



Fragile X Syndrome

The quest for targeted treatment

Shimriet Zeidler

Fragile X Syndrome

The quest for targeted treatment

Het fragiele X syndroom
De zoektocht naar een gerichte therapie

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Scope of this thesis

Fragile X syndrome is the most common hereditary cause of intellectual disability and autism. Currently, no targeted treatment is available. The identification of its causative gene, followed by the generation of animal models, has boosted research options. The past decades, preclinical research has focused on involved pathways as targets for therapeutic interventions. Targeting many of these pathways in animal models improved core features of the disease. These promising results were received with great enthusiasm, and led to clinical trials in patients with fragile X syndrome.

The aim of this thesis was to develop and evaluate therapeutic intervention strategies for fragile X syndrome with the ultimate goal to advance care and treatment options for patients with the disorder.

One aspect was to identify possible targets for therapeutic intervention using social behavior of *Fmr1* knockout mice as outcome measure. In addition, new strategies were explored using combination therapy (*chapter one - four*).

A second aspect was to investigate a potential target, the mGluR5, for its efficacy in clinical trials. The mGluR5 antagonist mavoglurant was studied in a multicenter randomized, double-blind, placebo-controlled trial (*chapter five*).

A third aspect was to center patient care, in order to advance clinical expertise and enable clinical research. An expert center and clinical guidelines are indispensable in patient care and further development of treatment for fragile X syndrome (*chapter seven*).

The findings were then placed in the light of current knowledge and future perspectives (*chapter six and eight*).

List of abbreviations

ABC-C	Aberrant Behavior Checklist – Community edition
ABC-CFX	Aberrant Behavior Checklist – Fragile X edition
ADAMS	Anxiety, Depression, and Mood Scale
ADD	attention deficit disorder
ADHD	attention deficit hyperactivity disorder
ADHD-RS	ADHD Rating Scale
ADOS	Autism Diagnostic Observation Scale
AE	adverse event
Akt	serine/threonine protein kinase
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPArs	AMPA receptors
ANOVA	analysis of variance
ASD	autism spectrum disorders
ASS	autisme spectrum stoornis
ATT	automated tube test
AVG	arts voor verstandelijk gehandicapten
BDNF	brain derived neurotrophic factor
bid	bis in die, twice daily
CARS	Childhood Autism Rating Scale
CBCL/ABCL	Achenbach Child and Adult Behavior Checklists
CELF	Clinical Evaluation of Language Fundamentals
CGI-I	Clinical Global Impression-Improvement
CGI-S	Clinical Global Impression-Severity
CI	confidence interval
cm	centimeter
CM	complete methylation
CNF1	cytotoxic necrotizing factor 1
CNS VS	Computerized Neurocognitive Test Vital Signs
CNT	Contingency Naming Task
CPRS	Conners' parent rating scale
CS-	unpaired conditioned stimulus
CS+	conditioned stimuli
CTRS	Conners' teacher rating scale
CY-BOCS-PDD	Children's Yale-Brown Obsessive Compulsive Scale modified for PDD
CO	cross-over
DAG	diacylglycerol
DB	double blind
DEC	Dutch Animal Ethical Committee
DGK κ	diacylglycerol kinase kappa
DHPG	(S)-3,5-dihydroxyphenylglycine
DIV	days <i>in vitro</i>

10| List of Abbreviations

e.g.	exempli gratia
eIF4E	eukaryotic initiation factor 4E
ECG	electro-cardiogram
EEG	electro-encephalogram
ERK	extracellular-signal regulated kinase
ERP	event-related brain potentials
EVT	Expressive Vocabulary Test
<i>FMR1</i>	fragile X mental retardation 1
<i>Fmr1</i> KO	<i>Fmr1</i> knock-out
FMRP	fragile X mental retardation protein
FXS	fragile X syndrome / fragile X syndroom
FXTAS	fragile X associated tremor and ataxia syndrome
FXPOI	fragile X associated premature ovarian insufficiency
FXPOF	fragiele X geassocieerde prematuur ovariumfalen
GARS	Gilliam Autism Rating Scale
GGZ	geestelijke gezondheidszorg
GABA	γ-aminobutyric acid
HEp-2	human epithelial type-2
HVLT	Hopkins Verbal Learning Test
ID	intellectual disability
i.e.	id est
iPSCs	induced pluripotent stem cells
IQ	intelligence quotient
ISI	inter-stimulus interval
IVF	in-vitro fertilisatie
KITAP	Test of Attentional Performance for Children
K-SADSL PL	Kiddie schedule for affective disorder and schizophrenia
KO	knock-out
LTD	long-term depression
LTP	long term potentiation
M1-5	muscarinic receptors
mACh	muscarinic Acetylcholine receptor
mGluR5	metabotropic glutamate receptor type 5
MMP-9	matrix metalloproteinase 9
MPEP	2-Methyl-6-(phenylethynyl)pyridine
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NKCC1	Na ⁺ -K ⁺ -2Cl ⁻ -co-transporter 1
NMDA	N-Methyl-D-aspartic acid
NMDARs	NMDA receptors
NOR	novel object recognition
NPI-Q	NeuroPsychiatric Inventory Questionnaire
OL	open label

