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THE EFFECTS OF PHARMACEUTICAL INTERVENTION ON NEURAL CONNECTIVITY AND COMPLEXITY IN FRAGILE X SYNDROME

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THE EFFECTS OF PHARMACEUTICAL INTERVENTION ON NEURAL CONNECTIVITY AND COMPLEXITY IN FRAGILE X SYNDROME

A DISSERTATION APPROVED FOR THE

DEPARTMENT OF PSYCHOLOGY

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Abstract

This study aims to investigate the effects of targeted pharmaceutical interventions on brain networks in individuals with Fragile X Syndrome (FXS). Focusing on three drugs tested in phase 2 clinical trials – (1) a metabatropic glutamate receptor-5 (mGluR5) antagonist in children with FXS, (2) single-dose racemic baclofen in adolescents and adults, and (3) a PDE4D allosteric inhibitor in adolescents and adults.

Previous studies suggested increased gamma connectivity, decreased alpha connectivity, and decreased complexity in FXS, which may be reversed with targeted treatment. However, none of the three drugs examined showed a significant reversal of these phenotypes. Some brain network measurements did show interesting patterns, which give insight into how the drug mechanisms affect brain function including: sedation effects on connectivity with racemic baclofen and potential minor changes in gamma connectivity with the mGluR-5 antagonist. Additionally, these measures provided insight into brain function within FXS populations. Specifically with increased alpha connectivity for males with FXS compared to females. While connectivity measures show promise in identifying potential drug effects, challenges in achieving consistent significance in small populations are evident.

Unfortunately, the small effect sizes and variability within both connectivity and entropy measures indicated that these measures are not robust enough to use as primary outcome measurements in FXS clinical trials given the small populations. Rather, they may be used more effectively in larger studies examining differences between populations or how pharmaceutical interventions work on a large scale rather than in determining individual change. This study underscores the need for further research to understand the unique dynamics of FXS,

emphasizing the importance of considering gender, age, and measurement in clinical trials and future investigations.

Chapter 1: An Introduction

Purpose

The purpose of this study is to examine the effects of targeted pharmaceutical interventions on brain networks in individuals with Fragile X Syndrome (FXS) in order to evaluate how changes in specific neurological mechanisms impact network-level brain function. The relationship between targeted treatments and network-level brain activity will have important implications for ongoing and future pharmaceutical development in FXS.

Research Objectives

The goals of this study are to determine the effects of three different drugs that were tested in phase 2 studies in FXS (1) a metabotropic glutamate receptor - 5(mGluR5) antagonist on children with FXS, (2) racemic baclofen in adolescents and adults with FXS, and (3) a PDE4D allosteric inhibitor on brain activity in adolescents and adults with FXS. Measures of brain activity include (a) functional connectivity strength, path length, and clustering and (b) permutation entropy and weighted symbolic mutual information (wSMI). (4) Additionally, comparisons between baseline populations will provide information on how brain activity measures may differ between study populations. (5) Lastly, comparison of brain activity changes between the studies will further determine how changes in brain activity are linked to mechanistic changes and symptom outcome measures.

(1) How does treatment with mGluR5 antagonist affect (1a) functional connectivity and (1b) entropy of brain networks in children with FXS?
(2) How does treatment with racemic baclofen affect (2a) functional connectivity and (2b) entropy of brain networks in adolescents and adults with FXS?

(3) How does treatment with a PDE4D allosteric inhibitor affect (3a) functional connectivity and (3b) entropy of brain networks in adolescents and adults with FXS?

(4) How are baseline measures of brain activity similar or different between studies?

(5) How are changes in brain activity similar or different between studies?

Justification

Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability and most common monogenetic cause of autism. It results from the silencing of the fragile X messenger ribonucleoprotein gene (FMR1) located on the X chromosome due to excess CGG repeats (200+). The phenotypes associated with FXS consist of a range of physical, cognitive, behavioral, and sensory deficits that can have severe impacts on quality of life (Hagerman et al., 2017).

Many different researchers are working on targeted treatments to treat Fragile X Syndrome (FXS). These treatments have been successful in mouse models of FXS but have often failed to show efficacy in human trials. The reasons for this failure in translation could include the choice of target mechanism, treatment plan, or measurement of outcome. What remains unknown is which of these reasons are the main contributing factors and if the contributing factors differ between the different drugs that have undergone human trials.

One possible reason for translational failure in clinical trials is the method of measuring outcomes. Neurodevelopmental disorders like FXS have wide-ranging symptoms that can vary from person to person. These include mood dysregulation, anxiety, hyperexcitability, and cognitive impairments, which can also encompass different aspects such as verbal intelligence and working memory. Clinical trials must choose one primary target outcome to assess significant change with intervention.

The first problem in choosing a primary outcome measure is knowing which outcome is likely to be affected by the mechanism that the drug is targeting. One method of informing this decision is to look to mouse studies, but mouse measurements of behavior do not easily map onto measures of behavior in humans. For instance, in one FXS mouse study examining reducing mGLuR5 in FMR1 knock-out (KO) mice, researchers found improvement in neuronal spine density and behavioral learning and memory. They measured learning and memory using an inhibitory avoidance and extinction task in which mice were trained that entering a dark space resulted in an adverse experience and then subsequently tested to see how long it takes for them to enter the dark space with no adverse experience. FMR1 KO mice learned not to enter the space at similar rates but had a faster extinction and in later tests entered the space faster compared to wildtype or MGluR5 reduced mice (Dölen et al., 2007). Another study found that FMR1 KO mice socialized more with a stranger mouse, and when treated with a mGluR5 antagonist, FMR1 KO mice reduced their social interaction with the stranger mouse to wild-type levels (Gantois et al., 2013). To translate these mouse experiments to human behavior directly is quite difficult, but findings may lead researchers to examine working memory retention or sociability more generally. However, researchers need methods that are available and have shown efficacy in assessing symptoms in FXS. One phase 2 clinical trial for a mGluR5 antagonist picked the Aberrant Behavior Checklist community edition (ABC-C) as the primary outcome, which examines a range of symptoms that all may be impacted by FXS including irritability, hyperactivity, social withdrawal, and stereotypic behavior (E. Berry-Kravis, Portes, et al., 2016). Another phase 2 trial assessing a mGluR5 antagonist in FXS used the Anxiety Depression and Mood Scale (ADAMS) (Youssef et al., 2018). Both studies picked primary outcomes that assess symptoms that are known to be affected by FXS but do not directly relate to

the mouse measures and could not be easily assessed in mice as indicators of target engagement. Neither study found significant results (E. Berry-Kravis, Portes, et al., 2016; Youssef et al., 2018).

The second problem in picking an outcome measurement is that many outcome measurements are often parent report surveys or scales such as the Clinician Global Impression – Improvement scale, which is a very general rating of improvement of symptoms (Busner & Targum, 2007). These measurements can be easily biased, are subject to placebo effect, and may not accurately capture the internal experience of the participant. Outcome measurements of this type could be missing significant cognitive changes that are not being captured by outside observation. Additionally, measures of cognition and working memory often encounter difficulties in assessing children or adults with intellectual disabilities due to floor effects and having the specificity required to detect significant change (E. M. Berry-Kravis et al., 2018). Measures need to be developed that can quantify different aspects of cognition in populations with intellectual disabilities and low IQ that are valid, reliable, and have adequate specificity.

Beyond measurement issues, the failure of translation could be due to the target mechanisms functioning differently in humans compared to mouse models. The reproducibility of preclinical results has been a problem in many different areas of clinical research. In Alzheimer's disease research from 2002 to 2012, 244 compounds were tested in clinical trials and only 1 was successfully approved for market use (Cummings et al., 2014). In the case of FXS mouse models, the FMR1 KO mouse causes complete loss of FMRP. However, in humans, some individuals may have repeat size mosaicism or methylation mosaicism and thus produce a small amount of protein despite CGG repeats exceeding 200 in many cells. Humans also have a more complex nervous system, and it may be the case that mouse models can not sufficiently

predict the complex and interacting mechanisms affected by FXS. Lastly, clinical trials are primarily done in adults before trials are extended to children or infants. However, animal studies are typically done on young animals (E. M. Berry-Kravis et al., 2018). This difference in age may account for some of the discrepancies between animal and human research. FXS researchers are divided on the continued utility of the FMR1 KO mouse model. Some have suggested that due to translational failures, the FMR1 KO mouse can no longer be considered a sufficient model for preclinical studies, and novel disease models in non-rodent species are needed (E. M. Berry-Kravis et al., 2018). Others point to problems in preclinical and clinical trial design as the reasons for translational failure and still believe the FMR1 KO mouse to be a valid model (E. M. Berry-Kravis et al., 2018).

A clear way to parse out the contributing factors in clinical trial failures is to use directly translatable tools to assess outcomes. Unlike behavioral outcome measurements, electroencephalography (EEG) measurements can be assessed similarly in humans and mice. For instance, FMR1 KO mice show the same increased resting state gamma-band power and decreased evoked gamma synchronization as is seen in people with FXS (Lovelace et al., 2018). Therefore, if preclinical studies show a change in gamma power with drug and no change is observed in human trials, then it would be clear that the FMR1 KO mouse model is not a sufficient model for preclinical evidence of efficacy. Beyond assessing the validity of preclinical FXS research, including EEG in clinical trial design, has many benefits. Examination of EEG outcomes could help to parse out if the drug is having an underlying effect on brain activity that is not being captured in the measures of behavior used or during the short time-line typical of a clinical trial. If a drug affects some EEG phenotypes and not others, then that inconsistency could provide insight into what phenotypes are being targeted by treatment or what

compensatory mechanisms may be influencing the success of treatment. Lastly, if EEG phenotypes are improved by pharmaceutical intervention in a trial that did not find behavioral efficacy, it could indicate that better outcome measures or trial design are needed, and the drug should be revisited.

Measures of functional connectivity would be particularly useful for assessing the extent of changes in brain activity. Connectivity measures provide more information than standard EEG power measures. In EEG, functional connectivity typically examines the strength of connection between sensors and so can provide information about communication within or between regions. It can be further examined using path length and clustering coefficients to determine the typical distance of significant connections for different frequency bands and the extent to which electrodes cluster together. Together these measures allow for the investigation of strength and spread of network function in the brain, which would be particularly beneficial for investigation FXS, a disorder that affects the balance of activation of brain networks.

