NEUROBEHAVIORAL FEATURES AND TARGETED TREATMENTS IN FRAGILE X SYNDROME: CURRENT INSIGHTS AND FUTURE DIRECTIONS

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UDK: 616.89-008.434.5-057

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Summary

Fragile X syndrome (FXS) is the leading known monogenetic cause of autism spectrum disorder (ASD) and inherited form of intellectual disability (ID). As the major and growing public health problem worldwide, ASD is purely behaviorally defined whereas FXS is a medical/genetic disorder characterized by ID and ASD in males and learning and behavioral/emotional problems (social anxiety, attention network) in both genders. FXS is caused by a mutation in the *Fragile X Mental Retardation 1 gene* (*FMR1*) that leads to the epigenetic silencing of the gene and absence of its encoded protein, the fragile X mental retardation protein (FMRP). FMRP selectively targets approximately 4% of the transcribed mRNAs in the brain. Namely, to date, 842 such target mRNAs in mammalian brains have been identified and many of them converge on the same pathway as nonsyndromic ASD. Thus, FXS is the most studied 'disorder of synapse' model for syndromic ASD in the field of neuroscience. There are currently no effective treatments for either FXS or ASD.

In this review article, we discuss recent progress and future directions in translating breakthrough preclinical findings that reveal potential therapeutic targets into clinical trials for humans with FXS of potential relevance for ASD. To date, a total of 20 double-blinded, randomized, placebo-controlled clinical trials in FXS have been identified through the FDA ClinicalTrial.gov website. The majority of these trials were completed between 2008–2015. These mostly phase II trials in adults and adolescents tested 13 compounds and have primarily targeted excitatory/inhibitory imbalance theory of FXS and ASD, namely a reversal of social/behavioral symptoms that are part of the core FXS phenotype but not the core synaptic dysfunction in FXS. Despite much progress in the field propelled by the preclinical advances, these clinical studies illustrate the gaps and challenges of translating therapies from animal models to humans with FXS and ASD ('building a bridge and walking on it'). Specifically, recent challenges in the clinical trials demonstrate the need to study for longer period of time (6–12 months), prepubertal age group(s), develop more objective clinical outcome measures (i.e., clinician-based, relevant learning paradigms, and desired biomarkers), and longer placebo run-ins to minimize the placebo impact for both FXS and ASD. Ultimately, a truly satisfactory FXS, and ASD, therapy may likely involve a combination of drugs (and learning paradigms), each targeting a different aspect of the core synaptic, cognitive and behavioral impairments in FXS.

Key words: Fragile X syndrome, autism, monogenetic

INTRODUCTION

***Fragile X Mental Retardation 1*: One gene, three different phenotypes.** Extraordinary progress has been made in illuminating the molecular pathogenesis and defining neurobehavioral features of fragile X syndrome (FXS) since the discovery of the *Fragile X Mental Retardation 1* (*FMR1*) gene in 1991 (reviewed in Budimirovic & Subramanian, 2015) [1]. Located on the Xq27.3 chromosome (*Fig. 1*), the *FMR1* gene normally has 30–45 CCG nucleotide triplets along the first exon of the (5' UTR) regulatory region.

Two types of genetic mutations that expand the number of CCG 'triplet' repeats cause clinical phenotypes under the umbrella of FX-associated disorders (FXD): full-mutation (FM, >200 triplets) when FXS occurs, the focus of this review, and not permutation (PM, 55–200 triplets). In between 45–54 CGG repeats is a 'gray or intermediate' zone, which has not been studied enough. In a few cases, deletions [2] of *FMR1* have been reported to be the cause of FXS. The prevalence rate of the FM is up to 1 in 2500 males [3, 4], whereas the PM is around 10 times more common (1 in 130 females and 1 in 250 males; reviewed in Tassone et al., 2012) [5]. The PM is not associated with FXS but with a "carrier" status (1 million in the USA and 20 million worldwide). Depending on the number of AGG repeats ('speed bumps' between the CGGs, in red *Figure 1*), the *FMR1* PM allele can be unstable and expands into the FM

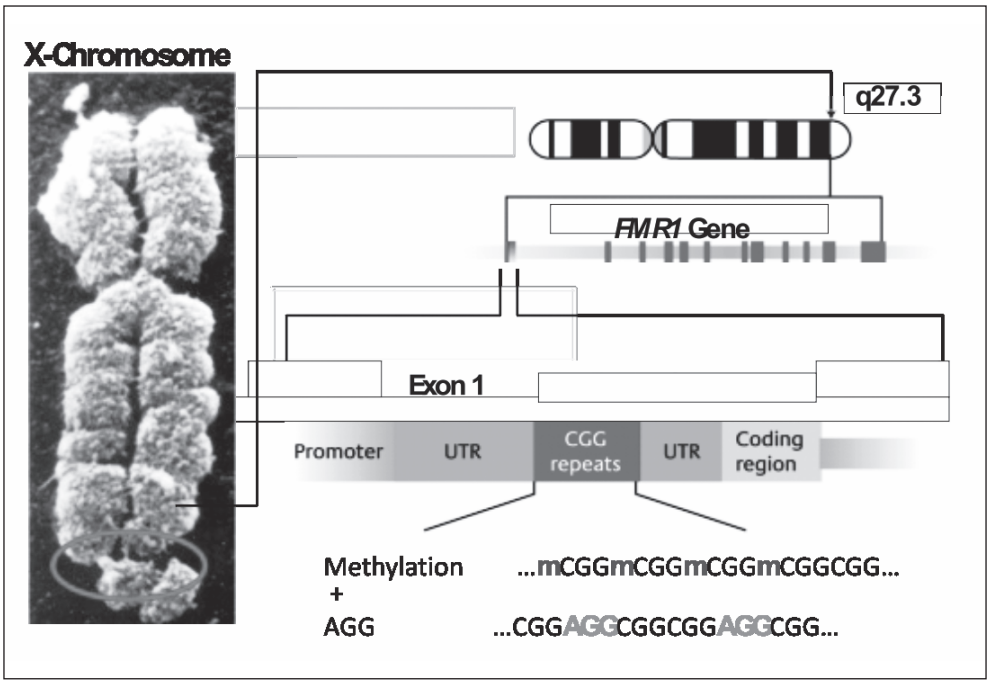

Figure 1 One gene, many markers. **An expansion of the number of CGG nucleotide repeats in the *FMR1* gene gives the appearance that the X chromosome can easily break (red oval). This region is responsible for FXS and other FXDs due to effects of the CGG repeats, AGG interruptions, and (hyper)methylation. Courtesy of Gary Latham, PhD, Asuragen Inc.**

(up to 50% chance in a mother PM carrier passing to her male offspring). For example, the presence of 2 AGG repeats increases such a risk of PM expansion into FM by as much as 3–4 times in carriers with 60–80 CGG repeats. [6]. The clinical presentation of the carrier PM can range from minimal-moderate clinical phenotypes (e.g., mild cognitive/behavioral) to severe such as FX-POI (fragile X-associated ovarian insufficiency) [7] and FX-TAS (fragile X-associated tremor syndrome) in some adults [8].