OSAS	obstructief slaap-apneu syndroom
PA	phosphatidic acid
PET	positron emission tomography
PARS	Pediatric Anxiety Rating Scale
PBS	phosphate-buffered saline
PGD	pre-implantatie genetische diagnostiek
PI3K	phosphoinositide 3-kinase
PLS-4	Preschool Language Scale—4
PM	partial methylation
PND	prenatale diagnostiek
PSD	post synaptic density
RhoGTPase	Rho family of guanosine triphosphate-ase
RM-ANOVA	Repeated Measures Analysis of Variance
PPI	prepulse inhibition
PPVT	Peabody Picture Vocabulary Test
RM ANOVA	repeated measures ANOVA
RBANS	Repeatable Battery for Assessment of Neuropsychological status
RBS	Repetitive Behavior Scale
S6K1p70	ribosomal subunit 6 kinase1
SBIQ	Stanford-Bine IQ
SD	standard deviation
SE	standard error
SNAP-IV	Swanson Nolan Pelham questionnaire parent rating scale
SPL	sound-pressure level
SRS	Social Responsive Scale
STEP	striatal-enriched protein tyrosine phosphatase
TEA-Ch	Test of Everyday Attention for Children
TVPS	Test of Visual—Perceptual Skills
US	unconditional stimulus
VABS	Vineland Adaptive Behavior Scale
VAS	Visual Analogue Scale
WAIS	Wechsler Adult Intelligence Scale
WJR	Woodcock-Johnson Tests of Cognitive Ability
WT	wild type



CHAPTER 1

Translational endpoints in fragile X syndrome

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Abstract

Fragile X syndrome (FXS) occurs in less than 10% of the intellectually disabled (ID) population. The cause of FXS is a CGG trinucleotide repeat longer than 200 CGG units within the first exon of the *FMR1* gene, which leads to hypermethylation and consequently silencing of the *FMR1* gene. The lack of *FMR1*'s gene product, the fragile X mental retardation protein (FMRP) in neurons is the cause of the ID in patients with FXS. FMRP plays an important role in local protein synthesis at the synapse including modulation of synaptic plasticity. The advancing knowledge about the cellular function of FMRP has led to the identification of translational endpoints for future therapeutic intervention strategies. This review highlights the challenging routes to the identification of reliable outcome measures in preclinical studies using both cellular models and *Fmr1* knockout mice. Finally, clinical studies carried out to correct intellectual and behavioral deficits in patients with FXS, using a variety of existing and new drugs, are discussed.

1. Introduction

In 1943, Martin and Bell described a family in which multiple male members showed abnormal social behavior and moderate to severe intellectual disability (ID) ¹. In 1969, Lubs *et al* described an abnormality of the X-chromosome using cultured leucocytes from mentally retarded males ². In 1977, Sutherland confirmed that a fragile site on Xq27.3 was associated with this anomaly, hence, the name fragile X syndrome (FXS) ³. By now, we know that silencing of the fragile X mental retardation gene (*FMR1*) leads to this type of X-linked intellectual disability ⁴. The silencing of the *FMR1* gene is caused by an extended methylation of the promoter region of the gene. The methylation is triggered by an abnormally large expansion (> 200 units) of a naturally occurring CGG repeat in the 5'-untranslated region of the gene. This ultimately leads to an absence of the protein product, fragile X mental retardation protein (FMRP), from early development onwards ⁵. Next to the physical, cognitive and behavioral symptoms of FXS, neuroanatomical abnormalities are also within the phenotypic spectrum. Microscopic analysis of autopsy material from FXS patients has shown abnormally sized and shaped dendritic spines ⁶⁻⁸. This morphological spine phenotype indicates a possible defect in synaptic plasticity, a molecular mechanism implicated in learning and memory. Notably, spine abnormalities in Golgi-stained brain sections have been described in Down's and Rett syndromes and in individuals with intellectual disabilities of unknown etiology ^{9,10}. Current preclinical research on FXS is focused on therapeutic interventions that target the underlying molecular mechanisms of these synaptic defects. Recently, several clinical trials have been conducted using existing and newly developed drugs designed to correct the behavioral symptoms of FXS. This review provides an overview of the current knowledge of the molecular mechanisms underlying FXS and the identification of translational endpoints in preclinical and clinical research to assess the efficacy of therapeutic intervention for future studies.

2. Clinical, behavioral and neuroanatomical phenotype of FXS

Diagnosis of FXS is usually made around the age of three years ^{11,12}, when children present with developmental or behavioral problems. Due to the X-linked nature of the disorder, females usually display milder features, because they are protected by the presence of a normal X-chromosome. Although the physical features are quite mild, the behavioral and psychiatric phenotype, together with the intellectual disability, cause significant limitations in daily life.

Intellectual disability is the most consistent and prominent feature of FXS, with an average IQ of 40-50 for adult men. Problems occur in language, working and short-term memory, executive functioning, mathematical and visual-spatial abilities ^{12,13}. Usually, adaptive and achievement skills are higher than predicted by the patients IQ. Young children typically present with developmental delay, especially speech delay ¹³. Physical features of FXS include mild physical and dysmorphic features, consistent with

mild connective tissue disorder ¹². The facial characteristics include long narrow face, high forehead, prominent jaw, highly arched palate and large prominent ears. They occasionally have strabismus, hyperextensible joints, soft skin, pes planus, mitral valve prolapse, scoliosis and hypotonia. Most post-pubertal males display macroorchidism. Medical issues may be recurrent otitis media in childhood and gastro-esophageal reflux in infants ¹²⁻¹⁵. The physical symptoms in females are usually milder than in males ^{14,16}.