As a complement to functional connectivity, measures of entropy, or network complexity, provides additional information regarding how brain activity is affected by the intervention. While connectivity and entropy are inversely related, they do capture slightly different aspects of network activity. For instance, entropy has been found to better differentiate between states of consciousness in typically developing individuals, children with Angelman's syndrome, and children with duplication 15q11.2-13.1 syndrome (Dup15q) compared to typical spectral or functional connectivity measures (Frohlich et al., 2022). Past literature has shown a complex relationship between functional connectivity and FXS with increased connectivity in gamma frequencies and decreased connectivity in alpha frequencies (Schmitt et al., 2020; Wang et al., 2017). However, entropy has only shown a general reduction of complexity in individuals with

FXS (Proteau-Lemieux et al., 2021). The dynamic relationship between functional connectivity and entropy in FXS will provide more information about the impacts of pharmaceutical intervention on brain activity in FXS than either measure would provide individually.

Measures of EEG connectivity and entropy are particularly useful in terms of practicality because they rely only on resting-state data, which is both easier to collect than stimulus-related data and more frequently utilized than any other task making it easy to compare between studies. Fortunately, clinical trials are beginning to regularly include exploratory EEG measures in trial design, which typically includes resting-state EEG. However, no study has yet examined the effect of pharmaceutical intervention on functional connectivity or entropy in FXS. Examining functional connectivity and entropy in the context of pharmaceutical intervention could determine the effect of the intervention above and beyond standard symptomatic measures as well as provide insight into what brain networks are affected and why they were or were not successful in changing previous outcome measures.

Chapter 2: Literature Review

Fragile X Syndrome (FXS) is a single-gene X-linked disorder associated with a variety of cognitive, behavioral, and sensory deficits and general intellectual disability that can range from moderately disruptive to debilitating. FXS results from an excess of CGG repeats of over 200 on the fragile X messenger ribonucleoprotein gene (FMR1). This excess of CGG repeats results in partial to full methylation of the gene, which limits or completely prevents transcription of the fragile X messenger ribonucleoprotein (FMRP), itself a translation regulator. In individuals with FXS, deficits can be severe and may include a wide range of mental and physical phenotypes, including intellectual disability, autism spectrum disorder (ASD), attention hyperactivity

disorder (ADHD), anxiety, epilepsy, insomnia, large ears, macroorchidism, and elongated faces. FXS is present in approximately 1 in 5,000 males and 1 in 8,000 females due to its location on the X chromosome (Hagerman et al., 2017).

Current treatment methods for FXS focus on managing the various symptoms of FXS such as anxiety or attention deficits. There are no currently approved medications that specifically target mechanisms involved in FXS. This is not due to a lack of research into treatments targeting specific mechanisms. FXS is the most common inherited cause of intellectual disability and autism. As such, investigation into the mechanisms of FXS may be more straightforward compared to other genetic or idiopathic ASD and may yield insights into other disorders. Research into mechanisms that can be pharmaceutically targeted to treat FXS has uncovered several different potential theories for how the absence of FMRP may lead to FXS symptoms. However, so far, no drug has found efficacy in a phase 3 trial. This could be due to many different factors including trial design aspects such as the length of treatment, the age at treatment, and measurement of outcomes, or a failure in the choice of target mechanisms (E. M. Berry-Kravis et al., 2018). Importantly, several phase 3 and earlier trials are currently ongoing, and hopefully, there will soon be an available treatment that can specifically target FXS. One thing that will undoubtedly help with this process and applying the successes of FXS to other disorders is a greater understanding of the network activity of the brain and how treatments affect brain activity.

FMRP Function and Pharmaceutical Interventions

FMRP has many functions including regulating and inhibiting the translation of many different proteins in neurons. Therefore, it has a broad impact on cellular functions. It is specifically involved in mRNA translation within dendritic spines and impacts synaptic plasticity (Bagni et al., 2012; Sidorov et al., 2013). In FMR1 KO mice, the absence of FMRP has been

linked to increased long-term depression (LTD) and decreased mGluR-dependent long-term potentiation (LTP) (Sidorov et al., 2013). Efforts to target the effects of FMRP have been focused on restoring synaptic plasticity.

The MGluR Theory

In the past decade, the predominant theory for how the absence of FMRP causes irregularities in plasticity that result in the phenotypes present in FXS has been the MGluR theory (Bear et al., 2004; E. M. Berry-Kravis et al., 2018). The mGluR theory asserts that deficits in network functioning in FXS are largely caused by group 1 MGluR-dependent protein synthesis, which is typically regulated by FMRP, but without FMRP, other proteins are produced in abundance. This overproduction causes increased internalization of AMPA receptors. Increased AMPA internalization results in increased long-term depression, altered spine morphology, and overall imbalance of excitation and inhibition in neural networks (Bear et al., 2004; E. M. Berry-Kravis et al., 2018; Pop et al., 2014).

The MGluR theory was originally supported by three basic findings: activation of MGluR1 causes protein synthesis at the synapse including synthesis of FMRP, MGluR - dependent long-term depression (LTD) depends on fast protein synthesis at the synapse, and LTD is exaggerated in FMR1 KO mice (Huber et al., 2000, 2002; Krueger & Bear, 2011; Weiler et al., 1997). Further evidence in support of the MGluR theory has been found in preclinical studies using animal models of FXS. Genetically reducing MGluR5 protein levels in Fmr1 KO mice corrected some FXS phenotypes (Dölen et al., 2007). Additionally, pharmaceutical interventions using MGluR5 antagonists have shown success in rescuing features of FXS in FMR1 KO mice and fly models (Gantois et al., 2013; Gasparini et al., 1999; Gross et al., 2010; Lindemann et al., 2011; Michalon et al., 2012; Pop et al., 2014; Porter et al., 2005; Yan et al., 2005). Unfortunately, several clinical trials in humans have recently shown that pharmaceutical

intervention on the MGluR system/mechanisms is not sufficient to impact FXS symptoms or behavior (E. Berry-Kravis, Portes, et al., 2016; Youssef et al., 2018). The cause for the discrepancy between the animal models of FXS and human trials has not yet been fully determined, although rapid tolerance of mGluR5 inhibitors has recently been documented in humans (Stoppel et al., 2021).

The GABA Theory

Another mechanism that could explain how the absence of FMRP impacts plasticity and FXS phenotypes involves the relationship between FMRP and the primary inhibitory neurotransmitter, gamma-aminobutyric acid (GABA). There are two classes of GABA receptors, $GABA_A$ and $GABA_B$. FMRP is involved in regulating the expression and catabolism of eight different $GABA_A$ receptor subtypes, which allow the flow of chloride ions across the cell membrane causing hyperpolarization and inhibiting cell firing. FMRP also regulates GABA transport proteins, proteins necessary for GABA receptor catabolism, and is involved in GABA synthesis. In the absence of FMRP, the GABA system is downregulated, and the chronic inhibition provided through GABA is decreased (Lozano et al., 2014). This effect is found particularly in the amygdala and could be related to increased sensory sensitivity, anxiety, and audiogenic seizures (Gao et al., 2018; Lozano et al., 2014).

One way to target this downregulation is to increase the activation of existing GABA receptors. Acamprosate, a broad-spectrum GABA receptor agonist, showed success in reversing some FX phenotypes in FMR1 KO drosophila and mouse models (Hutson et al., 2018; Schaefer et al., 2017). A small open-label trial in humans with FXS showed some improvement in the Clinical Global Impression – Improvement (CGI-I) scale but was not a placebo-controlled study (Erickson et al., 2014).

Researchers have also examined the effects of gaboxadol, which acts as a more specified subtype δ $GABA_A$ receptor agonist. In FMR1 KO mice, gaboxadol causes reversal of amygdala hyperexcitability and some behavioral phenotypes including hyperactivity and prepulse inhibition (Cogram et al., 2019; Olmos-Serrano et al., 2010, 2011). A phase 2a clinical trial showed potential improvements for adults and adolescents with FXS in several measures including the primary efficacy outcome, the Clinical Global Impressions-Improvement scale, but did not have a non-treatment placebo-controlled group (Budimirovic et al., 2021).

$GABA_B$ receptors are not directly regulated by FMRP. However, a reduction in GABA in GABAergic terminals in FXS may play a role in decreased inhibition from $GABA_B$ receptors (Gao et al., 2018). Additionally, $GABA_B$ receptors work to inhibit glutamate release. Glutamate receptors are upregulated in FXS, and hence, increasing $GABA_B$ receptors could work as a compensatory mechanism for the upregulation of the MGluR system (Lozano et al., 2014; Morin-Parent et al., 2019).

In FMR1 KO mouse models, arbaclofen, a $GABA_B$ agonist, has been shown to normalize protein synthesis and correct increased spine density (Henderson et al., 2012). In a phase 3 human trial, adults and adolescents showed no improvement in outcome measures, but children (ages 5-11) showed significant improvement in the ABC-C_{FX} Irritability subscale and Parenting Stress Index as well as trends toward improvement in hyperactivity and social avoidance (E. Berry-Kravis et al., 2017). Recent preclinical work has shown success in using racemic baclofen (RBAC), the enantiomer of arbaclofen, in FMR1 KO mice (Sinclair et al., 2017). A phase 2 study examined single-dose RBAC in both humans and FMR1 KO mice and found reduced gamma power in both humans and mice (Jonak et al., 2022).

Cyclic AMP

Another potential target for treatment of FXS is through its link to cyclic AMP (cAMP). The absence of FMRP is associated with a reduction in cAMP in both people with FXS and in animal models (E. Berry-Kravis et al., 1995; E. Berry-Kravis & Huttenlocher, 1992; Choi et al., 2015; Gurney et al., 2017; Kanellopoulos et al., 2012; Kelley et al., 2007). cAMP is a second messenger that is involved in many processes in the body. Importantly, it facilitates associative learning through activation of protein kinase A (PKA) after N-methyl-D-aspartate receptor (NMDAR) activation. Phosphodiesterase-4D (PDE4D) is a regulator of the cAMP involved in associative learning. Therefore, inhibition of PDE4D may increase cAMP and improve associative learning in FXS by allowing for increased utilization of existing cAMP. BPN14770, a PDE4D negative allosteric modulator, can act to inhibit PDE4D and increase use of PKA related cAMP. When treated with BPN14770, FMR1 KO mice showed improvements in both dendritic spine length as well as behavioral phenotypes, which included social interaction, nesting, and marble burying behaviors (Gurney et al., 2017). A phase 2 clinical trial in adult males showed significant improvements in cognitive outcomes as well as daily functioning measures with BPN14770 treatment. This study also explored the effect of BPN14770 on established electroencephalography (EEG) biomarkers and found a trend toward improved N1 amplitude in an auditory habituation evoked response potential (ERP) (E. M. Berry-Kravis et al., 2021).

EEG Measures in FXS

The state of FXS research is developing at several different levels: the understanding of cellular mechanisms involved, the understanding of brain activity and networks involved, and then the understanding and measurement of symptoms and behavior. There is also a consistently developing understanding of how the three levels fit together. Many of the aforementioned

clinical trials were designed with a clear theory for the mechanism of action but expected outcomes could only be informed by animal models and behavior. Biomarkers to examine how brain activity relates to symptoms and pharmaceutical intervention may offer insight into why preclinical data is not adequately predicting human response, and how the drugs may or may not be affecting brain activity, thus informing the next steps for clinical trial design and target mechanisms.