The FM and PM mutations give rise to two distinct clinical presentations and at different ages caused by two different pathogenic mechanisms (epigenetic silencing and mRNA-related sequestration toxicity, respectively). Specifically, FXS is a neurodevelopmental disorder caused by the loss of the Fragile X Mental Retardation Protein (FMRP) [9] that manifests at very early ages in boys whereas the PM generates an increased risk for neurodegenerative conditions that clinically manifests in the 40–50s. To expand on the aforementioned, on one hand, the FM leads to epigenetic (hypermethylation) silencing ("shut down") of the *FMR1* gene (*Fig. 1*), which results in the loss of its encoded protein (no FMRP) and clinically manifest as the most common cause of inherited form of intellectual disability (ID), and the leading known monogenetic cause of autism spectrum disorder (ASD) [10]. On the other hand, individuals with the PM present with a spectrum of unfolding genetic and clinical phenotypes [11], the best defined are early menopause due to ovarian failure (FX-POI) [7] (Sherman et al., 2014), and intention tremor, cerebellar ataxia, neuropathy and cognitive decline (FX-TAS) [8]. Clinically, FX-TAS occurs at ages 50–60 years in approximately 40–50% of male carriers and 16% of female carriers with an average age of onset at 62 years [8, 12]. Around 20% of females may develop FXPOI that manifest with early signs of menopause in the 30s. The American College of Medical Genetics and Genomics has recommended diagnostic testing for fragile X in symptomatic persons, women with ovarian dysfunction, and persons with tremor/ataxia syndrome. Importantly, unlike FXS that is caused by the loss of FMRP, FX-TAS is caused by *FMR1* mRNA-related sequestration that accumulates and becomes toxic to the cell [8, 13–14], which involves Sam68, DROSHA and DGCR8 [15,16].

***FMR1* molecular diagnosis and new PCR technologies.** Since the discovery of the *FMR1* gene in 1991, great advances have been made in the field of molecular diagnosis for FXS as well. Replacing cytogenetic analysis in the early 1990, molecular diagnostic testing of the *FMR1* mutation has historically been conducted by both Southern blot analysis and polymerase chain reaction (PCR) using genomic DNA. Advances in molecular methods have enabled the assessment of the degree of CGG expansion and promoter methylation as an aid in the diagnosis of FXS (reviewed in Tassone et al., 2015) [17]. Products under the AmplideX® and Xpansion Interpreter® *FMR1* methylation status (mPCR) brands by Asuragen offer a very successful suite (*Fig. 2*). The mPCR advanced method allows the quantification of the spectrum of methylation characteristics in patients with *FMR1* expansions [18]. This is important as mosaicism is well documented in FXS and this heterogeneity can confound molecular [18] and clinical interpretations [14]. Furthermore, by quantifying FMRP (qFMRP) – the

functional endpoint of *FMR1* gene expression – a more comprehensive understanding of FXS biology may help improve the diagnosis, prognosis, and treatment options for affected individuals. To demonstrate the potential of the qFMRP assay, Asuragen recently collaborated with the LaFauci and colleagues [19], who methodologically advanced an effort in the field of using dried blood spots (DBS) aimed to reliably quantify FMRP [19]. It is noteworthy that after they screened 2000 individuals of all ages, they found that *qFMRP test can identify PM females with low FMRP (methylated)*. This example demonstrates that to understand the complexity of the FXS and *FMR1* gene, values of not only the DNA (genetic) and the methylation status (epigenetic) are needed but also the encoded protein (FMRP). Intriguingly, the assays that recently analyzed fresh DBS in 2,000 anonymous newborns revealed as much as 7-fold higher levels of FMRP compared to fresh DBS in normal adults affected by FXS [20]. Reason(s) and significance of this apparent noteworthy differences are unknown. The assay is also an effective screening tool for aged DBS stored for up to four years in males and potentially females affected by FXS.

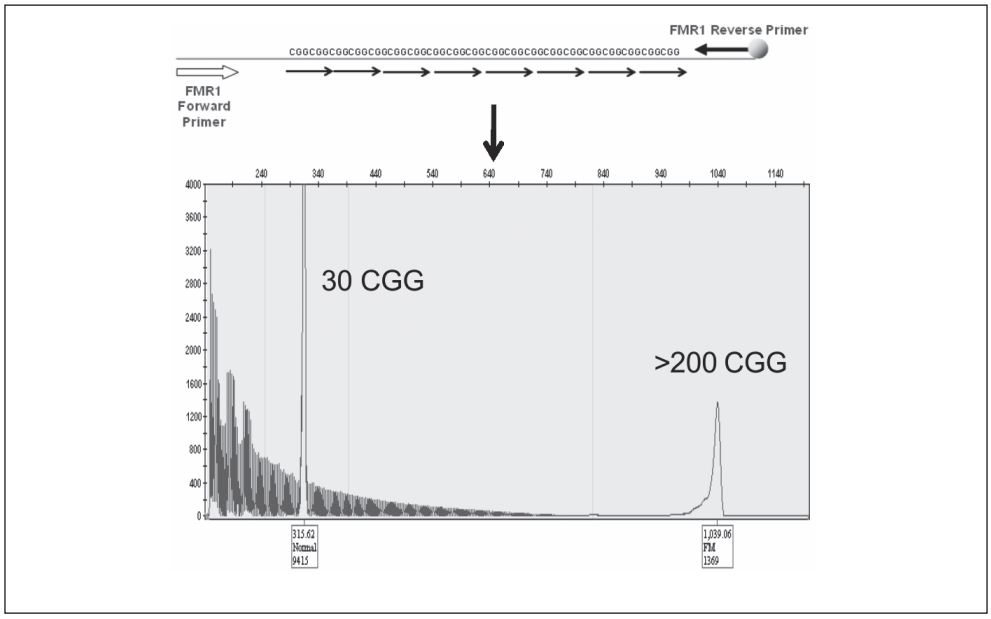

Figure 2. Workflow for amplification and detection of FMR1 amplicons using AmplideX® three-primer FMR1 PCR. **Input gDNA is amplified by two gene-specific primers (forward [Fwd] and reverse [Rev]) and a CGG repeat primer in a single tube. After amplification, the products, which include the full-length amplicon that completely encompasses the triplet repeat region and a multiplicity of CGG repeat primed products, are resolved by CE. The resulting electropherogram supports quantification of the number of CGG repeats, determination of the allele zygosity, and the sequence context of any AGG spacer elements.**
Courtesy of Gary Latham, Ph.D., Asuragen Inc

It is noteworthy that while the fragile X testing is currently not included in newborn screening (NBS) panels in the United States, the aforementioned progress in the *FMR1* molecular diagnosis and new PCR technologies are expected to facilitate such effort [21].