In male patients with FXS, autistic-like behavior is common, including avoidance of gaze, tactile defensiveness, hand flapping and biting, hyperarousal to sensory stimuli, irritability, impaired social skills and perseveration ¹². In general, 43-67% of the male patients fulfill the criteria of autism spectrum disorder ^{13,17}, with symptoms in the triad of impaired social interaction, language and communication deficits and repetitive or stereotyped behavior. Although FXS patients often display autistic like features, they do seem to have a strong social interest, combined with high social anxiety ^{13,18}. Often, hyperactivity, impulsivity, attention problems, mood disorders, aggressive behavior and generalized anxiety are present as well ^{12,13}. Individuals with FXS have been found to have aberrant eye-blink conditioning ^{19,20}. They also show deficits in prepulse inhibition of startle response (PPI) ^{21,22}.

Neurophysiological and neuroanatomical features include EEG anomalies and epileptic seizures with an incidence of 13-18% in male children with FXS ¹². Seizures often resolve during childhood. Postmortem neuropathological studies in patients with FXS have shown no gross abnormalities of the brain. However, microscopically dendritic spine anomalies have been demonstrated. There are longer, more tortuous and thinner spines as compared to controls, consistent with an immature spine phenotype. The spine contact surface is decreased with 35%. Together, this points towards a defect in development and maturation of the dendritic spines ^{6-8,23}. Also MRI neuroimaging studies show several abnormalities. Increased volumes of caudate nucleus, ventricular abnormalities and decreased cerebellar vermis volume, amygdala and superior temporal gyrus have been demonstrated ^{14,24-28}. Functional MRI studies show differences in brain region activation while performing tasks as compared to controls. This was the case for the amygdala ²⁹, the ventrolateral prefrontal cortex, right caudate head ³⁰, supplementary motor area, anterior cingulate and midcingulate cortex, basal ganglia, hippocampus, ventrolateral prefrontal cortex and striatum ³¹.

Recently, Kwan *et al* demonstrated structural alterations in the organization of minicolumns in the developing cerebral cortex of human FXS cases during a specific period in human embryonic development, that is important for the normal development and function of the neocortex, especially in processes like speech production, language recognition, decision making and complex social behaviors (e.g. empathy) ³².

3. Synaptic plasticity

The study of FXS has been facilitated by the development of several animal models. Preclinical studies have been performed on the fruit fly (*Drosophila melanogaster*)³³⁻³⁸, zebrafish (*Danio rerio*)³⁹⁻⁴³, mouse (*Mus musculus*)^{44,45} and recently a rat model (*Rattus norvegicus*) (SAGE Labs). Particularly the *Fmr1* knockout (KO) mouse has become invaluable in investigating the consequences of a lack of FMRP on a neuroanatomical, biochemical, electrophysiological and behavioral level.

In the normal brain, FMRP is found in spines, which are specialized dendritic protrusions that are involved in synaptic signal transmission between neurons. The protein contains three RNA-binding domains, and it has shown to associate with specific dendritic mRNAs as well as with polyribosomes responsible for protein translation^{46,47}. To date, it is evident that FMRP plays a role at the synapse in controlling local translation of specific mRNAs. FMRP blocks the initiation of ribosome assembly and stalls the elongation of actively transcribing ribosomes, which points at a negative regulator role for FMRP in mRNA translation^{47,48,49,50}. Consequently, the *Fmr1* KO mouse shows increased protein synthesis in total brain homogenates and isolated synaptoneurosome, which contain presynaptic and postsynaptic components⁵¹⁻⁵³. Since local or dendritic protein synthesis is required for long-term functional synaptic change, such as long-term potentiation (LTP) and long-term depression (LTD), this also supports a role for FMRP in synaptic plasticity. Synaptic plasticity is the continuous process where synapses are formed and retracted in response to external stimuli, a process believed to underlie learning and memory⁵⁴). During LTP, the strengthening of a synapse occurs by the recruitment of AMPA receptors (AMPARs) from cytosolic vesicles into the postsynaptic membrane of the spine after stimulation of the NMDA receptors (NMDARs). The AMPA receptors are then anchored to post synaptic density (PSD) proteins by actin. LTD is the weakening of the synapse as a result of a removal of AMPARs from the membrane through endocytosis and eventually subsequent degradation⁵⁵. Interestingly, one specific form of LTD is the group 1 metabotropic glutamate receptor (mGluR) dependent LTD in the CA1 region of the hippocampus. In this type of LTD, stimulation of the Group 1 mGluRs, with for example the selective agonist (S)-3,5-dihydroxyphenylglycine (DHPG), initially results in increased protein synthesis at the spine. One of the up regulated proteins in the LTD response was found to be FMRP itself. As a result, the AMPA receptor levels decrease persistently at the postsynaptic membrane^{56,57}. It is shown that *Fmr1* KO mice display an exaggerated hippocampal mGluR-LTD, and an increased level of AMPAR internalization^{58,59}. Based on these observations, the mGluR theory was proposed to explain many features of FXS. This theory states that in FXS, mGluR stimulation will lead to reduced synaptic strength due to increased AMPAR internalization as a consequence of the increased protein synthesis in the absence of FMRP⁶⁰. For more details about the role of metabotropic glutamate receptors in FXS, see⁶¹

