enhance thalamocortical signaling to enhance alpha activity and decrease asynchronous gamma. (TDC, typically developing controls; M, male; F, female).

Global decrease and "slowing" of alpha activity in FXS: A peak frequency of 10 to 13 Hz (upper alpha) has specific functional relevance for several major physiological systems in typically developing individuals[34-38]. As a radio receiver prefers specific electromagnetic frequencies, neurons and oscillatory networks also demonstrate frequency preferences[39, 40]. The "switch" to a dominant peak rhythm at 4-8 Hz in FXS (**Fig. 5A)** and reduced global alpha power (**Fig. 5B**) may be insufficient to drive neural ensembles with an alpha preference[35, 38, 41, 42]. Thus, the canonical role of alpha in

establishing inhibition-timing windows for optimal sensory and neurocognitive processing may be impaired in FXS.

The peak frequency of 10 to 13 Hz (upper alpha) is functionally important for several physiological systems in typically developing individuals[34-38]. Like a radio receiver, neurons and oscillatory networks also demonstrate frequency preferences[39, 40]. In FXS, there is a "switch" to a dominant peak rhythm at 4-8 Hz (theta range) and reduced global alpha power, which may be insufficient to drive neural ensembles with an alpha preference[35, 38, 41, 42]. Thus, the canonical role of alpha in establishing inhibition-timing windows for optimal sensory and neurocognitive processing may be impaired in FXS.

"Noisy" Elevated Gamma in FXS (**Fig 5C**): Asynchronous gamma oscillations (30 to 80 Hz) hold a special interest in NDDs because of their relation to cortical excitability[23, 43], their association with perception and cognition[44], and measurability in animal models[22]. The role of gamma oscillations is increasingly nuanced, such that precise synchrony in gamma power is associated with functional networks and higher-order cognition[36, 45], but also that a modest degree of asynchrony or "noise" is normal[46-48]. However, asynchronous (or background) gamma power above what is typically expected has been associated with disordered states[44] and, at a circuit level, is associated with reduced spike time precision and spectral leakage of spiking activities[49].

Evidence of Thalamocortical dysfunction:

Alterations in thalamocortical activity may unify the system-level hypothesis that underlies these EEG findings. Thalamocortical dysrhythmia (TCD) is an electrophysiological motif observed in several neuropsychiatric conditions (e.g., epilepsy, Parkinson's disease, tinnitus, depression, and neuropathic pain)[50-52] that reflects dysregulated cortical excitability. Like in FXS, patient groups that display TCD exhibit reduced alpha power, increased theta power, increased gamma power, and predominance of theta-gamma CFC. TCD-related EEG alterations have been associated with clinical features in these disorders. Importantly, in our dataset, abnormalities in alpha and gamma power demonstrate significant clinical associations with several core features of FXS, including cognitive function, anxiety, social communication, and auditory attention.

Feasibility of Statistical Learning Tasks (Aim 2): Extensive work by our team (Dr. Batterink) has reliability demonstrated in infant and youth populations[31, 53] that neural entrainment of SL can be tracked using EEG (see **Fig. 1C, pg. 13**). To date, SL has not been investigated in FXS, but is a highly salient area of study in FXS given the interest in language acquisition and as a cognitive proxy for learning in youth with FXS[19, 54]. Furthermore, neural entrainment is amenable to neurodynamic analysis, as embedded words gradually increase as the exposure progresses and predicts improvement in reaction time to test SL performance.

Evidence of neural entrainment by AAE in FXS (Aim 3): To date, neural perturbation, or entrainment to enhance cortical alpha activity, remains unexplored in FXS. The presence of hyperacusis (and EEG evidence of sensory hyperarousal) led the investigative team to consider using rhythmic alpha-frequency auditory signal to modulate cortical activity. We reasoned that an auditory entrainment signal could drive alpha oscillatory activity at both subcortical and cortical levels because encoded acoustic stimuli ultimately reach the temporal lobes via a thalamic intermediary (medial geniculate nucleus). Auditory entrainment has been used effectively as a neural probe, both for short-term suppression of tinnitus[55, 56] as well as eliciting physiological changes in EEG parameters[57]. Though audio entrainment has mixed evidence for durable effects as a therapeutic modality or cognitive enhancement[58], it has been shown in some clinical populations to have robust immediate or short-term physiological effects that can be measured by EEG[59-62]. We hypothesize that enhancement of alpha activity via AAE may activate neural circuits that have an alpha preference, and this enhancement can be observed by increased number of transient alpha spectral events, normalization of sensory auditory chirp response (increased ITC and decreased asynchronous gamma), and enhancement of SL (as measured by increase in word learning ITC). Closed-loop implementation: We have integrated AAE into a closed-loop perturbation paradigm in which alpha power can "self- correct" based on real-time responses. Closed-loop perturbation is a powerful framework for testing circuit hypotheses of complex neural phenomena and has emerging use

in clinical settings for epilepsy[63], Parkinson's[64], and Tourette's disorder[65]. A neural closed-loop system would ideally account for individual variability in response by automatically achieving the desired target by modulating the input signal based on empirical feedback[66].

AAE Feasibility data: Our approach is ideal for children as it is delivered at hearing threshold using headphones and the perturbation can be rapidly modulated in real-time. Unlike other non-invasive brain stimulation (e.g., TMS or TDCS), our approach is specifically tailored to a "bottom-up" approach by driving cortical oscillations through thalamocortical drive via auditory input (which we hypothesize is impaired in FXS). We developed a novel AAE stimulus ("baseline AAE") that creates a differential rhythm between the left and right ear starting at high theta range (7 Hz) through high alpha (13 Hz) in 2 Hz steps on a 500 Hz sine carrier tone.

AAE modifies alpha power in FXS: To demonstrate feasibility and obtain pilot data we performed baseline AAE with EEG on 8 subjects (TDC: 6M, 7M, 8F, 20M; FXS: 15F, 28M, 38M, 48M). All subjects completed the paradigm without any adverse events. The eventual goal of baseline AAE is to identify a starting frequency for closed-loop perturbation to account for baseline individual variability. Our pilot findings provide supportive evidence of our central hypothesis of alpha entrainment that may provide compensatory action within the FXS brain. 1) We demonstrate a high level of neural response variability to AAE (controlled by sham) in eight subjects (**Fig. 10A**), providing rational for the closed-loop approach