NEUROBEHAVIORAL FEATURES IN FXS

Clinical Presentation: It is not surprising that FXS is a global neuropsychiatric disorder given abnormalities in multiple neurotransmitter receptors and signaling pathways. Hence, neurobehavioral features of FXS consist of a wide range of variable cognitive and language impairments as well as associated neurobehavioral problems (i.e., attention difficulties, hyperactivity, anxiety, and autistic features) [14, 10]. Together, they constitute the major medical and educational concerns for patients with FXS. Because FXS is an X-linked condition, males are typically more frequently and severely affected, whereas females show substantial phenotypic variability because of variable X inactivation (i.e., some cells are able to produce FMRP). For example, ASD is almost exclusively a clinical problem in males who are typically more affected, and only 25% of females with FXS meet the criteria for ID; most have learning disabilities (i.e., math) and milder behavioral problems. Impaired executive function is particularly noticeable in females due to their better overall cognitive function. Expressive language is typically more affected than receptive language in individuals with uncomplicated FXS (i.e., no ASD). At present, the average age of FXS genetic diagnosis is at 35 to 37 months [22]. At birth, there are no apparent physical features in FXS, in contrast to some other genetic disorders (i.e., Down syndrome). Therefore, FXS is often "detected" after atypical behaviors and delays in skill acquisition begin to emerge [23, 24]. As noted above, recent advances in understanding of the mutations of the *FMR1* gene have enabled not only a model for neurodegeneration, but also more widely recognized the model for ASD [10, 25].

FXS as the model of ASD, from clinical management to biology. Characterized by early-onset impairments in social-communication and other impairments, ASD is a neurodevelopmental disorder that has become an enormous and growing public health [26] and economic [27] problem. Whereas FXS is a genetic diagnosis, ASD and autism are both broad terms for a set of complex disorders of brain development that are behaviorally-defined according to *the Diagnostic and Statistical Manual of Mental Disorders (DSM)*. The recent fifth edition of *DSM (DSM-5)* [28] has generated a single "umbrella" diagnosis of ASD and also consolidated diagnostic criteria. Since ASD is a very heterogeneous group of disorders of mostly unknown etiologies (idiopathic), treatments targeting the core deficits in ASD are also lacking. Despite an early controversy, it is now clear that ASD in FXS accounts for up to 6% of all cases of ASD [14]. Moreover, two out of three boys with FXS meet criteria for a diagnosis of the syndromic ASD (refers to ASD in FXS) [10] so that FXS has been extensively studied as the best-understood monogenetic cause of it. Together, as FXS is very homogeneous (expansion type of mutation in 99%), it has become the most meaningful genetic model of the syndromic ASD that offers hope to translate neuro-

biological breakthroughs into rational ASD therapy [14].

Clinically, there are significant overlaps between ASD and FXS. Clinical complexity in FXS manifests with a wide array of impairments in skills in individuals with FXS (i.e., ID-features of syndromic ASD, such as deficits in social interaction and communication (e.g., eye contact, peer relationships, social withdrawal-SW) [10], and restricted and repetitive behaviors [10,14]. There is also evidence of existence of neurobehavioral subgroups in FXS based on whether individuals meet criteria for ASD [29, 30], including subgroups based on severity of SW set of behaviors as a unifying factor of ASD and anxiety [31]. Indeed, the *FMR1* FM (FXS) confers an especially high risk for anxiety disorders compared to general ID [32, 33] (*Fig. 3*).

Budimirovic and colleagues (2006) has shown that deficits in adaptive socialization is the only significant predictor of ASD in FXS and that delay in the adaptive socialization skills and degrees of SW the two primary determinants of the severity of ASD diagnosis in boys with FXS [31, 33]. The most severe ASD phenotype is linked to both impaired adaptive socialization and prominent SW [31, 33].

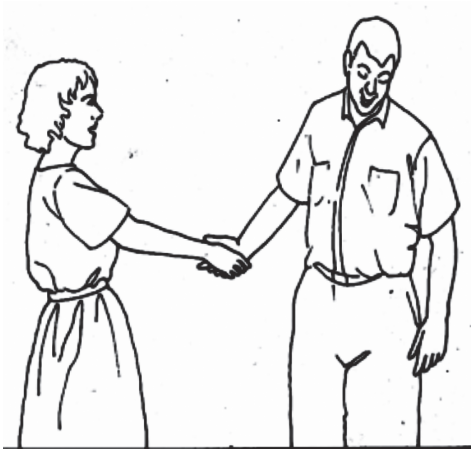

Figure 3. Fragile X handshake

Biological overlaps between ASD and FXS. There is not only a clinical overlap between FXS and ASD [10], but also a substantial overlap in the molecular pathology of the two disorders [34]. As detailed in a Fragile X Clinical and Research Consortium expert's consensus document, it may be helpful to think of ASD as a "cloud" which represents a final common pathway of abnormal patterns of brain wiring [35]. The cloud contains a common set of behavioral characteristics that are core DSM-5 features of ASD: social-communication and interaction deficits and restricted and repetitive patterns of behaviors. Individuals with ASD in FXS represent a spot in the cloud where an individual meets criteria for ASD with higher social anxiety, hyperarousal, and other FXS-related differences [35]. In terms of base for the molecular pathology of the two disorders, FMRP interacts with about 4% of total mammalian brain mRNAs [36] and regulates protein synthesis of many important proteins involved in dendritic brain synapses and signaling pathways that are associated with ASD in FXS [37–39], and with idiopathic ASD [40, 41]. To date, FMRP is an RNA binding protein with 842 target mRNAs in mammalian brain. Demonstrating that synaptic, transcriptional and chromatin genes are disrupted in idiopathic ASD, De Rubeis and colleagues [42] conducted a large exome sequencing study and found significant enrichment for genes encoding messenger RNAs targeted by two neuronal RNA-binding proteins: FMRP (34 targets [37], of which 11 are corroborated by an independent data set

[40]), and RBFOX(RBFOX1/2/3) (P50.0024, 20 targets, of which 12 overlap with FMRP [42]). The authors conclude that *de novo* mutations in ASD, ID and schizophrenia (Sch) cluster to synaptic genes such as *FMR1*. Since the deficits in FMRP seem to be the critical unifying factor linked at a molecular and synaptic level to dysfunction in brain pathways that lead to behavioral symptoms of ASD, therefore, current research is focused on identifying shared pathways and common therapeutic targets among patients with causal genetic defects of ASD in FXS [43].