Biomarkers

EEG has been used to identify several features of brain activity in FXS that differ in comparison to typically developing controls and that translate to mouse models. These biomarkers include an elevated resting-state gamma band power, decreased alpha band power, reduced phase-locking to auditory stimuli in the gamma range, and increased amplitude in auditory N1 ERP (L. Ethridge, 2016; L. E. Ethridge et al., 2017, 2019; Pedapati et al., 2022). These features support a general theory of imbalance of excitatory and inhibitory mechanisms in the brain, which has helped to inform target mechanisms and explanations for how a lack of FMRP may cause FXS phenotypes. Each of these biomarkers could also be a useful measure in assessing the effects of pharmaceutical intervention on brain activity. EEG has already begun being utilized in exploratory outcome measurements in several ongoing or recently completed phase 2 and 3 clinical trials, but it is still being established as a biomarker for clinical trial use.

The target mechanisms being explored for pharmaceutical intervention each involve the impacts of FMRP on plasticity and long-term potentiation or depression, which then have network wide impacts on level of excitability and connectivity. The EEG measures so far discussed can be particularly useful as biomarkers, and they provide some guidance as to what aspects of excitation and inhibition are affected by FXS. For instance, high resting gamma power may indicate disfunction in inhibitory parvalbumin neurons (Lovelace et al., 2018). In addition to

these measures, EEG can be used to examine the functional connectivity of brain networks. Functional connectivity can be used to investigate the same frequency band changes as previously mentioned measures, but it also allows for investigation of how those frequencies behave and interact across the brain, which can provide insight into how long range and local networks are affected by FXS and by interventions.

Functional Connectivity

The most basic measurement of connectivity between two signals is coherence, which quantifies the variance in one signal that can be explained by another signal as a function of frequency. If $S_{xx}(\omega)$ is the power estimate of signal x for frequencies ω, and $S_{xy}(\omega)$ is the averaged cross-spectral density of signals x and y, then coherence between signals x and y can be defined as: $coh_{xy}(\omega) = \frac{|S_{xy}(\omega)|}{\sqrt{S_{xx}(\omega)S_{yy}(\omega)}}$. Though relatively simple, coherence is only able to quantify if two signals are linearly related. It is not able to capture the directional relationship between two signals (Bastos & Schoffelen, 2016). To capture direction, the phase lag index (PLI) can be calculated by measuring the difference in the phase or slope of two signals irrespective of the magnitude and estimates the degree to which a signal leads or lags in relation to another signal. It, therefore, provides a measure of unidirectional relationships. Unfortunately, this measure can be influenced by noise, volume conduction, and small trial or sample size, which is common when working with developmental populations (Vinck et al., 2011). To account for these influences, PLI can be weighted by the magnitude of the imaginary component of the cross-spectrum to get the debiased weighted PLI (dbWPLI) (Vinck et al., 2011; Wang et al., 2017). dbWPLI is expressed as a number from 0 to 1 for each connection between two signals with relationships near 1 being very synchronous. It is commonly used because it provides a measurement of connectivity that accounts for unidirectional relationships and is

robust to common reference issues, differing signal-to-noise ratios, volume conduction, and small sample size (Vinck et al., 2011). To further evaluate connectivity, graph analysis on dbWPLI can provide information on local vs long-range communication and regional clustering by taking into account the cluster coefficients (how central a sensor is amongst significant connections), centrality (how many significant paths pass through each electrode), and path lengths (how distant significant connections are for each sensor) of dbWPLI outcomes across the head (Ortiz et al., 2012). Studies using dbWPLI to examine functional connectivity in adolescents and adults with FXS have found a pattern of increased local gamma connectivity and decreased long-range alpha connectivity in FXS compared to typically developing controls (TDC) (Schmitt et al., 2022; Wang et al., 2017). However, no studies have used graph analysis on dbWPLI in FXS participants.

Complexity

An additional way to examine network function is by measuring the complexity of signals through entropy. Entropy can be used to index functional interactivity between brain regions and in typically developing populations is frequently used to examine states of consciousness and has been used to examine emotional processing (Keshmiri, 2020; Patel et al., 2021). In ASD research, children with ASD have shown reduced complexity compared to TDC (Kang et al., 2019). Additionally, one study found that children with mild ASD had more brain complexity compared to children with severe ASD, which shows that entropy is sensitive to differences in broad symptom severity (Hadoush et al., 2019). FXS also displays this general reduction in complexity compared to TDC, and that higher signal complexity in FXS is related to lower irritability, lethargy, hyperactivity, and social avoidance (Proteau-Lemieux et al., 2021).

Entropy is used to examine the complexity of EEG time series for each electrode and captures the underlying predictability of brain activity. Although it is distinct from functional

connectivity there is a general relationship between the two with stronger connectivity related to decreased complexity, as signals that are strongly connected by definition are more similar (Ghanbari et al., 2015). Permutation entropy is calculated by parsing a time series of data into patterns or motifs, computing the probability of occurrence for each motif (p_i), finding the probability distribution of all motifs, and then calculating the permutation entropy using Shannon's formula of uncertainty:

$PE = -\frac{\sum p_i \times \ln(p_i)}{\ln(number\ of\ motifs)}$ (Bandt & Pompe, 2002; Olofsen et al., 2008). The results depend on the timescale chosen for the motifs and can be manipulated to examine different frequency ranges, for instance, a timescale of 4 ms results in entropy for frequencies 32 – 80 Hz whereas a timescale of 128 ms results in entropy for 1 – 2.5 Hz (Frohlich et al., 2022). Permutation entropy is particularly useful because it is more robust to noise than other measurements of entropy (Bandt & Pompe, 2002). While permutation entropy can calculate the complexity of a signal at each electrode, it does not capture the complexity of the relationships between signals. The weighted symbolic mutual information (wSMI) is founded in the calculations for permutation entropy but is unique in that it examines the joint probability of pairs of motifs between signals rather than the probability of one motif in one signal. Therefore, it provides a measurement of complexity for each signal pairing, making it similar to a combined measure of entropy and functional connectivity. One study has examined entropy in FXS and found reduced complexity compared to neurotypical controls (Proteau-Lemieux et al., 2021). However, this study did not compare frequencies and did not examine functional entropy.

Chapter 3: Methodology

Study Data/Participants

We have obtained resting-state EEG data from three phase 2 clinical trials examining the effects of three different drugs on FXS. These studies vary both in target mechanisms and in study designs and were not originally intended to be directly compared. All three studies have completed data collection and analysis for primary outcomes, which do not include resting-state EEG, and have published results (E. Berry-Kravis et al., 2023; E. M. Berry-Kravis et al., 2021; Jonak et al., 2022).

Study 1: The first study was a phase 2 clinical trial investigating the effects of AFQ056, a mGluR5 selective antagonist, on children with FXS. The study consisted of 110 children ages 32 months to 6 years with FXS of which 50 provided EEG data for at least one timepoint. All patients participated in a 4-month placebo lead-in period and then were randomized into treatment or control. The placebo-controlled treatment period was 8 months after which there was an 8-month open-label extension. All patients also participated in a 6-month language learning intervention starting 2 months into the treatment period. Study results found no significant improvements in language learning or in any primary or secondary outcome measurements (E. Berry-Kravis et al., 2023). For this analysis, we were able to obtain usable full EEG datasets for 15 participants, 13 males and 2 females. Data loss from this study was in part due to participant behavior, which is expected in young children with neurodevelopmental conditions, but additionally due to the impact of the global COVID-19 pandemic on in-person data collection for later timepoints.

Study 2: The second study was a phase 2 clinical trial investigating the effects of a single dose of racemic baclofen (RBAC), a $GABA_b$ selective agonist. The study was a placebo-

controlled cross-over design with a 2-week washout period. Seventeen adolescents and adults with FXS were randomized to receive control or treatment first or second. Treatment consisted of a single dose of 30 mg RBAC. Participants ranged in age from 16 to 43. Study results found improvement in gamma power with treatment (Jonak et al., 2022). For this analysis, we were able to obtain usable full EEG datasets for 15 participants, 7 males and 8 females. Data loss was minimal for this study, due primarily to participant behavior and electrical artifact.

Study 3: This study was a phase 2 clinical trial investigating the effects of a PDE4D allosteric inhibitor, BPN14770, twice daily at 25 mg. Participants included 30 males with full-mutation FXS, ranging in age from 18 to 42. The treatment period was 24 weeks and contained a cross-over at 12 weeks with no washout period. Participants were randomly assigned to start with placebo or treatment. There was a meaningful carryover effect, so the main analysis only included the first treatment group. The study found significant results for cognitive outcome measures (E. M. Berry-Kravis et al., 2021). For this analysis, we were able to obtain usable full EEG datasets for 13 participants. Data loss for this study was primarily due to participant behavior, however the majority of participants provided two out of three data collection timepoints, suggesting that the EEG was generally well-tolerated in adolescents and adults with FXS, as seen in study 2.

Procedure

Study 1: Approximately 3 minutes of eyes open resting state EEG was collected. Participants were asked to sit and watch a silent cartoon video during data collection. Six sites were included in the EEG portion of the study and had 4 different EEG systems: a Biosemi 32-channel system (1 site), an EGI 32-channel system (1 site), an EGI 64-channel system (1 site),

and an EGI 128 channel system (3 sites). All data were re-referenced to a standard 33-channel montage.

Study 2: Five minutes of resting-state EEG was collected while participants sat watching a silent video to facilitate compliance. Recordings were collected with a Phillips/EGI NetAmp 400 system using a 128-channel Hydrocel saline-based electrode net sampled at 1000 Hz referenced to Cz (Jonak et al., 2022).

Study 3: Continuous resting-state EEG was collected in which participants were instructed to sit for 1 minute with eyes open followed by 30 seconds with eyes closed and then each block was repeated. For consistency with the other studies, the present study will examine the eyes-open resting data. A 32-channel BioSemi ActiveTwo system was used and data was sampled at 512 Hz (E. M. Berry-Kravis et al., 2021). The BioSemi system utilizes a monopolar reference feedback loop connecting a driven passive sensor and a common-mode-sense active sensor, both located on posterior scalp.