4.1. Synaptic targets and therapeutic strategies in FXS

The first therapeutic strategies were based on reducing mGlu5 receptor signaling in *Fmr1* KO mice and in FXS patients; however by now it is clear that FXS is a result of more than just an altered mGluR signaling. Target proteins of FMRP, like phosphoinositide 3-kinase (PI3K) and matrix metalloproteinase 9 (MMP-9) and key signaling proteins such as endocannabinoid 2-arachidonoyl-sn-glycerol, mTOR or ERK are also excellent therapeutic candidates linked to synaptic plasticity and the pathophysiology of FXS⁶²⁻⁶⁷. In addition to the NMDA and AMPA receptors, the neuronal Kv4.2 potassium channels and the M1 muscarinic acetylcholinergic receptors are also known to be involved in FXS. Also, the inhibitory γ -aminobutyric acid (GABA) system with its two classes of receptors (GABA_A and GABA_B) has shown to be deregulated in FXS. Recently, a specific presynaptic function of FMRP has been revealed, namely the BK channel⁶⁸, that could possibly be used as a therapeutic target as well. A schematic overview of therapeutic interventions currently investigated in FXS is presented in Figure 1.

4.2. Translational endpoints in *Fmr1* KO mice

Several mouse models for FXS have been generated such as the *Fmr1* KO, *Fmr1* conditional KO and *Fmr1* conditional restoration, and a mouse model for the I304N mutation, *Fmr1* I304N⁶⁹. All are available in different backgrounds^{44,45,70-72}. To date, several translational endpoints have been described for *Fmr1* KO mice, including an altered dendritic spine phenotype (Figure 2). Although the spine density shows inconsistent results between brain areas and investigators, the immature spine phenotype with more longer and thinner spines has proven to be a consistent finding in *Fmr1* KO mice (see⁷³ for a recent review). In addition to the increased basal levels of protein synthesis, increased protein activity has also been detected in different brain areas of the *Fmr1* KO mouse^{51-53,65}. Increased ERK phosphorylation has been observed in the cortex of *Fmr1* KO mice^{74,75}. mTOR phosphorylation and activity are up regulated in the hippocampus of *Fmr1* KO mice as well as the levels of 110 β , a catalytic subunit of PIKE, and the PI3K enhancer PIKE-L^{63,65}. In addition, basal levels of striatal-enriched protein tyrosine phosphatase (STEP), a protein believed to play a role in synaptic plasticity, are elevated in *Fmr1* KO mice⁷⁶. As mentioned before, *Fmr1* KO mice show enhanced hippocampal mGluR-LTD as well as an enhanced cerebellar mGluR-LTD and, in line with these observations, excessive mGluR-mediated AMPAR internalization^{19,58,59}. However, not all *in vivo* studies on postsynaptic receptor levels show similar results^{77,78}.

Some studies reported unaltered levels or an increase in the postsynaptic scaffolding proteins SAPAP3 and PSD-95 in *Fmr1* KO mice⁷⁹⁻⁸¹. Whereas, other studies show a decrease in NMDA receptor subunits and the scaffolding proteins SAPAP3 and PSD-95 in the prefrontal cortex of *Fmr1* KO mice⁸².

Although these post synaptic measures would provide a good additional read-out for the defects in synaptic plasticity found in *Fmr1* KO mice, more research is needed to establish robust and reproducible biochemical outcome measures.

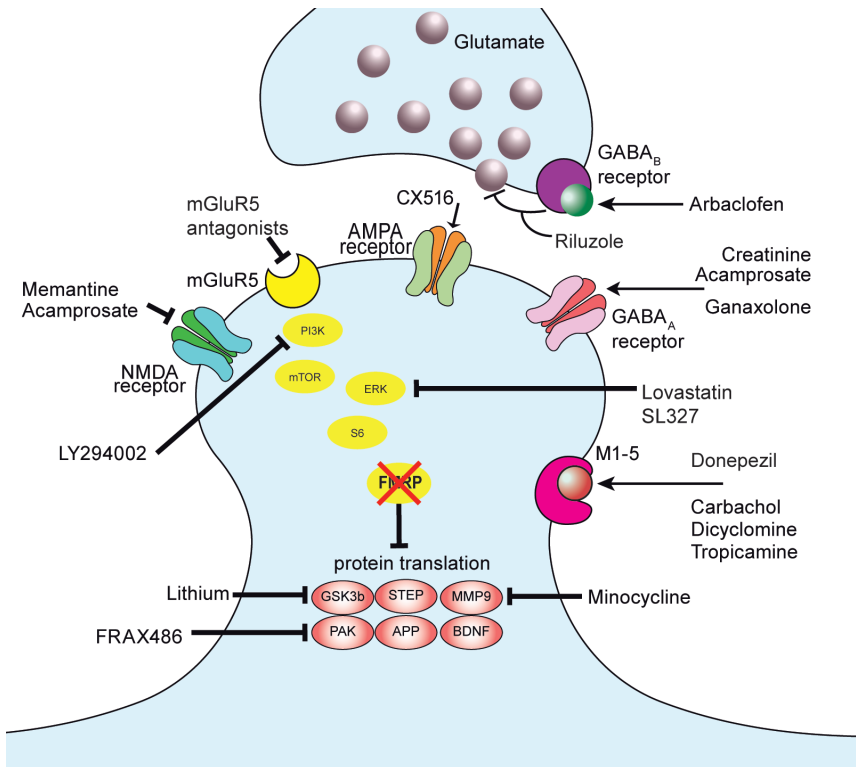


Figure 1: Synaptic targets of the therapeutic interventions in fragile X syndrome.