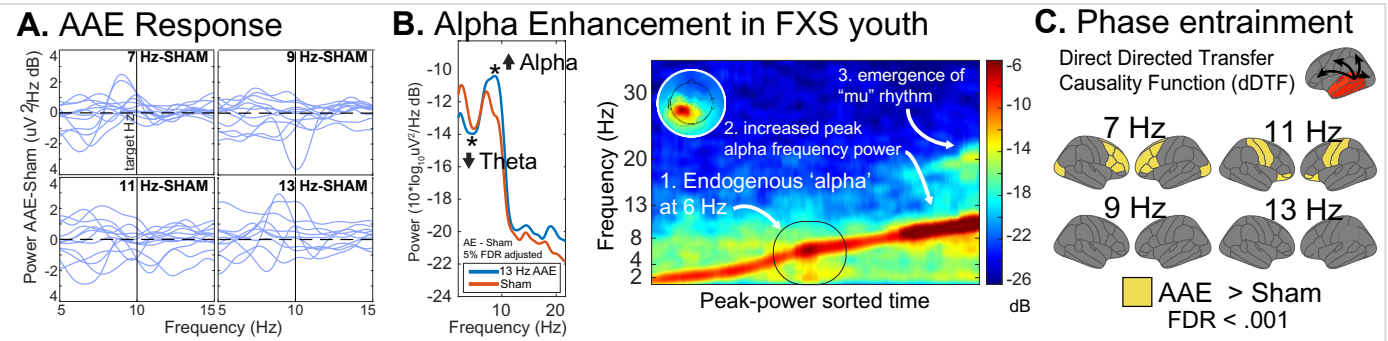


Fig. 10: Neural Entrainment by AAE. A. As expected, AAE resulted in high level of interindividual variability between stimulus frequencies. AAE power following subtraction of Sham. In our design, a starting frequency would be selected based on maximum response and then AAE would proceed in a closed-loop fashion individualized to each participant. **B. Left:** 13 Hz AAE vs. sham resulted in an increase in alpha power and a decrease in theta power in a 15-year-old female with FXS (paired-t test). **Right:** Peak Power Frequency (PPF) analysis following AAE baseline show endogenous "alpha peak" (~6-7 Hz) and AAE induced alpha peak (~9-11 Hz). Interestingly, the sensorimotor mu rhythm appeared following stimulation, suggesting normalization of alpha rhythms. **C.** Evidence of AAE information flow (phase-based) from L temporal lobe to frontal, central, and occipital regions using causality analysis.

and further emphasizing the importance of capturing individual variability in therapeutic development. 2) We show evidence of AAE's effects on resting-state EEG in a 15-year-old FXS female, wherein AAE appeared to increase alpha power and decrease theta power over sham AAE (**Fig. 10B Left**). 3) In the same subject, using peak power frequency analysis (PPF) we identified both an exogenous leftward-shifted "alpha" peak and increased alpha peak power between 9 to 11 Hz (**Fig. 10B Right**). 4) Using causality analysis (direct directed transfer function), we observed evidence of AAE information outflow (phase-based and controlled via sham) from the temporal lobes to frontal, central, and occipital regions in a 7-year-old male (**Fig. 10C)**

4.0 Study Endpoints

Aim 1: Sensory Auditory Studies

Primary outcomes of interest: 1) Neurodynamic features (peak power frequency, event rate, event duration), 2) 40 Hz ITC of the sensory auditory chirp, 3) Asynchronous gamma power.

Aim 2: Cognitive Studies

Primary: Word-learning Index (WLI) for the SL passive exposure task, i.e., EEG phase-synchronization at 1.1 Hz (word rate) compared to 3.3 Hz (syllable rate) over the exposure period.

Exploratory: Learning Effect (BLE) of the SL active performance task.

Aim 3: Neural Perturbation Studies

Primary: 1) AAE versus sham change in 40 Hz ITC, and 2) WLI during SL passive exposure task.

Exploratory: AAE vs. sham effect on reaction time (indicated by Behavior Learning Effect) during SL active performance task.

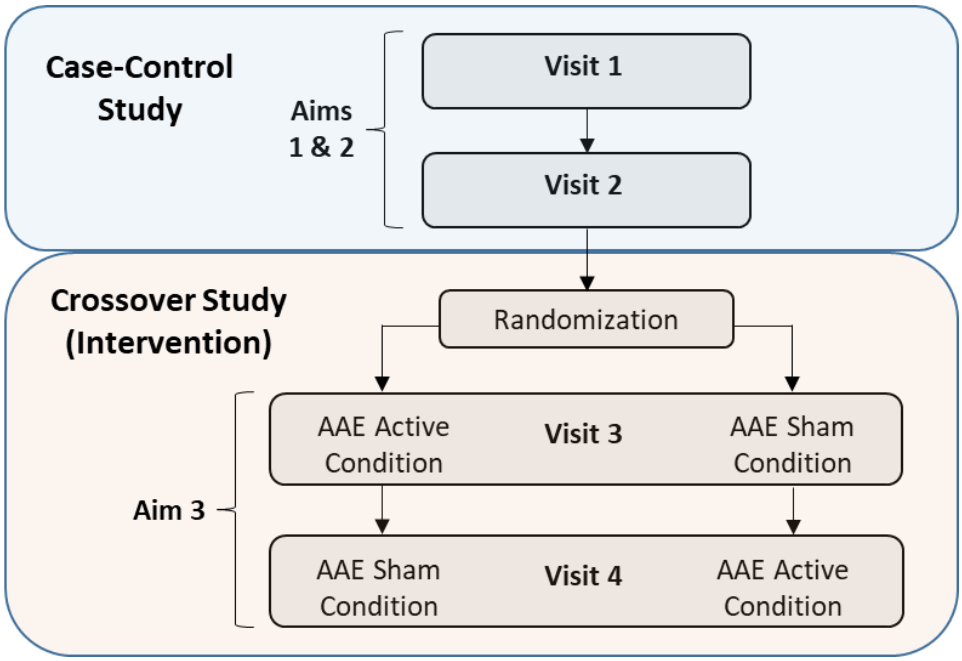
5.0 Study Design and Procedures

5.1 Study Design

Aims 1 & 2, combined, consist of a case-control study that will be completed over two visits lasting approximately 3-4 hours each, including: medical and psychological assessments, neurocognitive testing, and EEG/event-related potentials (ERP) procedures. Visits one and two may be completed up to three months apart. Following completion of Aim 1 and 2 studies, subjects and their guardians may be asked to participate in Aim 3.

Aim 3 (Intervention) consists of a two-visit, randomized controlled, crossover acute perturbation study to study the effect of AAE or sham stimulation on 1) the sensory auditory chirp (ITC and asynchronous gamma) and Passive SL Task (word learning ITC) and 2) in sufficiently cooperative participants, reaction time responses of an exploratory Active SL Task (word learning). AAE will be performed in two visits as a double-blind, sham-controlled crossover study such that each participant will receive AAE and sham in random order. For Aim 3, we estimate two 4-hour visits separated by at least a 1-week washout.

FX ENTRAIN Studies flow:



5.2 Procedures

The number of research visits for each subject may vary depending on subject age/functioning, tolerability of procedures, and family schedule. The research team will work to always fit the needs of

the subject/family. In line with this, subjects, caregivers, or parents may be given the option to complete appropriate surveys or questionnaires electronically through Redcap, over phone/video call with a member of the study team, or from home and mailed back to the research team. We may not administer all of the measures, assessments, or procedures, listed below. Measures administered will be at the discretion of the PI. If any data is unable to be collected, this will not be considered a protocol violation, but will be noted in the study chart. When applicable, assessments and measures previously collected from related, linked studies conducted by the Neurobehavioral Research Team and associated PIs can be transcribed and used for this study to reduce patient burden and the possibility of test-retest effects. Clinical data may include, but is not limited to, previous behavioral, neuropsychological, or other testing, and the results of any pertinent laboratory tests including previously conducted genetic chromosomal or microarray testing, vital signs, medication history, imaging history, CBC, metabolic panels, lipid panel, and other blood testing.