NEUROBIOLOGICAL FEATURES AND TARGETED TREATMENTS IN FRAGILE X SYNDROME

Neurobiological preclinical findings. Silencing of the *FMR1* gene leads to FMRP loss ("brake"), *up-regulated* metabotropic glutamate receptor 5 (mGluR5) and *down-regulated* GABA signaling [38, 44], which results in "runaway" translation of important synaptic proteins, and subsequently disrupting many neuronal signaling pathways. Other molecular pathways that are altered by FMRP loss include *up-regulated* mTOR signaling [44, 45] and *down-regulated* is dopaminergic systems [46]. Based on these neurobiological findings, research in the field of FXS throughout the past decade has displayed an enormous growth in terms of the study of new-targeted pharmacologic treatments [47]. The effort has focused on identifying agents that restore the aforementioned excitatory/inhibitory balance in the FXS brain based on the 'mGluR5 theory,' in which disruptions in mGluR5 signaling are thought to underlie a dendritic pathology [44] and clinical presentations of FXS [14]. Namely, the 'gold-standard' rescue of the dendritic pathology in FXS has been demonstrated in the Fmr1-knockout mouse that largely centered on the use of mGluR5 antagonists [48, 49] and GABA-B agonists [50]. These studies of mGluR5 antagonists further support the mGluR5 theory [44].

FMRP regulates local protein synthesis at the synapse including modulation of synaptic plasticity [51]. The values of FMRP correlate inversely with CGG repeat number in the PM (decrease with increased number of CGG repeats starting roughly after ~120 triplets [52] due to a deficit in translational efficiency [53]. FMRP is widely expressed throughout the embryonic brain development and its expression levels increase during neuronal differentiation [54]. Developmentally, FMRP is detected in microglia and oligodendrocytes while expression persists in mature atrocities and neurons. Thus, this is an ubiquitously expressed RNA-binding protein that is mostly expressed in the brain. FMRP is important for mRNA transport, mRNA stabilization and translation regulation of mRNA into protein at the synapse [55]. Within neurons, FMRP is expressed in the soma, dendrites, and spines [56]. Three RNA binding regions, two hnRNP K-homology KH domains and an RGG (arginine-glycine-glycine) box, allow FMRP to bind to the broad range of mRNAs in a selective manner [57] and transport mRNAs bound in the nucleus to the cytoplasm [58, 59]. As FMRP suppresses translation of its mRNA targets [60, 61], its loss leads to an "excess" of dendritic protein synthesis and increased density of dendritic spines [34, 62–65]. Unfolding evidence also show that the absence of FMRP in the

cortical neurogenesis [54] results in alterations of cortical neuronal differentiation and migration mediated at least in part via brain derived neurotrophic factor (BDNF) signaling. Importantly, alterations of BDNF/TrkB signaling caused by the absence of FMRP result in distinctive cellular and behavioral responses to fluoxetine in adult FXS mice [66], which requires further studies for identification of possible new treatment strategies. Furthermore, in the absence of FMRP, an increase in Rac1-GTPase-dependent NADPH-oxidase signaling leads to an excess of free radical production, which overtime produces oxidative stress that is a crucial factor in disrupting neuron, atrocity and microglia communication [67, 68]. Together, the *FMR1* gene FM typically alters the course of brain development, cognition, and behavior throughout life [1].

In addition to the potential therapeutic avenue of restoring excitatory/inhibitory balance in FXS, other molecular systems in FXS that carry such therapeutic potential are BDNF [69] and secreted amyloid precursor protein alpha (sAPP) [70]. BDNF is a protein that supports the maintenance of neurogenesis and synapses, and FMRP plays a role in BDNF-induced synaptic plasticity [71]. FMRP is known to repress the translation of APP RNA, and APP in plasma is known to be elevated in FXS [72, 73].

Potential role of FMRP in major mental illness. As eluded in the above, a set of unfolding evidence points toward a broader implication of FMRP role outside FXS and ASD, as abnormally low FMRP levels have also been linked to non-FXS disorders in those with a normal *FMR1* genotype (i.e., major depression (MDD) and bipolar disorder (BD) [74, 75] and schizophrenia [76]. Altered expression of four downstream targets of FMRP-mGluR5 signaling in brains of subjects with autism: homer 1, APP, ras-related C3 botulinum toxin substrate 1 (RAC1), and striatal-enriched protein tyrosine phosphatase (STEP) have been shown by Fatemi and colleagues [77]. Recently, the same group [78] investigated the expression of the same four proteins in lateral cerebella of subjects with Sch, BD, and MDD and in frontal cortex of subjects with Sch and BD. Together, their results provide further evidence that proteins involved in the FMRP-mGluR5 signaling pathway are altered in Sch and mood disorders. These findings support the potential use of the qFMRP test for much broader clinical applications other than just fragile X disorders [19].

TARGETED TREATMENTS IN FRAGILE X SYNDROME

A wide range of social deficits and maladaptive behaviors is common in individuals with FXS. These deficits cause enormous impairments in day-to-day function of these individuals and their family members [14]. FXS-nonspecific psychotropic drugs are often used to target different symptom clusters, but these medications have yielded only partial benefits [14]. The aforementioned FXS-specific targeted treatments that modify molecular substrates of the disease present as a potential solution to modify core social-communication and behaviors in FXS with ASD [79]. Table 1 depicts to-date controlled clinical trial studies in humans with FXS that used the most stringent controlled methodology (double-blind, randomized, placebo-controlled).

To date, total 20 such studies (13 compounds) in FXS, and the first study in FXTAS [80], have been identified through

the Food and Drug Administration (FDA) www.ClinicalTrial.gov and Medline/PubMed searches. The vast majority of these clinical trial studies have been completed between 2008–2015 and more than half of them have published their data to date. As expected, the vast majority of these studies (16/20, 80%) has targeted excitatory/inhibitory imbalance in FXS through either mGluR5 antagonists (mavoglurant-AFQ056 and basimglurant-RO4917523) or GABA agonists (Arbaclofen-GABA-B agonist and Ganaxolone-GABA-A agonist), which accounted for 11/20 (55%) studies. Reflecting that 15/20 (75%) of these studies were phase II, they have studied adults and adolescents, and only less than half of them (6/20, 30%) have studied children. It is noteworthy that reversals of social/behavioral symptoms that are part of the core FXS phenotype have been measured but not the core synaptic plasticity in FXS. Additional 3 compounds studied that also targeted glutamatergic system were either GABA/Glutamate 'normalizers' (acamprosate and riluzole) or targeted NMDA receptors (NNZ-2566, trofinetide). The rest of the reported studies studied a downstream target of FMRP such as MMP-9 (1), serotonergic (1) and cholinergic (1) systems, and a social brain neuropeptide (1). Of note, a Phase 2 trial of a highly selective extrasynaptic GABA-A agonist (SEGA) (Gaboxadol or OV101) in FXS is pending.