The NMDA and AMPA receptor equilibrium is deregulated in fragile X syndrome. Therapies aimed at lowering NMDA receptor signaling are memantine or acamprostate, while AMPA mediated neurotransmission can be modulated by the ampakine CX516. The muscarinic receptor subtypes M1 and M4 can be targeted by carbachol, dicyclomide or tropicamide or indirectly by the acetyl cholinesterase inhibitor Donepezil. The increased expression of p110 δ , a catalytic subunit of the key signaling molecule PI3K, can be lowered by exposure to a PI3K antagonist⁶⁵. Deregulated ERK expression can be altered by an MEK1/2 inhibitor like SL327 or more indirectly by lovastatin^{53, 75}. The increased GSK-3 activity found in different brain areas of *Fmr1* KO mice can be lowered by lithium. Increased p21-activated kinases (PAK) levels can be down regulated by PAK inhibitors such as FRAX486¹¹⁷. MMP-9 levels and activity have been shown to be increased in *Fmr1* KO hippocampus and can be lowered by administration of the MMP-9 inhibitor minocycline⁶⁶. The altered inhibitory GABA_A signaling in *Fmr1* KO mice can be targeted by the neuroactive steroids alphaxalone and ganaxalone. Acamprostate has also been shown to act as a weak GABA_A agonist as well. The presynaptic GABA_B receptors are of interest because they inhibit glutamate release and thus group 1 mGluR activity (e.g. Riluzole)¹⁸⁸. GABA_B agonists used in fragile X syndrome are baclofen and its active enantiomer arbaclofen (or R-baclofen).

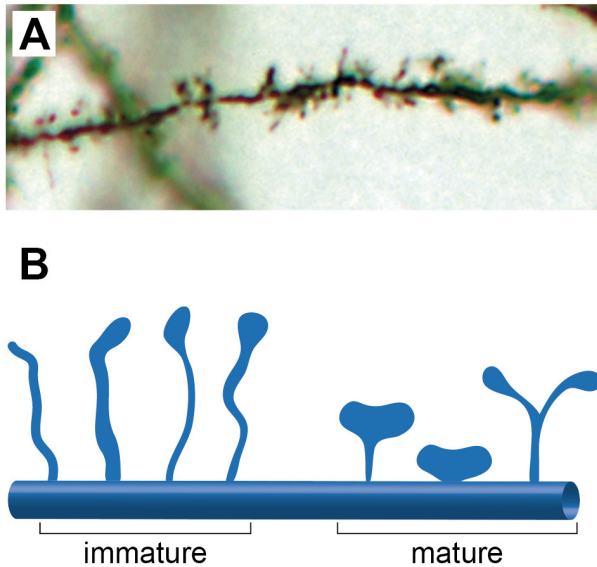


Figure 2: Morphology of dendritic spines.

Photomicrograph (A) shows a detail of the dendrite from a murine *Fmr1* KO hippocampal neuron stained with Golgi impregnation staining method. The Golgi technique selectively impregnates single neurons with silver chromate and allows visualization of dendrites and axons. Photomicrograph (B) shows graphical representation of dendritic spine morphologies defined as mature or immature.

In addition to the mGluR signaling pathway, the inhibitory γ -aminobutyric acid (GABA) system has shown to be involved in the pathogenesis of FXS as well. GABA is produced and released by inhibitory interneurons and acts on two classes of membrane-bound receptors namely GABA_A and GABA_B. In *Fmr1* KO mice, several GABA_A components were found to be expressed at a lower level in the cortex and hippocampus, including GABA_A receptor subunits and proteins and enzymes involved in GABA degradation and transport⁸³⁻⁸⁶. Electrophysiological experiments also suggested a decrease in efficiency of the GABAergic transmission in *Fmr1* KO mice^{87,88}. Reduced GABA concentrations were found in the synaptic cleft in the amygdala of *Fmr1* KO mice which point to altered GABA_B receptor levels, although no direct evidence has been found⁸⁹. However, until now no clear link between an abnormally functioning GABA system and synaptic plasticity has been shown in FXS.

As a functional read-out for the altered synaptic plasticity, different aspects of behavior of the *Fmr1* KO mouse have been explored. However, the wide variety of genetic backgrounds and protocols used in these studies may lead to different results for the same behavioral phenotype⁹⁰. Among the most robust behavioral phenotypes

are increased locomotor activity and increased exploratory behavior consistent with observations in human FXS patients^{70,74,91}. *Fmr1* KO mice also show deficits in behavior correlated with other mental disorders such as schizophrenia and autism spectrum disorder (ASD). For example, *Fmr1* KO mice show an aberrant prepulse inhibition of startle response (PPI) and high susceptibility to audiogenic seizures⁹²⁻⁹⁴. Autistic-like behavior such as repetitive behavior and aberrant social behavior has been shown in *Fmr1* KO mice as well^{71,72,95,96}. Subtle deficits have been detected in anxiety tests and learning tasks^{74,97}.