EEG Processing: Data from all 3 studies was processed using an identical procedure. Data was filtered using a low-pass filter of 100 Hz, a highpass filter of 0.5 Hz, and a notch filter at 60 Hz. Bad channels were interpolated (no more than 5%), and sections of extreme artifacts identified by visual inspection were removed. Independent component analysis was performed using EEGLAB (Delorme & Makeig, 2004), and components containing large amounts of high amplitude, ocular-motor, muscle tension, or cardiovascular artifact were removed. Lastly, data was segmented into 2-second epochs and subject to a 120 μV threshold to remove the remaining artifact. For study 2, only 32 electrodes that most closely match the 33 and 32 electrodes in studies 1 and 3 were used in the analysis. This was done in order to make comparison with the other studies more similar and reduce the number of comparisons between electrodes. Data from

studies 1 and 3 were rereferenced during initial processing to an average reference. For this analysis data was returned to the original reference, 'Cz'.

Analysis

Changes in all outcome measurements were analyzed using repeated measures ANOVAs in which time, condition, and the interaction of time and condition were examined as covariates. However, the meaning of condition differs for each study given the different study designs. In study 1 condition refers to whether the participant received placebo or drug, therefore time is a within-subjects variable and condition is a between-subjects variable. In study 2, each participant has a baseline and endpoint measurement for both drug and placebo, so condition refers to if the measurement was taken during the placebo period or the drug period and is a within-subjects variable. Study 3 had a crossover design in which there were three timepoints: baseline, crossover, and endpoint. Condition is a between-subjects variable and refers to if the subject received placebo first and then drug after the crossover or drug then placebo. For all studies the drug effect will be found in the interaction between time and condition. Given the signal-finding purpose of these Phase 2 studies, relatively small (although appropriately sized for Phase 2) samples, and exploratory nature of the analyses, although false-discovery rate is used to correct for multiple comparisons, figures are provided additionally showing all comparisons with $p<.05$ for all analyses.

Assumptions for use of repeated measures ANOVAs were examined. Firstly, participants in all three studies were randomly sampled from clinic populations and randomly assigned to placebo or control when applicable in studies 1 and 3. Therefore supporting independence within the samples. Connectivity measurements show a slight positive skew. Repeated measures models are robust to slight skew, but to further examine the impact, the distribution of residuals was

examined for each of the repeated measures analyses using Shapiro-Wilk tests. The vast majority of these tests do not show evidence of non-normality (average W = 0.90, average p-value = 0.35), and distributions of residuals are normal for all frequency bands and all studies. Small world values, permutation entropy, and wSMI are all normally distributed.

The sphericity assumption was examined for all tests using Mauchly's test for sphericity. For connectivity, the vast majority of tests showed equal variance between groups for all studies and frequency bands (average W = 0.76, average p-value = .41). However, permutation entropy measures did fail some sphericity tests. Study 1 showed unequal variances between groups for the 4-10 Hz ($W = 0.36$, $p < 0.05$) and 8-20 Hz ($W = 0.52$, $p < 0.01$) bands. Study 2 showed unequal variances for 2-5 Hz ($W = 0.36$, $p < 0.05$) and 4-10 Hz ($W = 0.27$, $p < 0.01$) bands. Lastly, for study 3, permutation entropy in the 8-20HZ band did violate the sphericity assumption ($W = 0.38$, $p < 0.01$). These violations increase the chance for type 1 error (Blanca et al., 2023). However, given that no significant results came from the permutation or wSMI analysis, no further actions were needed.

Connectivity Analysis

For all 3 studies, dbWPLI was calculated for 5 frequency bands: delta (2-3.5 Hz), theta (3.5-7.5 Hz), alpha (8-12.5 Hz), beta (13-30 Hz), and gamma (30.5-55 & 65-90 Hz) and each electrode pair (Cohen, 2014). At least 60 second of clean data were required to calculate connectivity. Data was down-sampled to 250 Hz and used 2 second epochs with a 50% overlap. Connectivity was calculated for each pair of electrodes in the data. ANCOVAs were run for each connection and a false discovery-rate of 5% was used to reduce type 1 error. Graph analysis was calculated using the Braph 1.0 Toolbox and was used to examine small world differences and to

plot path length and (Mijalkov et al., 2017). Small world differences were averaged over the head and analyzed for each frequency band.

Entropy Analysis

Permutation entropy was calculated for all 32 channels in all 3 studies. Permutation entropy was calculated using methods from Frohlich (2022). Data was first down-sampled to 125 Hz. Permutation entropy was computed using 5 second windows that overlap 50% with the number of motifs set to 3. It was calculated for 5 motif timescales, 8 ms (16-40 Hz), 16 ms (8-20 Hz), 32 ms (4-10 Hz), 64 (2-5 Hz), and 128 ms (1-2.5 Hz).

Functional entropy was assessed using wSMI. wSMI was calculated using methods from Frohlich (2022) adapted from King (2013)(Frohlich et al., 2022; King et al., 2013). All settings used in the permutation entropy calculation were repeated in the wSMI calculation. The relationship between all electrodes pairs was assessed. Calculations used for permutation entropy were repeated with the exception that probability between electrode pairs was examined rather than probability within individual electrodes. wSMI values were normalized by the corresponding permutation entropies of each electrode pairing. ANCOVAs were run for each connection and a false discovery-rate of 5% was used to reduce type 1 error.

Between Studies Analysis

Baseline dbWPLI and wSMI were compared between studies for each connection for each frequency band. A false discovery rate (FDR) of 5% was used to reduce type 1 error. Age and gender cannot be compared between studies as they are different between studies and are therefore confounded with study differences. However, we examined the effects of age on global dbWPLI within study 1 due to it being the only study with a developmental population (Ages 2-7, mean age = 5.43). Additionally, study 2 is the only study that contains balanced numbers of

males (N = 7) and females (N = 11). Only males were included in the between studies analysis. We additionally compared baseline global dbWPLI between gender for study 2.

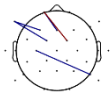
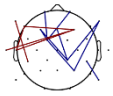
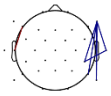
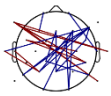
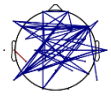
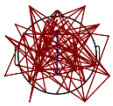
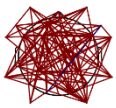
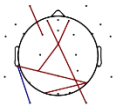
Chapter 4: Results

Connectivity

dbWPLI change between treatment groups for each frequency band was analyzed using a series of repeated measures ANCOVAs for each connection and for each frequency band. Study 1 contained 496 connections, Study 2 contained 465 connections, and Study 3 contained 465 connections. An FDR of 5% was used to reduce the chance of type 1 error. Significant differences prior to FDR correction for the interaction between time and condition were plotted on topographies to show potential patterns of change (see figure 1). For studies 1 and 3, no connections remained significant after FDR correction for the main effects of time and condition or the interaction between time and condition. Study 2 showed an increase in beta connectivity that remained in 3 connections after FDR and an increase in delta that remained in 2 connections after FDR. The significant beta connections are between Fp1, a left frontal electrode, and three electrodes: F7, T7, and O2, electrodes in left temporal and the right posterior region. The significant delta connections are between P5, a left posterior electrode, and two electrodes: E29 and E26, a face electrode and a right occipital electrode. In both frequency bands, plots showing significant differences prior to FDR indicate that this effect is likely global rather than specific to these connections (see figure 1).

Figure 1

Significant Interactions in Connectivity between Drug Condition and Timepoint prior to FDR

	Delta	Theta	Alpha	Beta	Gamma
Study 1					
Study 2					
Study 3					

Red lines indicate an increase in connectivity for treatment groups/periods relative to placebo. Blue lines indicate a decrease in connectivity for treatment groups/periods relative to placebo. All connections were insignificant after an FDR correction of 5%.

Mean small world values were examined for each frequency band to determine changes in organization of networks. Study 1 showed a significant interaction between time and condition for the delta frequency ($F(1,14) = 5.76$, $p < 0.05$) with small world decreasing for the placebo group and increasing for the treatment group (see tables 1 & 2). Study 2 also showed a significant interaction between time and condition for the delta frequency ($F(1,14) = 7.30$, $p < 0.05$) with small world decreasing for the placebo group and increasing for the treatment group (see table 1). Study 3 showed no significant differences in small world for time, condition, or the interaction of time and condition. All ANCOVA outcomes seen in table 2.

Table 1

Mean Small World Values for Each Timepoint

			Frequency Band									
			Delta		Theta		Alpha		Beta		Gamma	
		N	M	SD	M	SD	M	SD	M	SD	M	SD
Study 1 Time repeated measure	Baseline, Placebo	7	0.9995	0.0044	1.0000	0.0011	1.0008	0.0023	0.9999	0.0002	1.0001	0.0003
	End, Placebo	7	0.9968	0.0038	0.9980	0.0060	1.0014	0.0029	1.0000	0.00006	1.0000	0.0006
	Baseline, Drug	8	0.9973	0.0028	1.0012	0.0025	1.0011	0.0019	1.0012	0.00009	1.0000	0.0001
	End, Drug	8	1.0004	0.0054	1.0012	0.0019	0.9998	0.0013	1.0012	0.0003	1.0000	0.0002
Study 2 Time and Condition repeated measures	Baseline, Placebo	15	1.0019	0.0035	1.0009	0.0029	1.0012	0.0048	1.0000	0.0002	1.0001	0.0003
	End, Placebo	15	0.9984	0.0052	1.0018	0.0031	1.0008	0.0038	1.0004	0.0009	1.0002	0.0003
	Baseline, Drug	15	0.9987	0.0074	1.0013	0.0044	1.0003	0.0017	1.0004	0.0011	1.0002	0.0005
	End, Drug	15	1.0026	0.0036	0.9997	0.0034	1.0005	0.0027	1.0002	0.0004	1.0003	0.0013
Study 3 Condition repeated measure Condition1 = Placebo then Drug 2 = Drug then Placebo	Baseline, Condition 1	7	0.9998	0.0015	1.0003	0.0011	0.9999	0.0008	0.9998	0.00006	0.9999	0.0001
	Crossover, Condition 1	7	1.0008	0.0012	0.9998	0.0005	0.9999	0.0003	1.0000	0.0001	0.9999	0.00008
	End, Condition 1	7	0.9998	0.0015	0.9998	0.0007	1.0000	0.0009	0.9999	0.0001	0.9999	0.00007
	Baseline, Condition 2	6	1.0000	0.0029	1.0004	0.0012	1.0006	0.0009	0.9998	0.00009	0.9999	0.0001
	Crossover, Condition 2	6	1.0000	0.0010	1.0007	0.0013	1.0002	0.0009	0.9999	0.00009	0.9999	0.00003
	End, Condition 2	6	1.0005	0.0021	1.0005	0.0016	1.0000	0.0004	0.9999	0.00009	0.9999	0.00005

Table 2

ANCOVA Outcomes for Small World Comparison of Condition and Time for Each Study

		Frequency Band									
		Delta		Theta		Alpha		Beta		Gamma	
	df	F	p	F	p	F	p	F	p	F	p
Study 1											
Condition	1,14	0.14	0.71	3.91	0.07	0.32	0.58	1.70	0.21	0.89	0.36
Time	1,14	2.27	0.15	0.02	0.90	2.72	0.12	0.00	0.99	0.13	0.72
C x T	1,14	**5.76**	**0.03**	0.75	0.40	3.25	0.09	0.11	0.75	0.75	0.40
Study 2											
Condition	1,14	0.23	0.64	0.98	0.34	0.35	0.56	0.47	0.50	0.52	0.48
Time	1,14	0.03	0.86	0.27	0.61	0.01	0.91	0.10	0.75	0.17	0.68
C x T	1,14	**7.30**	**0.02**	2.20	0.16	0.18	0.67	2.79	0.11	0.00	0.96
Study 3											
Condition	1,16	0.79	0.39	0.81	0.38	0.80	0.38	0.79	0.39	0.79	0.39
Time	2,32	1.36	0.27	1.33	0.28	1.36	0.27	1.36	0.27	1.35	0.27
C x T	2,32	0.79	0.46	0.78	0.47	0.80	0.46	0.79	0.46	0.79	0.46

Results from the interaction of condition and time from the overall ANOVAs for small world. SW = b0 + bt +bc +bct + e.