These results indicate challenges in translating the preclinical success story into humans with FXS. Independent lines of preclinical evidence showed that modification of the FMRP-deficit driven dysre-

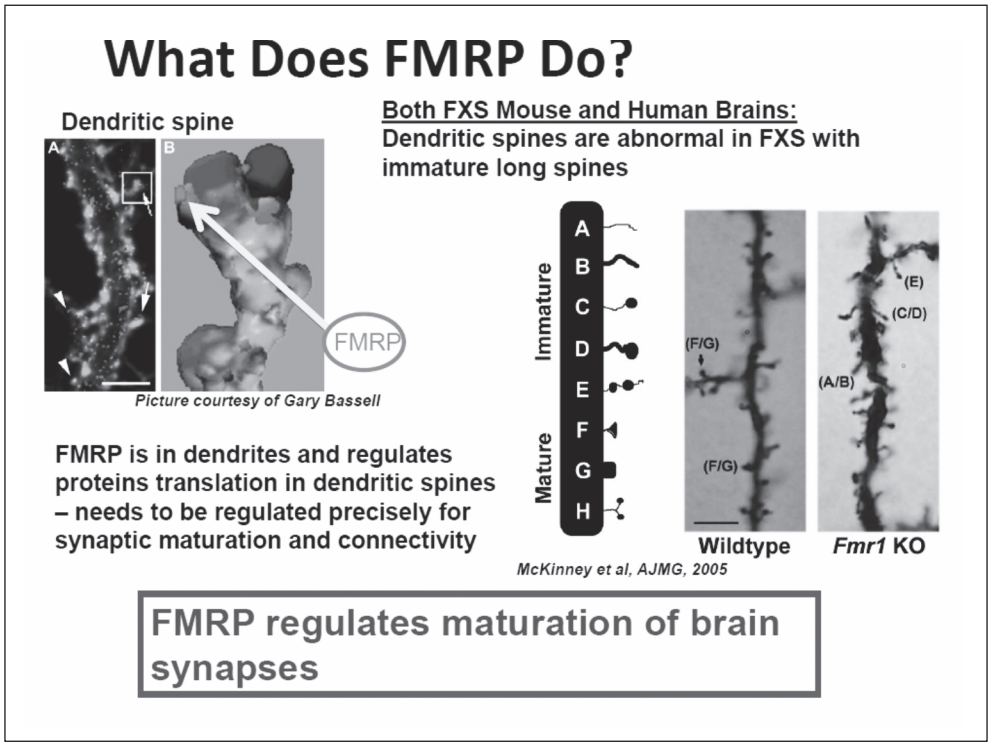

Table 1: Double-blind, randomized, placebo-controlled clinical trial studies of treatment of individuals with fragile X syndrome
Tabela 1: Dvostruko-slepe, nasumicno i placebo-kontrolisane klinicke studije tretmana lekovima osoba sa fragilnim-nestabilnim X hromozomom

FDA Registr # *Registar Broj*	Compound *Lek*	Mechanism *Mehanizam dejstva*	Population *Ispitanici*	Phase/sites/years *Faza/Lokacije/godine*	Status *Stanje*	Sponsor *Finansijer studije*	Results *Rezultati*
NCT00788073	Arbaclofen (STX209)	GABA-B agonist	Adults /adol	II multisite (2008-10)	Completed	Seaside (USA)	Berry-Kravis et al., 2012
NCT01282268	Arbaclofen	GABA-B agonist	Adults /Adol	III multisite (2011-13)	Completed	Seaside (USA)	Berry-Kravis et al., 2014
NCT01325220	Arbaclofen	GABA-B agonist	Children	III multisite (2011-13)	Completed	Seaside (USA)	Berry-Kravis et al., 2014
NCT01725152	Ganaxolone	GABA-A agonist	Adol /children	II two site (2012-15)	Not recruiting	Marinus (USA)	Pending/Iscekuju se
NCT02126995	Metadoxine	GABA transporter	Adults /adol	II multisite (2014-15)	Not recruiting	Alcobra (USA)	Berry-Kravis et al., 2015
NCT00718341	Mavoglurant (AFQ056)	mGlur5 antagonist	Adults /adol	II multisite (2008-10)	Completed	Novartis (Swiss)	Jacquemont et al. 2011
NCT01253629	Mavoglurant	mGlur5 antagonist	Adults	IIb multisite (2010-14)	Completed	Novartis (Swiss)	Berry-Kravis et al., 2016
NCT01357239	Mavoglurant	mGlur5 antagonist	Adolescents	IIb multisite (2011-15)	Completed	Novartis (Swiss)	Berry-Kravis et al., 2016
NCT01015430	Basimglurant (RO4917523)	mGluR5 antagonist	Adults	II multisite (2009-11)	Completed	Roche (Swiss)	Pending/Iscekuju se
NCT01517698	Basimglurant	mGluR5 antagonist	Adults /adol	II multisite (2012-15)	Completed	Roche (Swiss)	Pending/Iscekuju se
NCT01750957	Basimglurant	mGluR5 antagonist	Pediatric	II multisite (2012-15)	Completed	Roche (Swiss)	Pending/Iscekuju se
NCT01911455	Acamprosate	GABA/Glutamate	All ages	II/III multisite (2013-14)	Recruiting	Cinn Children's (USA)	Pending/Iscekuju se
NCT00895752	Riluzole	GABA/Glutamate	Adults	IV single site (2009-10)	Completed	Indiana Univ (USA)	Erickson et al., 2011
NCT01894958	Trofinetide	NMDA antagonists	Adults/adol	II multisite (2013-15)	Completed	Neuren (USA)	Neuren, Dec 2015
NCT00054730	CX516	Ampakine	Adults	II multisite (2003-05)	Completed	Cortex (USA)	Berry-Kravis et al., 2006
NCT00584948	Memantine*	NMDA antagonist	Adults	N/A (2007-10)	Completed	UC Davis (MIND) (USA)	Yang 2014
NCT01053156	Minocycline	MMP-9	Adol /children	II single site (2010-14)	Completed	UC Davis (MIND) (USA)	Leigh et al., 2013
NCT01474746	Sertraline	SSRI	Young kids	II single site (2011-15)	Not recruiting	UC Davis (MIND) USA	Pending/Iscekuju se
NCT01120626	Donepezil	Cholinergic drug	Adults/adol	II single site (2010-14)	Completed	Stanford Univ (USA)	Sahu et al., 2013
NCT01254945	Oxytocin	Brain peptide	Adults/adol	II single site (2010-14)	Completed	Stanford Univ (USA)	Hall et al., 2012
NCT01329770	Vitamins C&E	Antioxidants	Adol /children	II multisite (2011-15)	Completed	Imabis Foundation	Pending/Iscekuju se