4.3. Therapeutic intervention studies in *Fmr1* KO mice

The identification of reliable translational endpoints can be used to assess efficacy of therapeutic intervention in both pre-clinical and clinical studies. Several therapeutic candidates linked to synaptic plasticity and the pathophysiology of FXS have been tested in *Fmr1* KO mice. Many of these have been reviewed elsewhere^{98,99}. Most preclinical studies focused on mGluR5 antagonists and are extensively described in the chapter by D'Antoni *et al*⁶¹. Treatment with the GSK-3 inhibitor lithium, resulted in a normal dendritic spine phenotype and a normal LTD response in *Fmr1* KO mice^{100,101-104}. Administration of the MMP-9 inhibitor minocycline promoted spine maturation *in vivo*⁶⁶ and lowered MMP9 activity¹⁰⁵. In addition, lithium or minocycline administration normalized hyperactivity, susceptibility to audiogenic seizures and increased anxiety found in *Fmr1* KO mice. Autistic-like behaviors as repetitive behaviors and aberrant social interaction and cognitive measures were also reversed by lithium and minocycline which suggests broader effects than the inhibition of GSK-3 or MMP-9 alone^{100-103,106,107}. Treatment of *Fmr1* KO mice with the MEK1/2 inhibitor SL327 resulted in a reduced audiogenic seizure susceptibility⁶⁴. It was also shown that administration of a mix of serotonin agonists (5-HT1A/5-HT7) reverses the increased AMPAR internalization and the exaggerated LTD in *Fmr1* KO mice. By antagonizing the M1 or M4 receptors in *Fmr1* KO mice by treatment with dicyclomide or tropicamide, the repetitive behavior, susceptibility to audiogenic seizures, PPI and passive avoidance behavior was improved¹⁰⁸⁻¹¹⁰. GABA_A receptors are still sensitive to GABAergic drugs in *Fmr1* KO mice, and treatment with either alphaxalone or ganaxalone resulted in reduced anxiety levels and a rescue of the audiogenic seizure phenotype, respectively¹¹¹. GABA_B agonists used in FXS are racemic baclofen and its active enantiomer arbaclofen. Arbaclofen treatment resulted in normalized protein synthesis levels, reduced AMPAR internalization and a correction of the spine phenotype in *Fmr1* KO mice. Both racemic baclofen and arbaclofen treatment resulted in a reduction of the susceptibility to audiogenic seizures in *Fmr1* KO mice and R-baclofen improved social behavior¹¹²⁻¹¹⁵. Two drugs showed promising results in targeted therapeutic intervention studies. Lovastatin was shown to correct excess hippocampal protein synthesis and the susceptibility to seizures in the *Fmr1* KO mice probably by regulating the ERK1/2 pathway and the PAK inhibitor FRAX486, was shown to reverse the dendritic spine phenotype

as well as behavioral abnormalities such as hyperactivity, repetitive movements and audiogenic seizures ^{116,117}. Targeting BK-channels with a selective BKCa channel opener molecule (BMS-204352) resulted in rescue of hippocampal glutamate homeostasis and behavioral features ¹¹⁸. And targeting the endocannabinoid pathway improved learning and memory task ^{119,120}. Besides drug testing, also environmental enrichment has been investigated and some studies show improvement of memory, behavior and even biochemical features by this intervention alone ¹²¹⁻¹²³, which underlines that drug intervention might have to be combined with environmental interventions for optimal effect.

4.4. Translational endpoints in primary *Fmr1* KO neurons

Cultured neuronal cells provide an excellent *in vitro* system to measure effects of drugs on synaptic plasticity in a controlled environment. In cultured neurons several dendritic protrusion characteristics including length, width and total number per segment, can be used as a measure for synaptic plasticity. Based on their length and width, protrusions can be categorized into immature filopodia (long and thin protrusions) or mature spines (short and stubby protrusions) to facilitate the phenotypic analysis. Similar to human FXS patients and patients with other forms of ID, an immature spine phenotype is found in primary hippocampal and cerebellar *Fmr1* KO neuron cultures ^{66,92,124,125}. The abnormal spine phenotype can also be assessed at a molecular level by quantifying the expression levels of the different postsynaptic receptors, their scaffolding proteins or other key signaling proteins involved but up to now only a few studies have been published. *Fmr1* KO hippocampal neurons show increased AMPA receptor internalization, while the synthesis of an important PSD protein, Shank1, has been shown to be deregulated in cultured cortical *Fmr1* KO neurons ^{59,80}. The expression of p110 β is elevated in *Fmr1* KO hippocampal neurons ⁶⁵. In addition, cultured hippocampal *Fmr1* KO neurons were shown to have a reduced stimulus-induced localization of GABA α receptor δ subunit mRNA ¹²⁶.

4.5. Therapeutic intervention studies in primary *Fmr1* KO neurons

mGluR5 antagonists like MPEP, fenobam and AFQ056 have been tested in primary neuronal cultures showing a rescue of the immature spine phenotype ^{92,127,128}. Exposure to the NMDAR antagonist memantine, stimulated synapse formation and promoted dendritic spine maturation in cultured cerebellar granule cells of *Fmr1* KO mice ^{129,130}. After treatment with lithium, *Fmr1* KO mice showed a rescue of the spine phenotype ¹⁰⁰⁻¹⁰³. Administration of the MMP-9 inhibitor minocycline also promoted spine maturation *in vitro* ⁶⁶. Only a very limited number of studies have looked at the effects of therapies on the expression levels of the synaptic receptors or other plasticity proteins in primary neuronal cell cultures. Increased AMPA receptor internalization in *Fmr1* KO hippocampal

neurons was shown to be normalized by exposure to a PI3K antagonist. Exposure to this antagonist also resulted in normalized protein synthesis levels and spine density^{59,65}.