Pathlength was plotted using heat map topographies for each time period and condition as well as change in path length for placebo and drug conditions (see appendix). These topographies showed very little change that could be attributed to drug differences. In some cases, there were clear differences between periods regardless of condition which may indicate a sensitivity to state changes rather than drug changes.

Entropy

Permutation entropy was calculated for each node and then averaged across the head for each frequency band. For all three studies, no significant relationships were found for time, condition, or the interaction of time and condition for any of the frequency bands (see tables 3 &

4). Study 3 did show a trend towards significance for the interaction of time and condition in the frequency range 8-20, which encompasses high alpha and low beta, ($F(2,22) = 3.32$, $p = 0.054$) with an increase in entropy during treatment periods compared to placebo periods (see tables 3 & 4).

Table 3

Mean Permutation Entropy Values for Each Timepoint

			Tau (Frequency Range)									
			128 ms (1-2.5 Hz)		64 ms (2-5 Hz)		32 ms (4-10 Hz)		16 ms (8-20 Hz)		8 ms (16-40 Hz)	
		N	M	SD	M	SD	M	SD	M	SD	M	SD
Study 1 Time repeated measure	Baseline, Placebo	7	1.64	0.01	1.66	0.02	1.63	0.01	1.57	0.02	1.61	0.03
	End, Placebo	7	1.64	0.01	1.68	0.03	1.64	0.02	1.56	0.03	1.58	0.04
	Baseline, Drug	8	1.63	0.02	1.65	0.03	1.62	0.02	1.58	0.05	1.63	0.03
	End, Drug	8	1.64	0.01	1.66	0.03	1.63	0.03	1.58	0.07	1.62	0.05
Study 2 Time and Condition repeated measures	Baseline, Placebo	15	1.62	0.03	1.65	0.02	1.68	0.03	1.63	0.02	1.62	0.04
	End, Placebo	15	1.62	0.02	1.65	0.02	1.68	0.04	1.62	0.02	1.61	0.05
	Baseline, Drug	15	1.62	0.01	1.65	0.02	1.67	0.03	1.62	0.02	1.62	0.03
	End, Drug	15	1.62	0.02	1.65	0.02	1.67	0.03	1.62	0.02	1.62	0.03
Study 3 Condition 1 = Placebo then Drug 2 = Drug then Placebo	Baseline, Condition 1	7	1.57	0.02	**1.60**	**0.03**	1.62	0.03	1.60	0.02	1.59	0.03
	Crossover, Condition 1	7	1.58	0.02	**1.60**	**0.02**	1.62	0.02	1.60	0.03	1.58	0.03
	End, Condition 1	7	1.58	0.02	**1.61**	**0.02**	1.63	0.02	1.60	0.05	1.58	0.05
	Baseline, Condition 2	6	1.57	0.03	**1.59**	**0.01**	1.61	0.01	1.57	0.02	1.57	0.03
	Crossover, Condition 2	6	1.59	0.01	**1.62**	**0.01**	1.63	1.02	1.57	0.03	1.56	0.02
	End, Condition 2	6	1.59	0.02	**1.61**	**0.02**	1.63	0.01	1.59	0.02	1.57	0.03

Permutation entropy mean and standard deviation

Table 4

ANCOVA Outcomes for Permutation Entropy Comparison of Condition and Time for Each Study

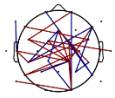
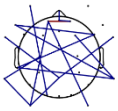
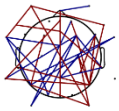
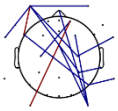
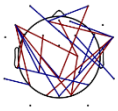
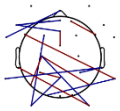
		Tau (ms) – Frequency Band									
		128 (1-2.5 Hz)		64 (2-5 Hz)		32 (4-10 Hz)		16 (8-20 Hz)		8 (16-40 Hz)	
	df	F	p	F	p	F	p	F	p	F	p
Study 1											
Condition		0.03	0.86	1.94	0.19	2.02	0.18	0.38	0.55	3.92	0.07
Time		0.34	0.57	1.19	0.30	2.23	0.16	0.07	0.79	1.30	0.28
C x T	1, 12	1.52	0.24	0.01	0.92	0.11	0.75	0.01	0.92	0.08	0.78
Study 2											
Condition		0.29	0.60	0.17	0.69	0.24	0.63	0.81	0.38	0.22	0.65
Time		0.61	0.45	0.20	0.66	0.07	0.80	0.56	0.47	0.80	0.39
C x T	1, 14	0.09	0.77	0.00	0.99	0.00	1.00	0.16	0.69	0.00	0.97
Study 3											
Condition	1,11	0.25	0.62	0.18	0.68	0.08	0.78	4.32	0.06	1.99	0.19
Time	2,22	0.12	0.89	2.19	0.14	0.08	0.92	0.10	0.91	0.33	0.72
C x T	2, 22	0.72	0.50	**3.32**	**0.054**	1.17	0.33	0.31	0.74	0.32	0.73

Permutation Entropy. Results from the interaction of condition and time from the overall ANOVAs for permutation entropy. Entropy = b0 + bt +bc +bct + e.

The change in wSMI was assessed using the same permutation approach used for dbWPLI for all connection and all frequencies, and an FDR of 5% was used to control for type 1 error. For all three studies, no significant connections remained after FDR correction for time, condition, or the interaction between time and condition. Significant connections prior to FDR for the interaction between time and condition for all 5 time scales are shown in figure 2.

Figure 2

Significant Interactions in Functional Entropy between Drug Condition and Timepoint prior to FDR

	128 ms (1-2.5 Hz)	64 ms (2-5 Hz)	32 ms (4-10 Hz)	16 ms (8-20 Hz)	8 ms (16-40 Hz)
Study 1			No significant connections	No significant connections	
Study 2					
Study 3					

Red lines indicate an increase in complexity for treatment groups/periods relative to placebo. Blue lines indicate a decrease in complexity for treatment groups/periods relative to placebo.

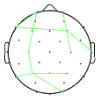
Baseline Comparisons

Connectivity

In order to compare connectivity between studies at baseline, the 18 electrodes present in all three net layouts were selected. Baseline connectivity was compared between all three studies using a one-way ANOVA for each electrode connection for each frequency band resulting in 152 tests per frequency band. An FDR correction of 5% was used, and after correction one significant connection remained for the delta band between P3 and T7 ($F(2,44) = 10.86$, $p = 0.0001$). All significant connections prior to FDR correction are shown in figure 3. Electrodes used for comparison were: Fp1, Fp2, Fz, F3, F4, F7, F8, C3, C4, Cz, O1, O2, P3, P4, P7, P8, Pz, T7, T8.

Figure 3

Connections with Significant Relationship between Connectivity and Study at Baseline prior to FDR

	Delta	Theta	Alpha	Beta	Gamma
Baseline Connectivity x Study					

Connections with a significant difference in baseline connectivity between studies. In this case, color does not represent value and therefore are shown as green.

To account for all original electrode connections, we also performed comparisons between baseline connectivity for data averaged over the entire head as well as for right and left frontal, temporal, and posterior regions and interhemispheric connections for each region. Although study 1 had five sites contributing EEG data, the majority of the clean EEG was collected at the two sites that study 2 and 3 were conducted through. Therefore, for this analysis differences in study and site can be further parsed by including a site covariate and removing study 1 participants for which data was collected at other sites. There were significant site by study interactions in whole-brain and frontal theta as well as whole-brain, frontal, and temporal alpha. These interactions showed a general pattern in which site 2 remained consistent between studies 1 and 2, but for site 1, study 3 had higher connectivity than study 1. Gamma frequency showed significant site effects for all regions with greater connectivity for site 1 compared to site 2. Lastly, connectivity in the beta frequency band showed a mix of site, study, and site by study interaction effects in the whole brain, frontal, and temporal regions. Differences seem to be primarily driven by increased beta connectivity in study 2, with the largest difference in beta connectivity occurring between frontal hemispheres. All means shown in table 5 and all ANOVA results shown in table 6.