Standardized Measures and Assessments

Participants may complete the following:

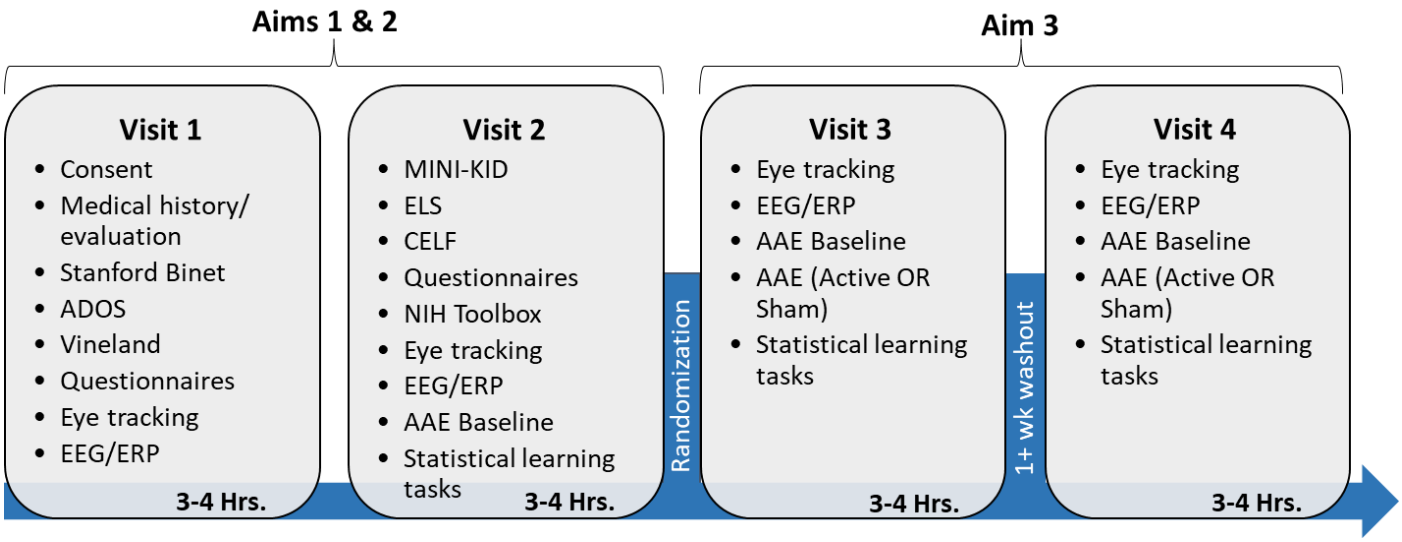
1. **Brief medical history and physical exam** to determine any exclusion criteria and medication. Medication history will contain medication details including duration taking the medication, time of last dose, history (with start and stop dates) of discontinued medications, and any non-pharmaceutical treatments such as herbals or complementary medications. Physical exam may include height, weight, head circumference, vitals and CNS data.
2. **Hearing and vision tests** to assess adequate hearing threshold using conventional threshold measurements from 0.25 to 8 kHz with simple behavioral responses, and vision tests to confirm corrected far visual acuity of less than 20/40.
3. **Social Communication Questionnaire (SCQ)**[67] which is a screener for autism spectrum disorder (ASD). TDC controls must score ≤8 to be included in the study and ASD controls must score < 15 to be included in the study.
4. **Autism Diagnostic Observation Schedule-2 (ADOS)**[68] will be completed to assess for co-morbid ASD in the FXS cohort, and to confirm ASD diagnosis in the ASD cohort. All ASD subjects and only FXS subjects scoring ≥ 12 on SCQ will complete the ADOS.
5. **Mini International Neuropsychiatric Interview Kid (MINI-KID)**[69]: Structural diagnostic interview to assess call co-occurring disorders in all subjects. The standard MINI Kid assesses the 30 most common and clinically relevant disorders or disorder subtypes in pediatric mental health.
6. **Stanford Binet IQ Scale-5th Ed (SB-5)**[70]. to provide a Full Scale, Verbal, and Nonverbal I.Q.
7. **Vineland Adaptive Behavioral Scales- Third Edition**[71], to assess adaptive skills.
8. **Expressive Language Sampling (ELS)**, conversation, and narrative[19] to assess naturalistic language abilities and heavily used in FXS.
9. **Clinical Evaluation of Language Fundamentals (CELF-5)**[72]**:** Comprehensive evaluation of language abilities to determine the level or levels of language (e.g., semantics, morphology, syntax, and pragmatics).
10. **Parent-report measures** of psychological functioning: Aberrant Behavioral Checklist (ABC)[73]; Anxiety, Depression, and Mood Scale (ADAMS)[74]; Sensory Profile[75]; Social Responsiveness Scale (SRS-II)[76]; Repetitive Behavior Scale-Revised (RBS-R)[77]; Pediatric Anxiety Rating Scale (PARS)[78]; Pediatric Quality of Life (PQL)[79].
11. **NIH Cognitive Toolbox**[80] for neurocognitive and neuropsychological skills.

Table 1: Detailed list of medical and standardized study assessments by cohort

Assessment	FXS	TDC	ASD	Remote Administration	Online Survey
Medical assessment (history, concomitant medication evaluation)	X	X	X	X	
Physical (height, weight, head circumference, vitals, CNS data)	X	X	X		
Hearing and vision screener	X	X	X		
Social Communication Questionnaire (SCQ)	X	X	X	X	X
Autism Diagnostic Observation Schedule (ADOS)	X*		X		
Mini International Neuropsychiatric Interview Kid (MINI-KID)	X	X	X	X	
Stanford-Binet Intelligence Scale, 5th Edition	X	X	X		
Vineland Adaptive Behavior Scale 3rd Edition	X	X	X	X	
Clinical Evaluation of Language Fundamentals (CELF-5)	X	X	X		
Expressive Language Sampling (ELS)	X	X	X		
Aberrant Behavior Checklist (ABC)	X	X	X	X	X
Anxiety, Depression, and Mood Scale (ADAMS)	X	X	X	X	X
Sensory Profile	X	X	X	X	X
Social Responsiveness Scale (SRS-II)	X	X	X	X	X
Repetitive Behavior Scale-Revised (RBS-R)	X	X	X	X	X
Pediatric Anxiety Rating Scale (PARS)	X	X	X	X	X
Pediatric Quality of Life	X	X	X	X	X
NIH Cognitive Toolbox	X	X	X		

* Only administered to FXS subjects with SCQ score ≥12

Detailed schedule of procedures by aim/visit



Neurophysiology Tasks

Table 2: Schedule of neurophysiology and neurocognitive procedures

Aim 1 (Visit 1): Sensory Auditory Studies	Aim 2 (Visit 2): Cognitive Studies	Aim 3 (Visits 3 & 4): Closed-Loop Neural Perturbation Studies
Resting State EEG	Resting State EEG	Resting State EEG
Sensory Auditory Chirp	Passive SL Task	AAE[1] Baseline
Auditory Steady State	Active SL Task	Sensory Auditory Chirp
AAE[1] Baseline	AAE[1] Baseline	Passive SL Task
		Active SL Task

[1]AAE, Alpha Auditory Entrainment Paradigm

EEG/ERP Procedures

EEG/ERP may be used to assess the electrophysiologic correlates of exposure to sensory stimuli (i.e. auditory/, visual, tactile) and/or during behavioral computerized testing. Recordings are acquired using 32 up to 256-channel cap (HydroCel Geodesic Sensor Net) with an amplifier. Data are recorded at a sampling rate of 1000 Hz with saline-based electrodes. Acoustic stimuli are generated and presented using MATLAB (MathWorks, Natick, MA) and synchronized with a pulse to mark the onset of each stimulus Video may be recorded to accurately identify non-brain artifact contamination of the recording. In order to facilitate accurate EEG electrode locations on the scalp during analysis any subject that receives an EEG may be photographed to determine electrode positions during recording; this may include a 5-10 minute procedure in which the EEG net and electrode positions are captured using photogrammetry software and a stereoscopic camera. Data will be used to generate a numerical coordinates file. All EEG collection will be completed by trained research staff members. Our team developed systematic behavioral preparation including social stories, visual schedules, and demo nets/parent training to optimize data collection. In our experience, >95% of study eligible individuals with FXS provide usable EEG data following our study protocols. To maintain cooperation with study procedures, resting and sensory EEG is performed while watching a standardized silent cartoon. Behavioral tasks will use response button boxes.