Abbreviations: FDA Register# (Federal Drug Administration Registration #s taken from the ClinicalTrials.gov website); GABA-Gama Amino Buteric Acid; Trofinetide (NNZ-2566). Adol=Adolescents, Vit=Vitamin, SSRI=Selective Serotonin Reuptake Inhibitor, GABA/Glutamate=GABA/Glutamate normalizer, MMP-9=matrix metallopeptidase 9 . *the only FX-Tremor Ataxia Syndrome study.

gulation of mGluR5 and GABA receptors reversed the underlying pathophysiology of FXS. However, recent targeted treatment studies propelled by preclinical findings have shown inconsistent, and, in light of the promise in initial pre-clinical stages, disappointing results. Specifically, multiple negative modulators of the mGluR5 receptor have been in trials for FXS. For example, phase IIb/III clinical trials studying social and other behaviors in individuals with ASD in FXS using a subtype-selective mGluR5 inhibitors (basimglurant and mavoglurant) have shown no therapeutic benefits in FXS patients for unknown reasons [81]. While preliminary molecular characterization of epigenetic (full-methylation) patterns of ASD in FXS in the Phase II trials [82] has suggested that methylation status may constitute a treatment-sensitive biomarker for predicting response to a mGluR5 inhibitor [83, 84], this finding was not replicated in two large phase III trials of mavoglurant [85]. Neither of these studies, as reported by Berry-Kravis and colleagues [85], reached the primary efficacy end point of improvement on behavioral symptoms measured by the Aberrant Behavior Checklist-Community edition using the FXS-specific algorithm (ABC-CFX) after 12 weeks of treatment with mavoglurant. These studies with mavoglurant also indicated that the methylation state of the *FMR1* promoter was also not able to predict drug efficacy. The authors [85] concluded that the preclinical results suggest that future clinical trials might beneficially explore initiating treatment in a younger population with longer treatment duration and longer placebo run-ins and identifying new markers to better assess behavioral and cognitive benefits. Data from basimglurant IIa/b studies of adult, adolescents and children (Roche) are not in public domain.

To expand on these clinical studies of mGluR5 antagonists in FXS, initially, 2-Methyl-6-phenylethynyl pyridine hydrochloride (MPEP) was developed. However, MPEP was found not to be well tolerated in humans, and thus other mGluR5 antagonists had to be developed, such as fenobam and mavoglurant (AFQ056). Fenobam single-dose was found to be safe in a small pilot trial of adults with FXS. The trial showed improvements in hyperactivity and anxiety, and in prepulse inhibition [86]. As noted earlier, this work is important to delineate neurobiological phenotypes within FXS but can be only demonstrated through double-blind, randomized, placebo-controlled studies. Yet, these clinical trials have also highlighted challenges such as the populations heterogeneity, the lack of specific and sensitive outcome measures capturing the full range of improvements of patients with FXS, and a lack of reliable biomarkers that can track whether a specific mechanism is responsive to a new drug within relatively short period of time, and whether the biomarker response correlates with clinical improvement [87, 83].

As for GABAergic dysfunction, GABA-B agonist arbaclofen reversed many abnormal phenotypes in animal models of FXS [50], presumably by lowering presynaptic glutamate release, resulting in reduction of group mGluR5s signaling. The major progress has then enabled a phase II double-blind placebo-controlled crossover clinical study in which arbaclofen showed improvement over placebo in the social withdrawal problem behaviors efficacy scores. Specifically, in a "lower sociability" subgroup (n=27), arbaflofen significantly reduced ABC-CFX Social Avoidance and Vineland Socialization scales (42% responders vs. 7% placebo as defined by CGI-Improvement 1 or 2 and ABC-SW subscale reduced for at least 25%) [88]. However, neither younger nor older FXS

or ASD patients in a large phase III 8-week placebo-controlled trial reached the primary efficacy end point of improvement on behavioral symptoms measured by the ABC-CFX [89]. While, there was no significant effect of arbaclofen over placebo for any measure in the older group and in the idiopathic ASD group 12-week study, in younger FXS subjects (ages 5–11 years), the highest dose produced significant improvement over placebo on the ABC-CFX Irritability subscale ($p<0.05$) and trends toward benefit on the ABC-CFX and Hyperactivity subscales ($p<0.1$). Together, while full analyses are pending, the younger group's effects size for a subset of the outcome measures showed encouraging results for several important measures (irritability, parental stress index, and global functioning) [89].

The clinical trial studies in humans have only managed to focus on reversing social/behavioral symptoms that are part of the core FXS phenotype. That is to say, none of the aforementioned most recent clinical studies have addressed the core FXS plasticity deficit that would translate to changes in cognitive and learning measures; to date, only one study published a decade ago by Berry-Kravis and colleagues (2006) [90] targeted cognition using CX516, an AMPA activator. AMPA compounds are potential treatments acting within the glutamate signaling pathway, which are excessively internalized as a result of increased mGlur5 signaling after the loss of FMRP. While the study did not show efficacy, it was used most likely at a subtherapeutic dose, a conclusion that would be supported by a suggestion of efficacy in patients co-treated with antipsychotics that are known to potentiate effects of CX516 [90]. Importantly, clinical observations from long-term extension studies with both arbaclofen and AFQ056 have suggested there may be long-term cognitive and functional benefits of these drugs that were not captured by formal measures employed in the trials [91].

Other studies from Table 1 show promise but await larger controlled clinical trials. For example, a phase 2 pilot placebo-controlled crossover trial of minocycline was carried out in children with FXS. The effect of this antibiotic that inhibits over expressed synaptic MMP-9 in FXS models showed mild global clinical improvement [92] and reduction of MMP-9 levels in the blood of responders [93]. As MMP-9 is over expressed in FXS, new unfolding compounds of relevance for reversing MMP-9 also hold promise such as eukaryotic translation initiation factor 4E (eIF4E) [94]. Acamprosate, currently FDA-approved for alcohol withdrawal, with agonist properties at both GABA-A and GABA-B receptors (GABA/Glutamate 'normalizer') has shown promise in an open-label trial for hyperactivity and social functioning in FXS [95]. Small phase 2 placebo-controlled studies of acamprosate (the Merck Foundation grant to Erickson) and Ganaxolone, GABA-A agonist, in FXS are underway [2016, cited January 15, 2016 available from www.clinicaltrials.gov].