Table 5

Mean Values for Connectivity at Baseline by Site

	Study	Site	Delta	Theta	Alpha	Beta	Gamma
			M (SD)	M (SD)	M (SD)	M (SD)	M (SD)
Whole Brain	**1**	**1**	0.30 (0.03)	0.21 (0.02)	0.15 (0.01)	0.09 (0.01)	0.08 (0.02)
		2	0.30 (0.03)	0.23 (0.04)	0.16 (0.02)	0.09 (0.01)	0.08 (0.02)
	2	**1**	0.31 (0.01)	0.22 (0.01)	0.19 (0.02)	0.10 (0.01)	0.08 (0.02)
	3	**2**	0.30 (0.02)	0.21 (0.02)	0.17 (0.02)	0.09 (0.01)	0.06 (0.01)
Frontal							
Between hemisphere	**1**	**1**	0.31 (0.03)	0.19 (0.02)	0.14 (0.03)	0.10 (0.01)	0.08 (0.01)
		2	0.30 (0.02)	0.22 (0.02)	0.16 (0.02)	0.09 (0.01)	0.06 (0.00)
	2	**1**	0.32 (0.02)	0.23 (0.02)	0.19 (0.03)	0.16 (0.01)	0.08 (0.02)
	3	**2**	0.29 (0.02)	0.20 (0.03)	0.16 (0.03)	0.10 (0.01)	0.06 (0.01)
Right	**1**	**1**	0.30 (0.03)	0.19 (0.04)	0.16 (0.02)	0.09 (0.01)	0.08 (0.02)
		2	0.30 (0.03)	0.22 (0.02)	0.16 (0.03)	0.09 (0.01)	0.06 (0.00)
	2	**1**	0.31 (0.02)	0.21 (0.02)	0.19 (0.03)	0.10 (0.01)	0.08 (0.02)
	3	**2**	0.31 (0.04)	0.21 (0.04)	0.18 (0.05)	0.10 (0.01)	0.06 (0.01)
Left	**1**	**1**	0.31 (0.03)	0.23 (0.04)	0.16 (0.03)	0.09 (0.01)	0.07 (0.02)
		2	0.31 (0.03)	0.23 (0.04)	0.16 (0.03)	0.09 (0.01)	0.07 (0.02)
	2	**1**	0.32 (0.02)	0.23 (0.03)	0.20 (0.04)	0.10 (0.02)	0.08 (0.02)
	3	**2**	0.30 (0.03)	0.20 (0.03)	0.17 (0.03)	0.09 (0.01)	0.06 (0.01)
Temporal							
Between hemisphere	**1**	**1**	0.31 (0.04)	0.22 (0.03)	0.15 (0.02)	0.09 (0.01)	0.08 (0.01)
		2	0.30 (0.02)	0.24 (0.05)	0.16 (0.03)	0.09 (0.01)	0.06 (0.00)
	2	**1**	0.32 (0.02)	0.22 (0.02)	0.19 (0.02)	0.11 (0.01)	0.07 (0.01)
	3	**2**	0.31 (0.04)	0.21 (0.05)	0.17 (0.03)	0.09 (0.02)	0.06 (0.01)
Right	**1**	**1**	0.27 (0.08)	0.20 (0.07)	0.15 (0.03)	0.08 (0.01)	0.08 (0.02)
		2	0.32 (0.03)	0.24 (0.03)	0.17 (0.03)	0.09 (0.01)	0.06 (0.00)
	2	**1**	0.30 (0.04)	0.22 (0.03)	0.20 (0.01)	0.11 (0.01)	0.07 (0.01)
	3	**2**	0.30 (0.04)	0.20 (0.03)	0.16 (0.03)	0.10 (0.01)	0.06 (0.01)
Left	**1**	**1**	0.32 (0.02)	0.21 (0.07)	0.17 (0.06)	0.09 (0.02)	0.09 (0.05)
		2	0.32 (0.04)	0.22 (0.03)	0.16 (0.04)	0.09 (0.01)	0.07 (0.02)
	2	**1**	0.31 (0.02)	0.22 (0.02)	0.20 (0.03)	0.11 (0.01)	0.07 (0.02)
	3	**2**	0.30 (0.03)	0.20 (0.03)	0.17 (0.04)	0.09 (0.01)	0.07 (0.01)
Posterior							
Between hemisphere	**1**	**1**	0.30 (0.05)	0.23 (0.05)	0.16 (0.04)	0.10 (0.01)	0.09 (0.03)
		2	0.30 (0.02)	0.23 (0.04)	0.17 (0.02)	0.09 (0.01)	0.06 (0.01)
	2	**1**	0.31 (0.01)	0.22 (0.01)	0.19 (0.02)	0.11 (0.02)	0.08 (0.03)
	3	**2**	0.30 (0.04)	0.22 (0.05)	0.18 (0.03)	0.10 (0.02)	0.05 (0.01)
Right	**1**	**1**	0.32 (0.02)	0.21 (0.07)	0.17 (0.06)	0.09 (0.02)	0.09 (0.05)

		2	0.31 (0.04)	0.22 (0.05)	0.15 (0.02)	0.09 (0.01)	0.06 (0.01)
	2	**1**	0.31 (0.02)	0.22 (0.02)	0.18 (0.21)	0.10 (0.01)	0.07 (0.02)
	3	**2**	0.31 (0.04)	0.21 (0.04)	0.18 (0.05)	0.10 (0.01)	0.06 (0.01)
Left	**1**	**1**	0.30 (0.04)	0.23 (0.03)	0.16 (0.03)	0.09 (0.01)	0.08 (0.03)
		2	0.30 (0.04)	0.23 (0.03)	0.16 (0.03)	0.09 (0.01)	0.08 (0.03)
	2	**1**	0.30 (0.01)	0.22 (0.01)	0.18 (0.02)	0.10 (0.01)	0.08 (0.02)
	3	**2**	0.30 (0.03)	0.21 (0.04)	0.18 (0.03)	0.10 (0.01)	0.06 (0.01)

Mean baseline connectivity values and standard deviations for each study and site for subjects used in baseline comparison between studies controlling for site. Study 1, site 1 N = 6. Study 1, site 2 N = 8. Study 2, site 1 N = 7. Study 3, site 2 = 21.

Table 6

ANOVA results for Mean Regional Connectivity at Baseline Between Studies

		Delta	Theta	Alpha	Beta	Gamma
		F	F	F	F	F
Whole Brain	**Study**	0.00	2.44	3.48	**6.05***	0.03
	Site	0.97	0.12	1.79	**10.94****	**17.48****
	S x S	0.86	**4.64***	**8.49****	**4.55***	1.06
Frontal						
	Study	0.52	0.52	2.07	**4.28***	0.12
Between hemisphere	**Site**	3.61	0.00	1.33	**6.88***	**14.61****
	S x S	2.10	**5.92***	**10.67****	0.05	0.00
Right	**Study**	0.21	0.42	0.76	**7.40****	1.18
	Site	0.26	0.23	**4.94***	4.29	**11.48****
	S x S	0.49	3.75	3.85	0.32	0.20
Left	**Study**	0.16	**6.11***	0.19	1.24	0.07
	Site	0.43	0.01	0.20	2.26	**14.46****
	S x S	2.61	**6.07***	**13.35****	3.33	0.55
Temporal						
	Study	0.54	1.44	2.34	0.55	**4.71***
Between hemisphere	**Site**	1.01	0.23	1.29	**5.55***	**20.24****
	S x S	0.19	0.58	**5.22***	2.05	**4.06***
Right	**Study**	0.25	**4.49***	0.58	**4.27***	0.10
	Site	1.57	0.61	2.10	3.55	**6.60***
	S x S	1.85	2.85	**8.91****	**13.50****	3.15
Left	**Study**	0.02	0.06	0.55	**6.06***	1.29
	Site	0.02	0.06	0.55	**6.06***	1.29
	S x S	0.04	2.13	**5.06***	**4.29***	0.09
Posterior						
	Study	0.04	0.77	2.33	1.77	0.71
Between hemisphere	**Site**	0.47	0.05	0.38	3.12	**11.11****
	S x S	0.14	0.01	0.82	1.09	1.70
Right	**Study**	0.02	0.08	**4.32***	2.75	0.56
	Site	0.53	0.00	1.60	1.64	**5.46***
	S x S	0.36	0.13	0.00	0.01	3.15
Left	**Study**	0.28	2.66	2.19	0.26	1.52
	Site	0.23	0.31	0.04	2.07	**13.10****
	S x S	0.38	0.18	1.11	1.98	**4.67***

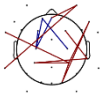
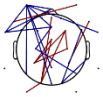
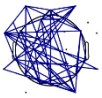
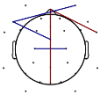
Results from the baseline ANOVAs for differences between studies at baseline for mean connectivity within regions. Connectivity = $b_0 + b_{study} + b_{site} + b_{study*site} + e$. Values represent F values from ANOVA with df = 2,32. * denotes significance at alpha = 0.05. ** denotes significance at alpha = 0.01.

Gender

Study 2 had a large proportion of females included in the study population in contrast to studies 1 and 3. Therefore, baseline connectivity was examined between genders using a series of ANOVAs for each connection and frequency band. No significant connections remained after a 5% FDR correction, but significant connections prior to FDR correction are shown in figure 4.

Figure 4

Connections with Significant Relationship between Connectivity and Gender within Study 2 prior to FDR

	Delta	Theta	Alpha	Beta	Gamma
Study 2					

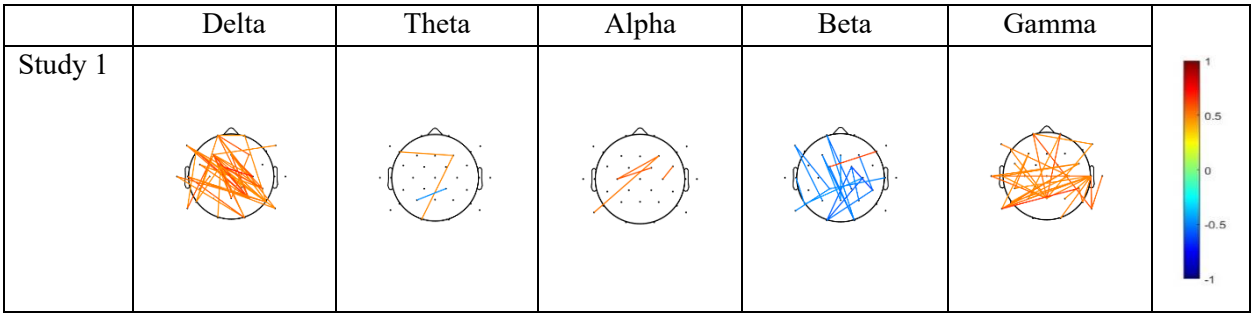
Red lines indicate greater connectivity for females relative to males. Blue lines indicate greater connectivity for males relative to females.

Age

The population of study 1 contained children ages 2-7. Given the changes in development during this period, we examined the correlation between connectivity and age for each connection and frequency band. No connections remained significant after a 5% FDR correction, but connections with significant correlations prior to FDR correction are shown in figure 5.

Figure 5

Connections with Significant Relationship between Connectivity and Age within Study 1 prior to FDR



Connections with significant correlation between connectivity strength and age at baseline in Study 1. The color of each line corresponds with the r value of the correlation.

Entropy

For entropy comparisons between studies at baseline, the 18 electrodes present in all three net layouts were selected. Baseline permutation entropy was then averaged across all 18 electrodes and compared between all three studies controlling for site using an ANCOVA. For 1-2.5 Hz, which corresponds to the delta frequency band, there was a significant relationship between baseline permutation entropy and site. Site 1 had decreased permutation entropy compared to site 2. For permutation entropy between 4-10 Hz, which corresponds to high theta and low alpha, there were significant study, site, and study by site interactions largely driven by low permutation entropy in study 1. Similarly, permutation entropy from 8-20 Hz, which contains alpha and low beta, there were significant effects of study and site that appear largely driven by lower permutation entropy in study 1. Lastly, for permutation entropy in a beta and low gamma range from 16-40 Hz, there was a site by study interaction in which site 1 had high permutation entropy in study 1 but not in study 2. All means shown in table 7 and all ANOVA results shown in table 8. Electrodes used for comparison were: Fp1, Fp2, Fz, F3, F4, F7, F8, C3, C4, Cz, O1, O2, P3, P4, P7, P8, Pz, T7, T8.