1. **Resting state EEG:** Continuous data will be recorded for 5-7 minutes.
2. **Sensory Auditory Chirp:** "Chirp" stimuli will be delivered through headphones/speakers with a 1000-Hz carrier tone amplitude modulated (AM) by a chirp sinusoid linearly increasing in frequency from 0–100 Hz over 2000 ms. One-to two-hundred stimuli will be separated by a 1.5-2 sec inter-trial interval, for 7.5-12.5 minutes of testing.
3. **Sensory Auditory Steady State:** As an exploratory outcome, we may test stability of synchronization at alpha (10 Hz) frequency and its relation alpha power phase. Stimuli will consist of white noise amplitude modulated by a sinusoid for 3 sec at 10 Hz. Fifty trials at each frequency (100 trials total) will be randomly presented and separated by a 1.5-2 sec inter-trial interval, for 8.3 minutes of testing.
4. **Alpha Auditory Entrainment (AAE) Baseline:** The AAE was generated in MATLAB 2019b with a 500 Hz sine carrier wave. We had five total conditions including silence (no stimulus), sham (500 Hz both ears), and linearly increasing alpha frequencies of 7 Hz (507 Hz in right ear), 9 Hz (509 Hz in right ear), 11 Hz (511 Hz in right ear), 13 Hz (513 Hz in right ear). The total stimulation length is twelve minutes with one minute of stimulation of each frequency in pseudorandom order.
5. **Dynamic AAE during closed-loop perturbation:** The requested frequency differential will be generated by a custom function in MATLAB to deliver AAE in the requested .5 Hz step. This stimulus will last for 60 seconds until the next iterative feedback. Combining AAE with paradigm: The AAE stimulus will be played back at background noise level alongside either the sensory auditory chirp or SL tasks. There is considerable literature on the combination of entrainment beats with other auditory stimuli (speech, music) maintaining the physiological response[81-83].
6. **Sham AAE:** The sham stimulus was generated in MATLAB with a 500 Hz sine carrier tone. The sham AAE condition will mimic the closed-loop AAE condition detailed above, however only the

carrier stimulus (500 Hz) is mixed with the paradigm audio during sensory auditory chirp or SL tasks.

Eye Tracking

Participants may be monitored or asked to make specific eye/eyelid movements. Behavioral and emotional tasks may be used in conjunction to monitor eye movements. This task involves the subject to look at a sequence of varying emotional faces and social sequences. The resulting ocular movements may be recorded using a Tobii Eye Tracker (Tobii Technology AB, Danderyd Sweden) or similar scanner-compatible technology.

Statistical Learning Tasks

Assessment of SL includes a 1) Passive SL Task and 2) Active SL Task. This approach allows us to capture data on all participants regardless of adaptive functioning. The Passive SL Task uses an EEG-based measure of neural entrainment involving ITC[31, 53] to calculate Word Learning Index (WLI). The Active SL Task based measure uses reaction time to estimate a behavioral measure of SL, the Behavioral Learning Effect BLE[32]. The tasks may be altered (e.g., speed reduced, additional instruction or practice given) to increase feasibility for participants.

Procedure Overview (all SL tasks)

Participants will be seated in front of a computer monitor with headphones compatible with EEG cap. If a subject is not able to tolerate headphones, alternative methods like speakers may be used instead. To facilitate cooperation and reduce motion artifact, standardized, silent non-social videos will be played during the task. Stimuli (see **Fig. 1**): Twelve syllables are combined to make four, three-syllable pseudowords. This miniature synthetic language resembles natural language as (1) transitional probabilities are higher for neighboring syllables within words than across word boundaries and 2) a given syllable may be present in more than one word. For example, a transitional probability of 1.0 for

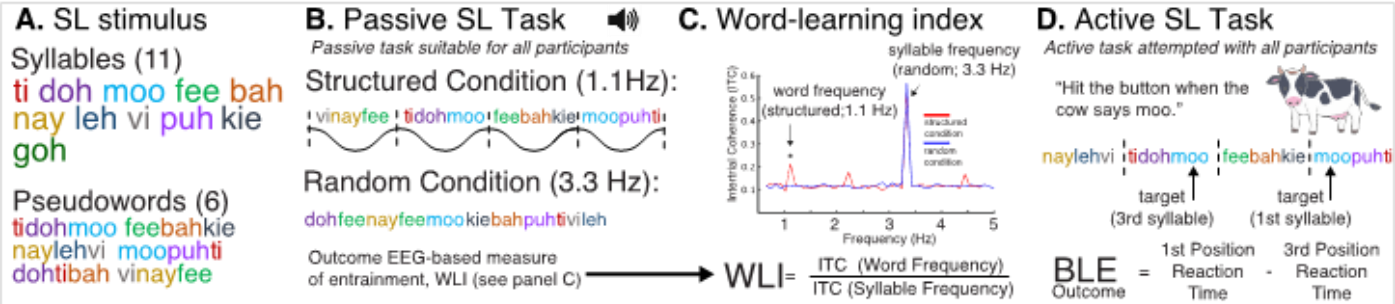


Fig. 1: Assessment of statistical learning (SL). A. Eleven synthesized generated syllables are combined into six trisyllabic pseudowords. **B.** A structured (experimental) and random (control) stream are played to the participant during EEG. **C.** Word learning index (WLI) is a previously validated EEG-based entrainment measure of learning. Similar our sensory index, it is also based on intertrial coherence (ITC). SL leads to perceptual grouping of trisyllabic words, indexed by a relative increase in phase synchrony at the word presentation rate and a decrease in synchrony at the syllable rate over exposure. **D.** Behavioral Learning Effect (BLE) is a reaction-time based measure of SL. Participants respond to a target syllable embedded in a continuous auditory stream of pseudowords.

a syllable pair such as "vinay" indicates that every "vi" in the stream was followed by "nay." SL occurs as the brain "discovers" hidden words in the speech stream by becoming sensitive to these linguistic patterns through repeated exposure.