Next, as FMRP is believed to mediate several receptor systems important for cognitive function, studies utilizing *in vivo* biochemical neuroimaging techniques in FXS are beginning to emerge. Such initial studies have identified disruption of the cholinergic system as a potential neurochemical target for intervention as well as serving as metrics for treatment efficacy [96]. Determining the downstream effects of FMRP deficiency on cholinergic receptor systems in humans provides direction for potential pharmacologic treatments for cognitive dysfunction in FXS. While a pilot open-label study of donepezil, an acetylcholinesteraze inhibitor, showed a promise [96], a 3-month double-blind,

placebo-controlled, randomized study showed no significant difference in IQ or behavioral scales [97].

Metadoxine extended release (Alcobra) has been studied as a treatment for ADHD and is also being studied as a potential treatment in FXS in a Phase IIb clinical study. Preliminary results from this 6-week study found no statistical difference on its primary measures compared to placebo [98]. While the study found a small improvement on the Vineland Adaptive Behavior Scale Daily Living Skills subdomain, these results need to be replicated.

Preliminary findings from sertraline treatment during six months for preschool children with FXS at the MIND Institute at UC Davis were associated with greater benefits in (1) global functioning; (2) improvements in hyperactivity, impulsivity and attentional behavioral issues; (3) marginal overall cognitive improvements and (4) increased social participation in community and family life when compared to placebo. A marginal treatment effect was also observed in the Cognitive Domain but no effects were observed on the outcome domains of language and most measures of Sensory Processing and ASD Symptoms [99].

Trofinetide (NNZ-2566), which has a unique mechanism of action very different from any other molecule that has been tested before in FXS, also holds a promise. Trofinetide is a synthetic version of the insulin-growth factor 1 (IGF-1), a naturally occurring neurotrophic factor generated in the liver that passes the blood-brain-barrier. Trofinetide is expected to normalize a number of biological processes in the brain that are impacted in FXS. In December of 2015, Neuren Pharmaceuticals has announced that their Phase 2 clinical trial of trofinetide in FXS has successfully established proof of concept. In addition to excellent tolerability profile of trofinetide fixed-dose 70 mg/kg and 140 mg/kg daily, the high dose demonstrated a consistent pattern of clinical improvement across core symptoms of FXS, observed in both clinician and caregiver assessments.

Open label studies include (i) riluzole (GABA/Glutamate 'normalizer') in a very small 4-week study was not associated with significant clinical improvement despite uniform correction of peripheral ERK activation [100]; (ii) lithium that in a 2-month pilot open-label proof-of-concept study in children and young adults with FXS resulted in significant improvement in behavioral scales, verbal memory, and abnormal ERK phosphorylation rates in lymphocytes [101]. This effect is supposedly mediated by reduced excess mGluR-dependent activation of translation by attenuating GSK3 activity and possibly phosphatidyl-inositol turnover, (iii) Lovastatin, an inhibitor of Ras-ERK1/2, also showed a promise in a 3-month small open label study in humans with FXS [102].

As for the PM studies, the first placebo-controlled, double-blind, randomized clinical drug trial in FXTAS was memantine in which 1-year treatment had significant effects on cued memory retrieval but not executive function [80]. Yet this study also demonstrated that well-designed cognitive event-related brain potential (ERP)/EEG studies tests might offer sensitive means to detect intervention effects that may not be evident in standard behavioral or clinical assessments. In the line of the aforementioned positive role of GABA-ergic modulation in FXS, Cao et al. 2012 [103] suggested allopregnanolone, a positive modulator of $GABA_A$ receptors, as a candidate therapeutic agent to ameliorate the abnormal mGluR1/5 signaling in *FMR1* PM neurons.

DISCUSSION

A significant progress in targeted treatments in FXS reflects the major preclinical breakthroughs [44] and shows promise in humans medical targeted therapeutics [82, 88], namely agonists of GABA-B receptors and antagonists of mGluR5 receptors. Specifically, the FXS mouse model (the *FMR1* knockout) has shown that FXS can be "cured" (reversal of the excess protein synthesis) of the core phenotype after using agonists of GABA-B receptors [50] or mGluR5 antagonists [48]. To date, a total of 20 double-blind, randomized, placebo-controlled clinical trials in FXS have tested 13 compounds primarily targeting excitatory/inhibitory imbalance theory of FXS and ASD. Despite much progress in the field propelled by the preclinical advances, these clinical studies illustrate the gaps and challenges of translating therapies from animal models to humans with FXS and ASD ('building a bridge and walking on it') and highlights the need for new paradigms. For example, the studies targeted a reversal of social/behavioral symptoms that are part of the core FXS phenotype but not the core synaptic plasticity in FXS.

The negative findings in the well-designed, properly powered studies serve as a model. These studies can provide an opportunity to reflect on future clinical trial design and implementation rather to conclude that these trials prove that a treatment is ineffective under all conditions or that the presumed underlying pathophysiological mechanisms are not valid [104]. Recent studies support also that derangement of the *mGluR* network may be responsible for increased rates of ASD seen in cytogenetically distinct forms of syndromic ASD]105]. Unfolding preclinical studies of GABA-B agonist arbaclofen [106] that measured *in vivo* regional rates of protein synthesis [107] and advanced our understanding of interaction between the arbaclofen and FMRP suggest that the GABA-B agonist, arbaclofen, has merit to be further studied in also better designed clinical trials. An effort is under way by Fragile X Clinical and Research Consortium's group led by Dr Elizabeth Berry-Kravis from Rush University in Chicago to analyze Arbaclofen FXS Trials – Patient Breakdown by Drug Response (drug responders and placebo nonresponders). Dr Budimirovic and his group from Kennedy Krieger Institute/Johns Hopkins have contributed significantly to this effort. While full analyses are pending, preliminary findings showed that many areas of positive response have not been well captured by the primarily parent-based outcome measures; thus, we are seeing only part of response. Arbaclofen 1 year (Vineland) data that showed change in the FXS disease trajectory in terms of participant adaptive skill has much relevance given that longitudinal have shown that the acquisition of adaptive skills slows, especially in male, as individuals with fragile X syndrome age [108].