Table 7

Mean Permutation Entropy Values for Each Timepoint

			Tau (Frequency Range)									
			128 ms (1-2.5 Hz)		64 ms (2-5 Hz)		32 ms (4-10 Hz)		16 ms (8-20 Hz)		8 ms (16-40 Hz)	
		N	M	SD	M	SD	M	SD	M	SD	M	SD
Study 1	Site 1	5	1.63	0.02	1.64	0.02	1.62	0.01	1.60	0.03	1.64	0.03
	Site 2	8	1.64	0.01	1.66	0.01	1.63	0.02	1.56	0.02	1.61	0.03
Study 2	Baseline Site 1	7	1.62	0.01	1.65	0.01	1.69	0.02	1.62	0.01	1.60	0.02
Study 3	Baseline Site 2	18	1.63	0.02	1.65	0.02	1.68	0.02	1.64	0.03	1.62	0.03

Permutation entropy mean and standard deviation by study and site for baseline comparison.

Table 8

ANCOVA Outcomes for Permutation Entropy Comparison Between Studies at Baseline controlling for Site

		Tau (ms) – Frequency Band									
		128 (1-2.5 Hz)		64 (2-5 Hz)		32 (4-10 Hz)		16 (8-20 Hz)		8 (16-40 Hz)	
	df	F	p	F	p	F	p	F	p	F	p
Study	1,32	3.24	0.08	0.37	0.54	**52.02**	**<0.01**	**53.34**	**<0.01**	0.36	0.55
Site	1,32	**7.94**	**0.01**	0.95	0.34	**11.17**	**<0.01**	**7.34**	**0.01**	0.05	0.83
Site X Study	1,32	0.60	0.44	0.91	0.35	**13.23**	**<0.01**	0.57	0.45	**6.26**	**0.02**

Results from an ANCOVA examining the relationships between study, site, and permutation entropy. Entropy = $b_0 + b_{site} + b_{study} + b_{site*study} + e$.

Chapter 5: Discussion

The primary goal of the current investigation was to evaluate how pharmaceutical treatments undergoing phase 2 clinical trials to treat patients with FXS affect the neural connectivity and complexity of the patients. This examination can add to the understanding of the function of the drugs and their impact, the understanding of the functioning of the brain in FXS, and the usefulness of functional connectivity and entropy measures in a clinical setting.

Drug Effects

Previous studies examining functional connectivity in FXS compared to TDC have shown a general pattern of increased gamma connectivity and decreased alpha connectivity (Schmitt et al., 2022; Wang et al., 2017). Investigation of entropy has shown a general decrease in complexity in people with FXS compared to TDC (Proteau-Lemieux et al., 2021). For each of the three studies examined and their different target mechanisms, we should expect to see a reversal of these patterns if the drug has the expected effect, so decreased gamma connectivity, increased alpha connectivity, and increased entropy. However, these patterns were not observed in this data.

For the analysis of connectivity, only study 2 retained significant connections for the interaction of condition and time after FDR correction in both delta and beta frequency bands. This interaction showed an increase in connectivity for patients during treatment period compared to placebo periods for both delta and beta frequencies. Plots of significant connections prior to FDR correction show that the effect is likely global rather than specific to the remaining connections. Study 2 was examining racemic baclofen, a $GABA_B$ agonist. GABA is the primary inhibitory neurotransmitter in the brain; the GABA system is theorized to be downregulated in FXS (Lozano et al., 2014). While delta and beta frequency bands have not been found to differ in

FXS compared to TDC, the effect may be due to sedation resulting from increased inhibition. GABA agonists have previously been found to cause sedative effects (Li et al., 2013). Pharmaceutical sedation has been linked to increased connectivity in delta and beta frequency bands (Lee et al., 2017). Additionally, beta desynchronization is associated with motor planning (Espenhahn et al., 2017). Therefore, an increase in beta connectivity could indicate a decrease in hyperactivity. These results do not discount the usefulness of racemic baclofen in treating FXS. People with FXS often experience hyperactivity and trouble sleeping (Hagerman et al., 2017). Instead of specifically addressing mechanisms involved in FXS this may instead bolster compensatory mechanisms for some aspects of the disorder. Furthermore, the mechanism in which racemic baclofen addresses FXS is slightly indirect. GABAB receptors are not directly affected by the absence of FMRP, but rather by the decrease in GABA caused by the lack of FMRP (Gao et al., 2018) . Also the drug was theorized to target upstream mechanisms that would reduce the increased glutamatergic functions (Lozano et al., 2014; Morin-Parent et al., 2019). These patterns in connectivity do not suggest that this aspect has been successful. Evidence for a downstream regulation of the metabotropic glutamate systems would have included improvement in alpha and gamma connectivity. This study, unlike Studies 1 and 3, was limited to a single dose, and thus may not provide enough therapeutic effect to enhance longer-term effects in connectivity and entropy. Still, these results encourage further research examining its effects on hyperactivity and sleep in FXS.

While the connections that displayed potential significant drug effects for studies 1 and 3 did not survive FDR correction, the plots of the connections prior to correction show patterns that may indicate underlying effects that we were not able to capture with the number of

participants in these studies. As a signal-finding analysis, these patterns are worth examining here.

Study 1 showed a potential decrease in gamma connectivity between hemispheres for the treatment periods relative to placebo. This decrease in gamma connectivity matches our expectations for a successful pharmaceutical intervention in FXS. However, this successful decrease in gamma connectivity somewhat subverts expectations for this study. The drug treatment in Study 1 was a metabotropic glutamate receptor antagonist given to children ages 2-7. Treatment occurred over approximately 12 months in conjunction with a language learning therapy given to both treatment and placebo groups (E. Berry-Kravis et al., 2023). Previous studies in FXS had found little success with metabotropic glutamate receptor antagonists in adults (E. Berry-Kravis, Des Portes, et al., 2016; Youssef et al., 2018). Additionally, this trial found no drug effects in primary or secondary outcome measurements (E. Berry-Kravis et al., 2023). The lack of change in alpha connectivity and in the primary cognitive outcomes may indicate that the drug is only partially targeting mechanisms involved in FXS and may not be sufficient to see changes in cognition or behavior. It may prove useful if used in conjunction with another treatment. However, at present, this connectivity measure is not sensitive enough to capture the change in gamma connectivity or to fully determine a lack of change in alpha given the current data.

Study 3 had very few significant changes in connectivity prior to FDR. There was a somewhat suggestive pattern of decreased delta and increased gamma connectivity with treatment. While this finding may seem counter to expected results based on previous work (Schmitt et al., 2022), previous work confined relationships between gamma connectivity and executive function to frontal brain regions only. The pattern here for both delta and gamma band

connectivity shows a more posterior shift, with relatively few frontal connections showing a drug effect. Coupled with the primary outcomes of increased executive function in patients with drug in this study (E. M. Berry-Kravis et al., 2021), this finding may suggest a more complex relationship between cognition, connectivity, and brain region specificity than a simple overall directional change.

Organization of functional connectivity networks was examined using small world, which encompasses both the pathlength and clustering coefficient of networks. Both studies 1 and 2 had an increase in small world values for delta frequency with treatment and therefore an increase in efficient organization of delta networks. Changes in strength and organization of connectivity do not necessarily go hand in hand. Study 2 had a significant increase in delta connectivity which is coupled with the increase in small world organization showing an overall increase in both strength and organization of delta networks. This again may be an effect of sedation with the drug and may or may not be helpful in treating FXS. In contrast, study 1 showed no change in delta connectivity but an increase in organization of delta networks. This could still indicate an improvement in delta network function. However, the original study found a slight increase in sleep problems with treatment, which would imply that these changes in delta are not indicative of improved sleep issues in FXS (E. Berry-Kravis et al., 2023).

Entropy has only minimally been used to explore brain activity in FXS. Previous findings indicate that complexity is generally decreased in FXS compared to TDC (Proteau-Lemieux et al., 2021). Other research indicates an inverse relationship between functional connectivity and entropy (Ghanbari et al., 2015). However, there were no significant drug effects in nodal permutation entropy nor effects in functional entropy using wSMI after FDR. Connections showing significance prior to FDR did not contain discernable patterns of change. Study 3 did

show a trend toward increased permutation entropy during treatment periods for 2-5 Hz. In FXS, peak alpha is slowed (Pedapati et al., 2022). This may indicate that when people with FXS have trouble utilizing alpha band they utilize theta to compensate. This possible increase in theta entropy may be an indication of improvement in that compensation. The theta frequency band is also associated with cognition, which is consistent with the improvements in cognition and executive function found in the primary outcomes for this study (E. M. Berry-Kravis et al., 2021).

Connectivity and Entropy in FXS

The populations of the three studies vary in several ways: the EEG systems used, the ages of the participants, the gender of the participants, and the treatments. Therefore, comparisons between the studies at baseline, prior to any study differences related to treatments, focused on a single gender, may also be useful in understanding differences in brain networks and the impact of these factors on their measurement within a range of ages and systems for individuals with FXS. In comparing baseline measures of brain activity, we cannot directly assess age and gender differences due to confounding variables and differences in populations, but differences between or within studies may point to potential targets for future research.

Some clear differences exist between the studies and sites, which can aid in interpretation of their similarities and differences. Study 1 contained children, while participants in studies 2 and 3 were adolescents and adults. Site 1 used an EGI EEG system, and site 2 used a BioSemi EEG system. Lastly, studies 1 and 2 used a silent video during data collection while study 3 did not. However, there may be other unknown differences between the studies that contribute to the results.

Comparison between studies and sites at baseline showed connectivity in the theta, alpha, beta, and gamma frequency bands differed consistently. Alpha and theta bands showed a pattern in which connectivity differed between studies within site 1 but not within site 2 driven by increased connectivity in study 2. Study 2 did not differ in EEG system from study 1 or in age from study 3. Therefore, this increased connectivity could be a combination of increased connectivity due to video watching compared to study 3 and age compared to study 1. Beta connectivity had a mix of effects, but they were also primarily driven by higher connectivity in study 2 and overall site effects.

Connectivity in the gamma range showed significant site effects for all regions with greater connectivity for site 1 compared to site 2. This could indicate that the EGI system was better for obtaining clear and clean high frequency data, but it could also reflect other differences in site environment.

All females were dropped from this comparison to reduce the differences between studies. Unfortunately doing so reduced the study 2 participants from 15 to 7 for this analysis. However, it also allowed us to examine the impact of gender within study 2.