Passive SL Task: Each participant will be presented with both a structured and random stream of syllables. The structured stream will consist of the four generated words, concatenated in pseudorandom order without any pauses or acoustic cues between words (e.g., *vinayfeetidomoo…*). Each word will be presented 200 times, for 800 total word presentations. Order will be constrained to avoid successive syllable and word repeats. The random stream contains the same total number of syllables, which are ordered pseudorandomly to avoid syllable repetitions but will otherwise not follow

any hidden structure. Words will be presented at a rate of 1.1 per a second (thus, syllable rate is 3.3 Hz), resulting in 10 minutes of each structured and random continuous stimuli. As previously published, the expected outcome of this study is an increase in EEG phase-locking at 1.1 Hz over the exposure period—present in the structured condition only—indicative of gradual word discovery and successful word segmentation resulting from statistical learning.

Word-learning Index (WLI) for Passive SL: WLI is the primary EEG outcome measure for the exposure task and, similar to our sensory studies, is based on intertrial phase coherence (ITC). As the participant "learns" the four words in the structured stream (i.e., uses statistical probabilities across neighboring syllables to segregate the speech stream), their EEG typically shows increased phase-locking (increased ITC) at the word presentation rate (1.1 Hz) and decreased phase-locking at the syllable presentation rate (3.3 Hz). Thus, patterns of EEG phase-locking, corresponding to a shift in processing from raw syllable units to cohesive words, reflect gradual statistical learning in the brain. The effect can be quantified by creating a ratio of the ITC for words versus syllables, as follows:

$$\text{WLI} = \text{Intertrial Coherence}_{\text{word rate}} / \text{Intertrial Coherence}_{\text{syllable rate}}$$

A higher WLI indicates a relatively stronger response to tri-syllabic nonwords compared to raw syllables, reflecting stronger word segmentation due to statistical learning. The WLI predicts subsequent performance on behavioral tests of statistical learning and increases over time in the structured condition only, and not in the random condition[31].

Active SL Task (Exploratory): Following the exposure task, each participant may attempt to perform a reaction-time based task that provides a behavioral index of SL. Participants will be instructed to press a button when they hear a given target syllable (e.g., "moo"). Prior to the main task, the participant may complete 10 practice attempts on non-experimental syllables to assess feasibility. The four words from the exposure task will then be presented a total of 20-45 times per word across a total of five, one-minute blocks, each corresponding to a separate target syllable. Within each one-minute block, each of the six words will be repeated 4-9 times. A visual reminder of the target (e.g., a picture of a Cow for "moo") will be displayed for the entirety of the block. Targets presented within later positions within a nonsense word, (i.e., those syllables that are more predictable) elicit faster reaction times than less predictable, word initial syllables, providing a reaction-time based measure of statistical learning. Thus, the Behavioral Learning effect can be quantified as follows:

$$\text{BLE (in ms)} = (\text{Reaction time}_{\text{target syllable position 1}})-(\text{Reaction time}_{\text{target syllable position 3}})$$

Larger BLE values indicate greater proportional facilitation to predictable words in the stream, indicative of stronger SL at the individual level. Group comparisons can be facilitated by dividing BLE by the $\text{Reaction time}_{\text{target syllable position 1}}$ to normalize to an individual's baseline reaction time. Larger BLE values indicate greater proportional facilitation to predictable words in the stream, indicative of stronger SL at the individual level.

Interventional Agent: Alpha Auditory (Neural) Entrainment

Randomized, sham-controlled acute neural perturbation studies will test the hypothesis that enhancing alpha oscillations in FXS will enhance thalamocortical drive in FXS and normalize observed neuro-oscillatory alterations in sensory and cognitive paradigms. As this is a highly novel approach, we have attempted to reduce the complexity of the protocol to be feasible in youth and simplify interpretation for future chronic dosing studies.

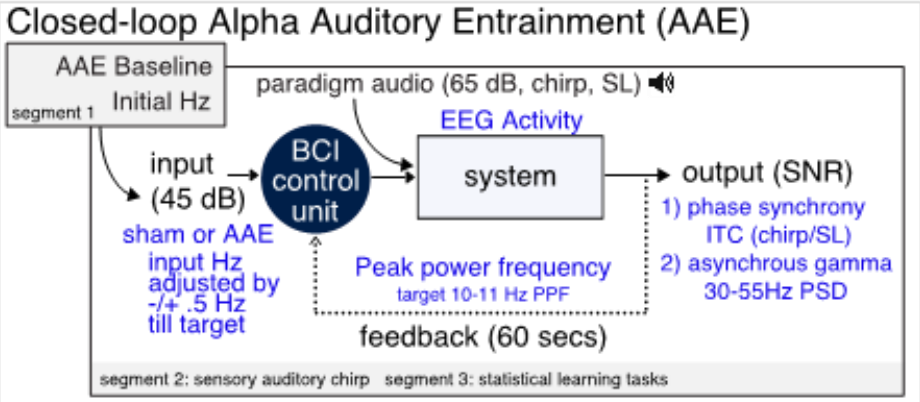


Figure 12: Controlled closed-loop alpha auditory entrainment (AAE). Session is divided into 3 segments. Youth will have earphones, wearing EEG cap, and watching a silent video. **Segment 1:** AAE base line will be played to identify a starting frequency that provides maximal shift in peak power frequency (PPF). **Segment 2 and 3:** Closed-loop feedback with AAE or sham will be active during sensory auditory chirp and passive SL task. A moving average is calculated of peak power frequency (PPF) over 60 s. The BCI control unit will either hold the current frequency or advance or decrease by .5 Hz within the boundaries of the alpha band (8 to 13.5 Hz). The cycle is repeated until the task is complete. Sham stimulus is carrier frequency only without alpha entrainment. For successful proof of concept in 15 year old female with FXS see **Fig. 10.**

Procedure Overview

Three segments (approximately 15 minutes each) will be conducted to 1) identify the baseline AAE, 2) conduct closed-loop perturbation sensory studies, and 3) conduct closed-loop perturbation SL studies. Similar to Aim 1 and 2 studies, subjects will be seated comfortably and allowed to watch silent, non-social videos during setup, segment 1, and the passive portion of segment 2 to facilitate cooperation and reduce movement artifact. Following the first segment, the subject will be offered a 5 to 10-minute break.

Baseline AAE (Segment 1): To avoid extensive "frequency finding" during the closed-loop segments we have opted to perform a linear increasing auditory entrainment paradigm to identify an optimal starting frequency. We will record 3 to 5 minutes of resting state EEG prior to the AAE baseline paradigm. The brain computer interface (BCI) will provide real-time detection of peak-power frequency and magnitude of the target frequency range at each frequency step and will be recorded with subject data. Following completion of the baseline AAE paradigm for each subject, the investigator(s) will evaluate output curves and determine optimal starting frequency. Segment 1 is performed during AAE or sham treatment conditions.

Closed-loop segments 2 and 3: Paradigm procedures for both sensory auditory chirp and SL paradigm will be identical to Aims 1 and 2, with the addition of the AAE stimulus (either active or sham) to the paradigm audio. As the SL Tasks syllables can be varied, the subjects will learn a new “miniature” language on each visit. In the closed loop condition, prior to starting paradigm sixty seconds of the selected baseline AAE will be delivered via earphones before the start of the paradigm to capture sufficient data for feature feedback. Following initiation of the paradigm, every 60 seconds a moving average of peak power frequency will be broadcast via LSL to MATLAB for adjustment of entrainment frequency. If the peak power frequency is less than the target frequency entrainment stimulation will advance by .5 Hz. If the peak power frequency is higher than the target frequency, the entrainment stimulus will decrease by .5 Hz. If the peak power frequency is within the target, no change in frequency will be performed. As in Aim 2, all subjects will receive the SL exposure task, however, subject feasibility will be assessed for completion of the SL performance task. Following completion of paradigm, subjects will be allowed to watch a video of their choice (with sound) as the EEG cap is removed to facilitate cooperation and serve as a reward.