4.6. Other models for studying fragile X syndrome

Thus far, the identification of reliable biomarkers in the *Fmr1* KO mouse has been the starting point for putative biomarkers that may aid in the development and evaluation of therapeutic interventions in FXS patients. An option to expand the search for potential therapies is the use of a high-throughput screen using animal systems, including *Drosophila melanogaster* (fruit fly) and *Danio rerio* (zebrafish). For example, homozygous *Fmr1* null fruit flies show many phenotypes associated with FXS. However, if embryos were exposed to small molecules in a chemical genetic screen, three compounds involved in the GABAergic inhibitory pathway were shown to rescue multiple phenotypes¹³¹. In addition, the vertebrate zebrafish can be used for high-throughput screening as well. An *Fmr1* mutant KO fish lacking *Fmr1* expression did not show any phenotype at a young age³⁹. However, adult *Fmr1* KO fish seem to show behavioral and synaptic abnormalities⁴³. Although this model holds potential benefits for drug screening, more research is necessary to fully characterize the molecular and behavioral deficits in the adult *Fmr1* KO fish.

In 2008, it became possible to model FXS, as well as other genetic neurodevelopmental disorders such as Rett syndrome, *in vitro* by creating patient-specific induced pluripotent stem cells (iPSCs)¹³². Recently it was shown that fragile X iPSCs have an inactive *FMR1* gene and lack FMRP expression in the pluripotent state as well as in differentiated states¹³³⁻¹³⁵. To date, several protocols are available for differentiating iPS cells into functioning mature neurons^{136,137}. Thus far, FXS iPS cells and the derived neurons have only been used to test gene-reactivating therapies. However, these neurons show great potential as a human model system to study FXS phenotypes and the effects of potential existing and new drugs.

5. Translational endpoints in patients with fragile X syndrome

The identification and validation of reliable biomarkers in patients with FXS is an important pre-clinical aspect in the drug discovery process. These biomarkers can directly be translated into translational endpoints in clinics.

In the past years, extensive work has been done to develop targeted treatment strategies for FXS, in order to alleviate the symptoms and maybe eventually providing a cure. Recently several phase 1 and 2 trials have been conducted with drugs targeting the mGluR5 pathway, NMDA and AMPA receptors, the GABAergic system, MMP-9 and cholinergic pathway, all with variable results.

In this section the translational endpoints that have been used in clinical trials are described. In addition, new and promising reliable outcome measures will be discussed.

Table 1. Most used outcome measures in clinical trials with fragile X syndrome patients

Test	Characteristics	Measured function	Reference
Eyeblink	Repeated exposure of the cornea to an air puff combined with a sound leads to a conditioned blinking response to the sound alone.	Conditioning response, coordinated by cerebellum and mediated by LTD.	19,20
Prepulse inhibition (PPI)	A weak auditory stimulus precedes a loud startling noise, leading to an inhibited startle response.	Sensorimotor processing glutamatergic and GABA-ergic pathways	21,22
Aberrant Behavior Checklist – Community edition (ABC-C)	Caregiver-rated 58-item score list. Subscales: Irritability, Lethargy/social avoidance, Stereotypy, Hyperactivity, Inappropriate speech.	Different behavior aspects in persons with developmental disability	138
Clinical Global Impression-Severity (CGI-S)	Clinician rated scale: global severity of illness compared to other patients with diagnosis. 1=normal, 2=borderline mentally ill, 3=mildly ill, 4=moderately ill, 5=markedly ill, 6=severely ill, 7=extremely ill.	Overall severity of patients illness in mental disease	165
Clinical Global Impression-Improvement (CGI-I)	Clinician rated scale: 1=very much improved, 2=much improved, 3=minimally improved, 4=no change, 5=minimally worse, 6=much worse, 7=very much worse.	Improvement of patients illness in target symptoms which are defined at baseline	165
Vineland Adaptive Behavior Scale (VABS) PL)	Caregiver or teacher input. Categories: Communication, Daily living skills, Socialization, Motor skills, Maladaptive behaviour.	Asses daily functioning and adaptive behavior	141
Visual Analogue Scale (VAS)	Caregiver rated continuous visual scale on which agreement or severity is pointed.	Level of impairment in reciprocal social behavior and autistic spectrum symptoms	167
Social responsive scale (SRS)	Caregiver rated, 65 items score list on severity of autism spectrum symptoms.	Assess expressive vocabulary and development	179
ADHD Rating Scale (ADHD-RS)	Clinician rated.	Score ADHD symptoms	168
Peabody Picture Vocabulary Test (PPVT)	Measurement of single word receptive vocabulary.	Communication and language, receptive vocabulary and verbal ability	169,170

Table 1. Most used outcome measures in clinical trials with fragile X syndrome patients (continued)