FXS is an X-linked disorder. It is both more common in males and presents with more severe cognitive deficits in comparison to females with FXS (Bartholomay et al., 2019). Previous findings on gender and functional connectivity in FXS have been mixed but may indicate increased connectivity in females for cross-hemispheric connections (Schmitt et al., 2022; Wang et al., 2017). Study 2 had both male and female participants, which allowed for the examination of gender differences at baseline. Patterns of connectivity prior to FDR correction indicate greater alpha connectivity for males compared to females. This suggests that the phenotype of decreased alpha connectivity in comparison to TDC may be more extreme in females with the

disorder. This is unusual given that females typically have reduced symptoms compared to males in FXS (Bartholomay et al., 2019; Hagerman et al., 2017). However, it supports the idea that pharmaceutical interventions may have different impacts on symptoms and brain function in males and females. There may need to be different approaches to treatment or different measurements of outcomes depending on gender.

No previous studies have shown significant effects of age on connectivity in FXS, but no studies have examined connectivity in FXS children in the age range of study 1, 2-7 years old (Proteau-Lemieux et al., 2021; Schmitt et al., 2022; Wang et al., 2017). Therefore, we examined the relationship between connectivity and age within study 1. Correlations between connectivity and age prior to FDR correction showed patterns of increased delta and gamma connectivity with age and decreased beta connectivity with age. Without a typically developing comparison group or at minimum adequate research on typical development of EEG connectivity in this age range, this is difficult to interpret meaningfully. The increase in gamma connectivity with age may indicate a worsening of gamma hyperexcitability, as gamma connectivity is significantly increased in FXS adults compared to TDC and associated with worsening cognitive function (Proteau-Lemieux et al., 2021; Schmitt et al., 2022; Wang et al., 2017). On the other hand, although no studies have compared functional connectivity in children with FXS and TDC, a recent study found decreased connectivity in children ages 3-10 with ASD compared to TDC (Geng et al., 2023). So, the higher connectivity in delta and gamma with age could possibly be due to compensation for lower levels of connectivity in childhood.

Differences in baseline entropy between studies can also provide some insight into how complexity of brain networks in FXS may differ with age or measurement. Alpha and low beta frequencies showed strong study effects driven by low entropy in study 1, in which the primary

difference is the young study population. Therefore, it is likely that childhood alpha and possibly beta entropy is reduced compared to adulthood in FXS. The reverse was found in the high beta, low gamma range in which study 1 site 1 had elevated entropy. There is evidence that complexity increases with age from childhood to adolescence in typically developing populations across frequency bands (Van Noordt & Willoughby, 2021). So, the decreased alpha and beta may be expected, while the lack of change in delta and the increase in high beta / low gamma may be indicative of cortical disfunction in FXS that worsens with development. However, without a direct comparison group it is difficult to say whether the rate of change observed in alpha and beta is on par with typical development.

Connectivity and Entropy as Clinical Trial Measurements

Neither entropy nor functional connectivity measures have ever been used to assess treatments in FXS. One goal of this analysis was to investigate the utility of connectivity and entropy measures in measuring change with treatment in FXS.

The measures of connectivity were helpful in identifying potential drug effects. In particular, the effects of racemic baclofen on delta and beta connectivity helped in further identifying the capacity of the drug as a potential compensatory treatment, but not to directly target typical FXS network phenotypes. However, only a few of the significant connections were able to pass the FDR correction needed to compensate for the many ANOVAs used. The measure loses much of its usefulness without examining pre-FDR significance. None of the other patterns discussed had large enough effects to surpass correction, and therefore can only be discussed as potential avenues for future research. In clinical trials, an effect needs to be large enough to be found significant in small populations (n=15-30) that are typical of phase 2 trials like the ones used in this analysis. Further, they need to be able to track treatment progression on

an individual level with some consistency. This requires that the measure be able to detect variation that is caused by brain changes, but that is robust to variability due to noise or state changes. In this case, if the patterns seen are true effects, the effects are not large enough or are too variable to detect significance in a phase 2 trial, and therefore too variable to be confident in individual effects.

Small world values showed some differences with treatment in delta in studies 1 and 2. However, we also examined plots of pathlength across head topographies in order to further examine the change in organization of networks and found an overall pattern of period effects rather than treatment effects. This may indicate that path length and by extension small world values may be more sensitive to changes in state and less sensitive to treatment related changes in FXS. This is supported by research that found path length was subject to change depending on state of consciousness between fully awake and stage 1 sleep states (Uehara et al., 2014). Indicating that pathlength captures variation in networks on a short-term state level, which may make long-term variation in functional or anatomical organization much more difficult to detect.

The use of entropy measures in FXS research has been limited, and so its use in examining drug effects was particularly exploratory. None of the drug analyses were significant for permutation entropy or wSMI. The most interesting finding was a trend toward increased permutation entropy in theta with treatment in study 3. This is made more interesting given that study 3 showed efficacy in primary and secondary cognitive and behavioral outcome measurements, and this was the only finding near significance for study 3. If it is a true effect, then the effect is still too small or variable to be useful for implementing in future clinical trials unless changes in states of consciousness are the primary target of investigation. The usefulness

of entropy as a clinical trial measure can also be examined in reference to the relationship between the connectivity and entropy results.

One of the most interesting measurement focused aspects of these results was the lack of consensus between connectivity and entropy measures. Entropy measurements should in theory go hand in hand with measurements of connectivity. In general, the stronger connections in a network, the less complex it is. However, none of the connectivity results were reflected in the entropy results.

Frohlich et al. (2022) found that measures of entropy were better able to distinguish between states of consciousness in neurotypical children, children with Angelman syndrome, and children with Dup15q compared to measures of connectivity. This result indicates two things. First simply that entropy and connectivity measures are not directly interchangeable. Second, entropy may be more sensitive to changes in state and consequently connectivity may be more sensitive to changes in treatment.

One could imagine that if a network increases in strength it may also be interrupted less causing a decrease in complexity, but a network may be able to increase strength of connections but retain the amount in which it is implemented therefore retaining the complexity of mixed network signals. The reverse could be true when thinking about states of consciousness, networks in a system may have set levels of strength, but during alert states the networks may switch rapidly causing increased complexity while in sleep states the networks may switch less rapidly resulting in a more simplistic signal. The variation in entropy with state changes may be too overwhelming to capture changes in entropy due to network organization or strength changes unrelated to state. While this is a theoretical exploration into why we were not able to capture changes with treatment in entropy measures that we were able to capture in connectivity

measures, it is further supported by previous uses of entropy in research, which are largely related to anesthesia and consciousness (Azami et al., 2023; Frohlich et al., 2022; Liang et al., 2015; Olofsen et al., 2008).

An alternate explanation for the differences in connectivity results and permutation results may be that the disconnect between the two measures is unique to FXS. The abnormalities in neural organization may affect FXS in such a way that decreased alpha connectivity is not accompanied by increased complexity in alpha. Rather the alpha that exists is more focused to compensate, and when alpha is improved the existing simplicity does not change. Previous research did find a relationship between functional connectivity and entropy in children with ASD (Ghanbari et al., 2015). This does not necessarily mean that this relationship exists in children with FXS. Further research on the relationship between functional connectivity and entropy in EEG that includes a neurotypical population is necessary in order to determine if this decoupling is unique to FXS. However, one more alternative explanation for the discrepancy between connectivity and entropy measures may be the parameters used for calculating each set of data. Connectivity was calculated using 2 second epochs and 60 seconds of clean data at a 250 HZ sampling rate, while entropy was calculated on 5 second windows from 30 seconds of clean data at a 125 HZ sampling rate. It is possible that these parameters allowed for the connectivity measures to capture changes that were not captured in the entropy measures.

Conclusion

The primary goal of this investigation was to assess the impact of pharmaceutical treatments undergoing phase 2 clinical trials on neural connectivity and complexity in individuals with FXS. The analysis encompassed three different studies, each targeting different mechanisms implicated in preclinical FXS research. While the results provided valuable insights

into the potential effects of these treatments, it is essential to interpret them within the broader context of FXS research and clinical trials.

The examination of drug effects revealed patterns with functional connectivity across the head, notably with study 2, which investigated the GABAB agonist, racemic bacloden. The increase in connectivity observed in the delta and beta frequency bands with treatment may be indicative of sedative effects. These effects may or may not address issues related to hyperactivity and trouble sleeping that can be present in FXS. However, these results suggest that the drug's effects are not directly affecting dysfunctions in FXS brain networks. Nonetheless, the findings do encourage further research into the drug's effects on hyperactivity and sleep disturbances in FXS.

Studies 1 and 3 presented connectivity patterns that did not survive FDR correction, but the trend observed in their pre-correction data may indicate underlying effects that warrant further investigation, in particular the decrease in gamma connectivity seen in study 1. The analysis also examined the small world values, which indicated potential changes in the organization of delta brain networks with treatment.

Entropy measures, a relatively unexplored area in FXS research, did not yield significant results in assessing drug effects. However, a trend toward increased permutation entropy in the theta frequency band in study 3 could indicate improvements in compensation mechanisms.

Comparison between the studies at baseline revealed consistent differences in connectivity patterns across the alpha, beta, and gamma frequency bands, which may be influences by factors such as age, recruitment strategies, data collection settings, or collection

protocols. The gender-based differences within study 2 underscore the need to tailor treatment approaches based on gender, as FXS presents differently in males and females.

Lastly, this analysis shed light on the potential utility of connectivity and entropy measures as tools for assessing treatment effects in clinical trials for FXS. While connectivity measures showed promise in evaluating and understanding the effects of treatment, both connectivity and entropy measures did not have enough sensitivity to capture effects in small populations such as phase 2 trials, particularly after accounting for data loss due to participant behavior, which is always a concern in neurodevelopmental studies. Specifically, data loss in study 1, while unavoidable during the global pandemic, was also particularly impacted by age-normative and FXS-related behaviors, suggesting that future FXS clinical trials incorporating EEG in this age range should focus on the simplest EEG set-ups (e.g. minimal electrodes and task time) with the most robust outcome measures in order to compensate for data loss inherent to this population. Connectivity and entropy likely do not meet these criteria. The lack of consensus between connectivity and entropy measures raises questions about the relationship between the two and how they are affected both by FXS and by treatment or state related changes in brain function. Additional research is necessary to address these questions.

In summary, this investigation provided valuable insights into the understanding of neural connectivity and complexity and their relationship with pharmaceutical treatments in FXS. Although connectivity and complexity measures do not seem suitable for further development as clinical trial outcome measurements. They did show utility in being able to evaluate how drug mechanisms influence network level brain activity and may be useful in larger studies or for evaluating differences between populations rather than individual change. As we continue to explore new avenues of measurement and treatment for FXS, it is crucial to refine our

understanding of the disorder and develop more effective strategies to improve the lives of individuals living with FXS.

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