5.3 Blindness and Procedure for Breaking the Blind

This is a double-blind, sham-controlled study. Investigators, study staff, and study subjects will be blinded to the randomized study treatment assignments. Unlike drug studies, the “treatment” in this study is the AAE stimulus (alpha auditory entrainment) versus Sham (carrier frequency alone) without an alpha entrainment rhythm. Each subject will get either AAE or sham, but the order will be

randomized and blinded. A balanced, coded randomized list of treatment orders for sixty subjects will be generated via software. The key list will be kept with a designed study personnel (who is not involved in running subject visits or data analysis). The blinded list will be coded with BCI software to deliver the correct treatment order of Sham or AAE. As the stimulus will be delivered via headphones or speakers at background noise levels and generate identical triggers study personal will remain blinded until after study completion. Though we do not anticipate any need for an emergency unblinding, it will only be considered in situations where the knowledge of the treatment code has an impact on the planned treatment of the emergency. Once the randomization code is broken for a subject, he/she must be withdrawn from the study. The investigator must record the reason for the emergency unblinding in the source documents and recorded in the electronic database.

5.4 Protection Against Risks (all aims)

All experimental procedures will be carried out in a setting and among staff who are carefully trained and experienced in working with individuals with developmental disabilities including FXS and ASD. All human research procedures take place on the clinical campus of CCHMC. In addition to the on-site presence of an attending physician who is PALS certified, the clinical resources of CCHMC will be available in the case of an adverse event which includes access to AED equipment and the Emergency Department. See **Risks to Subjects** section for detailed precautions against risks.

6.0 Sharing of Results with Subjects

Findings from assessments will be made available to agents of the participants (parents, schools, treating psychologists, physicians) only with the expressed, written consent of the parents/guardians and if appropriate for the study participant.

The study investigator team is joined by a pediatric neurologist (S.W.) with expertise in clinical EEG and brain stimulation and extensive collaboration with the P.I. on pediatric TMS studies[84-89]. Since ASD patients have a higher risk for epilepsy than the general population, the neurologist's role will be to review all research resting-state EEGs for abnormalities, including evidence of seizure activity. If any abnormalities are found, these findings' clinical relevance and urgency and the need for a neurological exam will be discussed with the principal investigator. The findings will be directly discussed with the family with appropriate clinical referral. In addition, any features of clinical significance within the EEG recordings will be considered in subgroup and co-variate analysis.

7.0 Study Timelines

Individual subjects' duration of participation in this study is estimated to be approximately 16 hours over the course of up to two years, with up to three months of separation allowed between visits 1 and 2 (descriptive study), and 3 and 4 (clinical trial), respectively. It is expected that participants' duration of participation will be significantly shorter than is stated above, however, considering many FXS families travel great distances to be seen in our lab clinically, high flexibility in scheduling is imperative for ensuring equal opportunity to those who wish to participate in research.

We plan to enroll all study subjects by end of year 4 and estimate the primary analyses will be completed by end of year 5.

8.0 Inclusion and Exclusion Criteria

Inclusion Criteria (all aims):	**FXS**	**ASD**	**TDC**
Aged 5-15 years, inclusive	X	X	X
Patient has full FMR1 mutation confirmed by genetic testing	X		
Have no known genetic mutation		X	
Have documentation of ASD diagnosis		X	
Score ≤ 15 on SCQ screen		X	

Be in good health per investigator		X	X
Patient has met normal developmental milestones			X
Patient has no family history of heritable neuropsychiatric disorders			X
Patient has an IQ greater than 85 on the Stanford-Binet			X
Score ≤8 on an SCQ screen			X

Exclusion Criteria (all aims):	**FXS**	**ASD**	**TDC**
Patient has auditory or visual impairments that cannot be corrected	X	X	X
History of substance abuse or dependence within the past 6 months	X	X	X

Vulnerable Populations

- Inclusion of women and minorities: We will include males and females in numbers consistent with the distribution expected in the clinic populations. We will not exclude any participant based on race or ethnicity. The distribution of minorities will be consistent with the distribution expected in Cincinnati.
- Inclusion of children: A central aim of the proposed research is to evaluate the underlying mechanisms responsible for brain development in children with FXS. This can only be done by studying affected individuals in that age group. The minimum age of 5 was chosen because of the level of cooperation required to complete procedures, and the upper age limit of 15 was chosen because of common age-related changes in behavior and brain function beyond that age.
- 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 event-related potential (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.

9.0 Local Number of Subjects

The target enrollment for the case-control portion (Aims 1 & 2) is 120 subjects and 60 subjects for the clinical trial (Aim 3), for a total of 180 subjects across all aims. We plan to recruit a total of 60 subjects with Fragile X Syndrome with full FMR1 mutations (>200 CGG repeats; at least partial FMR1 gene methylation). Of these FXS subjects, 40 will be recruited for the case-control portion and 20 will be recruited into the clinical trial. The overall project will recruit an additional two cohorts of control subjects for a total of 60 IQ-, sex-, and chronologically aged matched non-syndromic (i.e., idiopathic) ASD controls, and 60 sex-and chronologically aged matched TDCs. Following completion of the case-control portion, subjects and their guardians may be asked to participate in the clinical trial portion of the study.

Retention Plan: We do not anticipate a high drop-out rate since almost all testing is non-invasive, and each portion of the study (case-control and clinical trial, respectively) can be completed in 2 visits, with ample time allowed between visits. Thus, we expect close to 90% retention of all recruited subjects. Subjects unable to complete study procedures or who fail to consent/withdrawal following enrollment will be replaced to maintain our enrollment target of 180 total subjects.

10.0 Recruitment

Recruitment Methods and Materials

Potential participants may be recruited for the study at CCHMC, from the general public, and at special community events or lectures. In some instances, subjects who have consented to be contacted for

future studies may be sought if they qualify for this study. Participants may also be recruited from local and national databases intended for recruitment, EPIC queries, and OCTR database queries.

Fragile X subjects will be recruited from the Cincinnati Fragile X Research and Treatment Center by treating physicians and their staff. The clinical team will present the project to patients and family members when they are at our clinics or treating physicians may contact them directly about their interest in participation.

Participants with a history of Developmental Disability will be recruited from the CCHMC Developmental Disabilities Psychiatry Service (inpatient and outpatient services), the CCHMC Division of Developmental and Behavioral Pediatrics outpatient clinics, other CCHMC outpatient clinics, CCHMC inpatient units, at special events and from the general public.

The study will be advertised in hospital/clinic and general public areas via print, email, electronic and social media. The Cincinnati Fragile X Research and Treatment Center will be sending out a monthly newsletter providing resources, research findings, upcoming events, and research opportunities to families who have expressed interest in clinic/research; information about research opportunities will also be distributed in the form of a brochure. All recruitment materials mentioned above will be IRB-approved prior to send-out.