The gaps include a lack of models for clinical trials in FXS. For example, there were no significant moderate/large clinical trials in FXS with any "standard" drug before 2002, and there is no defined measure of behavioral improvement, "gold standard" outcome measure, or template from any developmental disability about measuring cognitive outcomes when attempting to treat the underlying disorder [87]. In order to improve the understanding of FXS and ASD, future clinical trials should take into account the study length, timing of intervention, appropriate clinical endpoints, use of a combination with psyc-

hopharmacological interventions, patient stratification into endophenotypic subgroups within FXS though different models [83]. Further molecular-clinical studies are needed to understand the full value of *FMR1* as a diagnostic and therapeutic marker. This is possible only with innovative assays in the *FMR1* gene diagnostics [109, 110], as well as other methodologies to further investigate other mediators of the dendritic dysregulation in FXS by profiling at baseline which persons with FXS may best respond to a particular treatment [95]; both aim to develop personalized medicine options for this disorder.

The compelling need for new paradigms. Published descriptive studies of severity of SW set of behaviors as a unifying factor of ASD [31, 33] and/or anxiety [32], emerging investigation of the biological basis of FXS through imaging-behavioral [29], molecular-behavioral using next generation fragile X PCR that also allows fragile X testing to be simple and more efficient [35] are examples of such helpful models. In parallel, an effort at identifying potential molecular mediators of dentritic overgrowth in FXS as new potential targets (sAPP and BDNF) of treatment [70] are needed. Integration of molecular and neurobiological data in FXS with ASD (i.e., FMRP, CYFIP1, mTOR, MMP-9), possibly eIF4E [94] and it's still unknown and/or adequately understood targets [43] will ultimately be necessary. For example, CYFIP type 1 intermediate phenotype link between ASD in FXS [111, 112] and subsets of idiopathic ASD such as 15q11-13 duplication [113, 114] emerges as a compelling example of the shared neurophysiology. Next, studies of educational, behavioral, and therapeutic interventions are also needed to generate evidence on which to base recommendations about supportive interventions and the similarities and differences between those recommendations for patients with FXS and ASD. As PM is also associated with ASD, further studies are clearly needed in this area, especially given the 10-fold higher frequency of PM vs. FM. The integration of all these pieces of data is a major challenge and will be better addressed when additional data becomes available. Overall, future clinical trials implementing the aforementioned not only hold a hope but a meaningful clinical and functional progress in FXS and ASD, and improved quality of life for affected individuals and their families.

In conclusion, there are currently no effective treatments for FXS. The negative findings in the recent well-designed, properly powered studies can serve as a model. Specifically, recent challenges in the clinical trials demonstrate the need to study for longer period of time (6–12 months), prebubertal age group(s), develop more objective clinical outcome measures (i.e., clinician-based, relevant learning paradigms, and desired biomarkers), and longer placebo run-ins to minimize the placebo impact for both FXS and ASD. Ultimately, a truly satisfactory FXS, and ASD, therapy may likely involve a combination of drugs (and learning paradigms), each targeting a different aspect of the core synaptic, cognitive and behavioral impairments in FXS. Combining a learning paradigm with one drug is realistic at this stage, which is a hard enough study to design. While drug combinations algorithm is likely important in the future, we're clearly not ready to do those trials. Therefore, using multiple drugs together and a learning paradigm is too much to suggest at this stage of our understanding as we don't have data on what each individual drug does.

NEUROBIHEJVIORALNE KARAKTERISTIKE I CILJANA TERAPIJA FRAGILNOG X SINDROMA: DANAŠNJE SPOZNAJE I BUDUĆA USMERENJA

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Kratak sadržaj

Fragilni (krhki) deo X hromoyoma sindrom (FXS) je vodeci poznat monogenetski uzrok autističkog poremecaja spektra (ASD) i nasleđene forme intelektualne zaostalosti u razvoju (ID). Kao glavni i rastući javni zdravstveni problem u svetu, ASD je definisan kao čisto biheviоralni premećaj, dok je FXS medicinski / genetski poremecćaj koji je karakterističan po ID i ASD kod dečaka u učenju i ponašanju / emocionalnim problemima (socijalne anksioznosti, u rasponu pažnje) kod oba pola. FXS je uzrokovan mutacijom Fragilnog X Mentalnom Retardacijom 1 gena (*FMR1*) koji dovodi do epigenetskog gašenja gena i odsustva njegovog kodiranog proteina, Fragilnog X Mentalna Retardacija Proteina (FMRP). FMRP selektivno reguliše produkciju oko 4% transkribovane mRNK u mozgu. Naime, do sada, 842 takvih ciljnih mRNK u mozgu sisara su identifikovani i mnogi od njih se ukrštaju na istom putu kao i izgleda bar neke forme ASD. Tako je, FXS najviše proučavani "poremećaj u okviru sinapse" model za sidromski (poznati uzrok) ASD u oblasti neuronauke, modela za sindromsku ASD u oblasti neuronauke. Trenutno ne postoje ciljani lekovi ni za FXS kao ni za ASD. U ovom članku, govorimo o nedavnom napretku i budućim pravcima u prevođenje vrlo značajnih za neuronauku prekliničkih dostignuća radi otkrivanja potencijalnih terapeutskih meta u kliničkim studijama za ljude sa FXS od potencijalnog značaja za ASD. Do sada je ukupno 20 dvostruko slepih, randomiziranih, placebo-kontrolisanih kliničkih studija sa FXS identifikovano putem sajta FDU ClinicalTrial.gov. Vecćina ovih ispitivanja su završena između 2008–2015. To su uglavnom faza II ispitivanja kod odraslih i adolescenata pri kojima je testirano 13 lekova i uglavnom su bile usmerene ka ekscitatornoj / inhibitornoj teoriji disbalansa FXS i ASD. Napredak je da su sržni simptomi socijalnog/behavioralnog ili ponašajnog karaktera u FXS modifikovani ali ne i sržni, najviše preokretima simptoma društvenih / ponašanja koji su deo osnovnog FXS fenotipa, ali nisu suština sinapsi disfunkcije kod FXS. Uprkos mnogim dostignućima u oblasti pokrenute velikih prekliničkim napretkom, te kliničke studije ilustruju nedostatke i izazove prevođenje terapije sa životinjskim modelima na ljude sa FXS i ASD ('izgradnju mosta i hod po njemu'). Naime, nedavno izazovi u kliničkim ispitivanjima ukazuju na potrebu da ove kliničke studije traju duži vremenski period (6–12 meseci), u prepubertetskoj starosnoj grupi (s) ili grupama, razviju objektivnije mere kliničkog ishoda (npr, zasnovane na lekaru, relevantne paradigme učenja, i željeni biomarkeri), i duže placebo faze da bi se sveo na minimum uticaj placeba za FXS i ASD. Na kraju krajeva, zaista zadovoljavajuća FXS i ASD terapija moraće da uključi kombinaciju lekova (i odgovarajućih testova i učenja), koji ciljaju različite aspekte tog sržnog nedostatka u okviru sinapse, učenja, i ponašanja u FXS.

Ključne reči: fragilni X sindrom, autizam, monogenetski

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