Interested families or potential participants may contact the research team directly by the phone number or email address given in the advertisements, at which time a research coordinator will follow up by phone or email. When individuals contact our research staff or provide consent to be contacted, we will provide them with basic information regarding study procedures and aims, inform them that this research is voluntary, and answer any questions about the study. Family and medical history will be reviewed in person or over the phone to determine study eligibility. If no exclusionary factors are identified in the review of family and medical history, then subjects will be scheduled for a meeting to obtain informed consent.

Potential participants and their families may be given the option to have a practice cap/beanie mailed to them to aid with sensory familiarity in preparation for a potential future visit. For some participants with a history of Developmental Disability, the optional EEG tasks may present an uncomfortable sensory experience, especially to those unfamiliar with the EEG cap. To allow for adequate practice and preparation time, this option may be presented before the potential participant has been consented to the study. The practice beanies fit snugly around the head, offering a similar sensation to a real EEG cap. If the family already owns a similar cap the subject can practice with they may be asked to use that instead. The caps will not be used to screen subjects and are only being provided to increase subjects' comfortability with EEG. Subjects who opt to receive a practice cap are not required to practice wearing the cap prior to the first visit. It will be explained to potential subjects/caregivers that receiving the practice cap does not require them to later enroll in the research study or to complete EEG tasks at a subsequent research visit. The practice caps are of negligible monetary value and are not believed to unduly influence subjects' consideration of participating in the study.

Compensation

Subjects will receive $50 for each completed visit. If a participant cannot complete any study visits, they will be compensated at a rate of $15/hr. based on what they have completed.

Payment will be in the form of a reloadable debit card (ClinCard). We will provide the card and load money onto the card after each completed visit. We will also administer a handout that will explain how to use it.

Because this research study involves payment for participation, we are required by federal Internal Revenue Service (IRS) rules to collect and use participants' social security or tax ID number (SSN) in order to track the amount of money that we pay. We will only use the participants' SSN to keep track of how much money we pay to them and their SSN will not be used as part of this research.

Families traveling significant distance to our clinic may be reimbursed for travel costs at the discretion of the PI. This may include mileage, hotel, airfare and/or other associated costs.

11.0 Withdrawal of Subjects

Any participant may be discontinued from the study at the discretion of the investigators if this is deemed to be in the best interest of the participant or if they are not compliant with study-specific requirements. A subject may voluntarily withdraw from the study at any time and for any reason. If a subject withdraws, at his or her request or at the request of his or her caregiver or legal representative, the reason(s) should be documented in the subject's source document. Any research information recorded for, or resulting from, participation in this research study prior to the date that the subject formally withdrew their consent will be retained and may continue to be used and disclosed by the investigators for research purposes; however, no new data will be collected.

12.0 Risks to Subjects

Confidentiality of records and results: The neurophysiological, medical, psychological, and behavioral measures will be made anonymous and labeled only by a unique identification number. Appropriate precautions will be taken to protect the confidentiality of all data. All subjects will be informed of this, including the HIPAA privacy of information act.

Behavioral and cognitive testing: There are minimal risks anticipated to participating in observational, clinical behavioral assessments. In the rare case that an individual might become overly fatigued or distressed during the assessment session, the session will be discontinued and rescheduled. Evaluation procedures are designed to be compatible with the attention span of a person with intellectual disability, greater activity level, and need for access to his or her parents or guardians. The NIH Cognitive Toolbox specifically created downward developmental extensions to accommodate for these needs. In addition, specialized procedures will be accompanied with specialized approaches such as using social stories and visual aids. There will be praise for task attention and overall emphasis on reducing language demands. The clinical investigators have extensive experience working clinically with developmentally disabled patients and their families.

EEG: EEG testing is considered minimal risk. The most common risk associated with these tasks is boredom. The EEG nets do not use an electrode gel, but rather a saline solution and sponges which has a very low risk of skin irritation. The candidate and EEG technicians are highly experienced with doing EEG with sensitive populations such as children and subjects with neurodevelopmental disorders.

13.0 Potential Benefits to Subjects

Direct benefit to study participants is minimal. The major immediate benefit of participation in the proposed studies would be screening for symptoms of ASD and related disorders. Subjects may receive benefit from a complete evaluation including diagnostic, psychiatric, psychological, and medical evaluations in the form of a report. For individuals who are diagnosed with ASD or new diagnosis of FXS, appropriate referrals can be made if desired by study participants and their guardians. Caregivers and guardians may receive information regarding understanding the subject's behavior which may benefit them. We may also provide resources to connect them to local, regional, and national support organizations for FXS. Families and subjects may appreciate contributing to the discovery of innovative FXS treatments that will benefit other individuals. Healthy subjects often have various motivations and different rewards for participating in the study. Some subjects will be primarily motivated by the participant compensation. Others may want to participate to learn more about their skills or are personally committed to developmental disability research.

14.0 Data Management and Confidentiality

Data Storage and Sharing

All records will be handled in a confidential manner. All hard copy data will be stored in code-labeled binders in locked storage facilities with limited access. Any electronic files containing patient identifiers will be either stored on a secure server or password protected database. Only the Principal Investigator and his designees will have access to hard-copy or electronic data.

Unique numeric identifiers that do not include any PHI will be assigned to each participant. Identification numbers will code data entered for computer analysis, and the designated study staff will keep all names and code numbers in a password-protected folder. EEG scans recordings will be anonymized by using only the participant study IDs and the date and time of scanning/recording and will be stored on a secure server.

Sociodemographic information collected from this research may be utilized to create a Universal Identification Number (GUID) for the National Database for Autism Research (NDAR). Data that is collected from each research visit may be shared with NDAR. Before this data is sent to NDAR, information such as name and birthplace city will be removed and replaced with a GUID.

Research data gathered as part of this study may be shared and provided to other investigators affiliated with the Neurobehavioral and Neurology Research Teams at CCHMC for the purpose of data sharing. If information is requested by an investigator, the PI must approve the request. If participants are enrolled in multiple or linked studies, their research data will be shared across studies to reduce participant burden and avoid duplication of procedures. All shared data will be de-identified, and results will be published as group data without the use of characteristics that would identify individual participants.

Quality Control

At point-of-entry, data values will undergo consistency edits (e.g., ID validation, range verification, duplicate detection) and personnel will be required to correct errors. Data management staff will run logic error programs to check for accuracy and irregularities within and across data structures and within and across sites. Quality assurance checks will be conducted regularly by site personnel as well as by data management staff. Regularly scheduled, and as needed, communications between the study team, the neuroimaging team and PI will clarify any inconsistencies and ambiguities in the data.

Data Analysis

Power Analysis: **Aim 1A:** Based on differences in PPF in FXS vs. TDC in our resting state EEG data, we expect an effect size of approximately .8 to 1.1, similar to effect sizes in low gamma power and N1 amplitudes in FXS[10, 12, 90, 91]. **Aim 2:** The effect size for WLI differences between structured and random conditions is approximately .8[53]. The primary comparisons for both Aim 1A and Aim 2 will be between diagnostic groups, FXS vs. TDC and FXS vs ASD, Therefore, to be statistically conservative, the nominal alpha of .05 will be divided by 4, i.e. .0125 per hypothesis. Using the available FXS vs. TDC effect size of 0.8, 40 subjects in each of the three diagnostic groups yields 84.3% power at a level of significance = .0125. Effects size data for FXS vs. ASD for these measures is not available but may be estimable for future studies. **Aim 3:** This aim is novel, without a strong basis for estimating an effect size. AAE effect size of PPF enhancement (vs. sham) in our preliminary data is between .89 and .97 (obtained peak frequencies of 9.3 to 9.6 Hz). For the within-groups crossover, an AAE trial to detect treatment differences in a 2 X 2 crossover design (20/group) yields approximately 80% power to identify group differences with an effect size of .9 at an alpha of 0.05.

Statistical analysis: PPF (**Aim 1A**) and WLI (**Aim 2**) were selected as primary response variables since they will be individually optimized as starting parameters in **Aim 3**. Planned comparisons include FXS vs. TDC and FXS vs. ASD. FXS vs. TDC will be of primary interest, but confirmation of FXS vs. ASD can weaken the inference that differences in intellectual ability and genetic liability alone account for group difference. Covariates: FXS vs. TDC model will include age, sex, and non-verbal IQ. FXS vs. ASD will additionally include co-occurring psychiatric diagnoses, psychiatric medication(s) (4 categories), and SRS score. A group by sex interaction will be added to each model given known sex

differences in FXS[15]. **Aim 1B:** Murine data will consist of a parallel model to Aim 1A with three neurodynamic variables (PPF, event duration, and event number) and independent variable of group (KO or WT; 80 in each group). **Aim 3:** A linear model with repeated measures will test a 2-treatment, 2-period, 2-sequence balanced-crossover design (Williams, 1989) with Kenward-Roger adjustment[92] applied. Further, as in Aim 1A and 2, two separate models will be examined for FXS vs. TDC and FXS vs. ASD. The responses of interest in Aim 3 are 40Hz ITC and WLI change (AAE vs. sham). The fixed effects of diagnostic group, treatment, the interaction between diagnostic group and treatment, treatment sequence, and period will be examined. Potential carryover effects will also be assessed. Covariates: Covariates for each comparison will be identical to models for Aim 1A and 2 above. The adjusted (least squares) means for the diagnostic group by treatment combination will allow to compare within and between diagnostic groups. Within the FXS group, for example, the treatment effect will be by testing the contrast (FXS treatment – FXS sham) at the 0.05 level of significance.

Exploratory analyses: **Aim 1A:** Other neurodynamic response variables (event rate and event duration) and chirp response (40Hz ITC and gamma ERSP) will be modeled as PPF. If the sex x diagnostic group interaction is significant ($p < .05$), subsequent post-hoc inter-diagnostic comparisons will be made for each sex. Lastly, for Aim 1A, the five variables representing neurodynamics and chirp response (PPF, event rate, event duration, 40 Hz ITC, and gamma ERSP) will be examined as a multivariate response across two paradigms (rest and chirp). As in the models above, diagnostic group will be the independent variable along with the appropriate covariates as described above. **Aim 1C:** To identify which FXS genotype (males with or without mosaicism and females) has similar alterations in neurodynamic variables as the Fmr1-/- KO (vs. WT) we will derive 95% confidence intervals for the effect sizes for each of the three neurodynamic variables for each of the three FXS subgroups (vs. their respective matched controls) and compare to the confidence interval for the effect size based on the Fmr1-/- KO vs. WT comparison. Lastly, significant differences between the FXS effect sizes and the murine effect size will be explored using bootstrap methodology where the resampling will be within each subgroup (human and mouse).

15.0 Provisions to Monitor the Data to Ensure the Safety of Subjects

T**here are no known risks or side effects associated with auditory entrainment; this stimulation is considered minimal risk.** However, given the potential of neuromodulation and our site's long history with TMS neuromodulation and clinical trials in FXS and ASD populations, we have opted to implement standardized safety measures and review study progress through a data safety monitoring board (DSMB).

Dr. Sam Vaughn, Outpatient Clinical Director with the Division of Psychiatry at CCHMC, will serve as the DSMB Chair. Dr. Vaughn assumes this role for the Pedapati lab neurodevelopmental disorders interventional trials, and he is divisionally supported to execute this work. The monitor will review recruitment and adverse events every 6 months and report their assessment to the P.I. The independent monitor will also review any SAEs and significant unanticipated events as they occur.

A physician will monitor data quality and adverse events at each visit. Any adverse events will be reported to the PI. 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. Any SAE during any study related activity (assessments, EEG, etc.) will be immediately reported to the DSMB and CCHMC IRB. In addition, the PI will review with the primary mentorship team if any future changes are deemed necessary to the study protocol to minimize future risk.

16.0 Provisions to Protect the Privacy Interests of Subjects

All subjects will be assured that any information collected about the participants as part of this study, including their identity, will be kept confidential. Data will be identified only by a unique identification number and not by the subject's name. No information about the participant, or provided by the participant during the research, will be disclosed to others without his/her written permission, except if necessary to protect his/her rights or welfare; or if required by law.

In reports of our findings from this study, data will only be presented as group averages that make no reference to individual participants. Participant information will not be used for any other purpose than this study unless specific informed consent is obtained beforehand.

17.0 Economic Burden to Subjects

Subjects will not incur any costs to participate in this study.

18.0 Consent Process

Consent will be obtained according to the following procedures prior to the start of any study procedures. For every subject under 18 years of age, their parent(s)/legal guardian(s) will be required to give voluntary written informed consent. Written assent will be obtained from subjects who are at least eleven years of age and cognitively able to give assent. Due to the age range for this study (5 to 15), some children will not be able to sign an assent form.

Informed consent procedures for the study will be conducted by research staff or investigators prior to enrollment. Legally authorized representatives together with participants initially will receive written materials and be given an oral explanation of the study. After an LAR has read the consent and expressed willingness to enroll in the study, the individual obtaining the informed consent will review any points about which the potential participant/LAR is unclear, and the participant/LAR be invited to ask questions. Participants and their LARs will be informed during their initial consent, and on several additional occasions throughout their experience in the study, that refusal to participate in the study will in no way influence or restrict treatment services at CCHMC, and the participant is free to withdraw at any time with no negative consequences. One signed and witnessed copy of the consent and assent forms will be stored in secure storage and the participants and LARs will be given a copy of the signed forms.

Consent may take place via in-person electronic consent (using REDCap for the eConsent) or over the phone or video call (via paper or REDCap for eConsent). REDCap is a secure web application for building and managing online surveys and databases. While REDCap can be used to collect virtually any type of data (including 21 CFR Part 22, FISMA, and HIPAA-compliant environments), it is specifically geared to support online or offline data capture for research studies and operations. The CCHMC REDCap team has templates available for research use for eConsenting which includes Consent to be a Research Subject and Authorization to Use or Disclose (Release) Health Information that Identifies Participant for a Research Study (HIPAA). The REDCap electronic consent format does not accommodate the current CCHMC formatting which includes headers with logos and stamps on each page and will therefore require some modifications. E-consenting can be conducted following CCHMC Clinical Research Procedure Number 41-1.4 Section 3.5.2.

19.0 Process to Document Consent in Writing

We will follow SOP guidelines for written documentation of consent (HRP-091).

20.0 Setting

All research procedures will take place at Cincinnati Children’s Hospital Medical Center.

21.0 Resources Available

We believe we have all the resources available to conduct this research study. Our recruitment goal is feasible considering the size of the target population, and we have sufficient amounts of time, facility space, medical resources, and trained staff to conduct and complete the study.

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