Test	Characteristics	Measured function	Reference
Conners Parent Rating Scale (CPRS)	45 item questionnaire based on caregiver/teacher report. Domains: Oppositional, Cognitive problem, Hyperactivity, inattention, Executive functioning, Peer relations, ADHD index	Assessing ADHD and autism spectrum symptoms	171
Conners Teacher Rating Scale(CTRS)			
Swanson Nolan Pelham questionnaire parent rating scale (SNAP-IV)	Caregiver and teachers rated scale on 90 items	Assesses oppositional defiant, inattentive, hyperactive-impulsive and combined ADHD symptoms	172
Repetitive Behavior Scale (RBS)	Caregiver rated score list, 44 items, 6 domains: Stereotypic, Self-injurious, Compulsive, Ritualistic, Sameness behavior, Restricted interests Clinician, teacher or caregiver rated, based on observation information of persons autism symptoms Observation on social interaction, communication and play during administration of structured modules Clinician rated, 10 item score list	Measures repetitive behavior, a key feature of autism	173
Childhood Autism Rating Scale (CARS)		Recognition of autism and differentiation from developmental disorders	174
Autism Diagnostic Observation Scale (ADOS)		Diagnosing, assessing and classifying autism	175
Children's Yale-Brown Obsessive Compulsive Scale modified for PDD (CY-BOCS-PDD)		Measure repetitive an compulsive behavior	176
Kiddle Schedule for Affective Disorder and Schizophrenia (K-SADS PL)	Clinician scored based on interviewing caregivers and caregiver scores by parent, child and teacher.	Child specific diagnostic criteria for psychopathology	177
Clinical Evaluation of Language Fundamentals (CELF)	Child specific clinical test, administered by clinician, consisting of several subtests.	Communication and language skills	178
Expressive Vocabulary Test (EVT)	Clinical test with 1-word synonym response to visual stimuli	Assess expressive vocabulary and development	179
Repeatable Battery for Assessment of Neuropsychological status (RBANS)	Repeatable test battery to measure attention, language, visuospatial ability, constructional ability, auditory/visual recall (immediate and delayed)	Neuropsychological status	180

5.1. Rating scales and clinical tests

In human patients, translational outcome measures usually consist of score lists or cognitive tests which assess improvements in the patients symptoms and functioning. The amount of different tests available to measure intelligence, cognitive functioning, behavior, emotional capacity, learning capacity and autistic features, is extensive. Tests are mostly performed by the physician or consist of caregiver-related questionnaires and the outcome thus relies on their interpretation. Table 1 summarizes tests that have been proposed as outcome measures in clinical trials for FXS. Standard IQ-tests (e.g. WAIS) are practically always used as baseline measurement, but not as outcome measure.

The most extensively used test in FXS clinical trials is the Aberrant Behavior Checklist – Community edition (ABC-C), a 58 item score-list, rated by the caregiver, that was especially developed to assess medication and treatment effects in developmental disorders ¹³⁸. It assess behavior on 5 subscales, namely irritability, social withdrawal and lethargy, stereotypy, hyperactivity and inappropriate speech. It has a confirmed reliability, validity, distribution of scores and sensitivity to change in developmental disability. Recently, a modified version of the ABC-C to better fit the FXS population has been developed after validation in 630 individuals with FXS ¹³⁹. This modified ABC, named ABC-FX, has now been applied in many clinical trials ¹⁴⁰.

The clinician rated Clinical Global Impression (CGI) scale provides three questions to rate the severity of the patients mental illness (CGI-S) and the improvement of this patient after initiation of treatment (CGI-I).

A third often used test is the Vineland Adaptive Behavior Scale (VABS) ¹⁴¹. It measures the patients adaptive skills, by assessing the daily living skills on the three domains communication, daily living and socialization. It is often used in intellectual disability and autism diagnosis and care.

The Visual Analogue Scale (VAS) consists of a continuous line between two points, which are predefined, for example, from no problem to the worst imaginable problem. The responder points to the level of severity of a symptom, or his agreement to a statement, on this line. VAS has been validated in ADHD, autism and psychiatric trials and shows good reliability ^{142,143}. It is often used in questionnaires. In the clinical trials mentioned, VAS was rated by the caregiver and often regarded the patients cognition or specific target behaviors that were chosen by the caregiver. Besides these tests, a wide spectrum of other tests were used to measure different FXS symptoms (see Table 1).

5.2. Neurobiological markers

In recent years, more objective outcome measures have been described. One of these is the prepulse inhibition of startle test (PPI), first described by Frankland *et al* in males with FXS ²¹ and later by Hessl *et al* ²². The PPI measures the brains sensitivity to sensory stimulation and it largely depends on neurons in the forebrain (nucleus accumbens,

hippocampus, amygdala or prefrontal cortex)¹⁴⁴. PPI shows disruption in several psychiatric and neurological diseases, like schizophrenia, ADHD, Huntington's disease, Tourette's syndrome and autism, all characterized by abnormalities in inhibitory control²². The test comprises of a weak auditory stimulus, that precedes a loud startling noise and leads to a reduced startle response by the test subject. Frankland *et al* was the first to test this in young males with FXS (10 patients and 7 controls)²¹. This study showed that FXS patients have obvious impairments in sensorimotor gating. Moreover, the impairment was correlated with the cognitive and behavioral deficits, suggesting a common core mechanism. Hessel *et al* used 61 individuals with FXS and 63 controls of different ages (8-40 years) and showed sensorimotor deficits in FXS males and females, with a good retest reliability, even in high functioning females²². Thus far, the PPI has been used once in a clinical trial and demonstrated to be a reliable measure that is correlated to the clinical features of the patients¹⁴⁵.

A second endophenotypic test in FXS is the eye blink test¹⁹. In this test, repeated exposure of the cornea to an air puff combined with a sound, creates a conditioned blinking response to the sound alone. The precise timing is coordinated mostly by the cerebellum and is mediated by LTD of the parallel fiber-Purkinje cells and is thought to be linked to the mGluR5 pathway²⁰. FXS patients showed delay in eye blink conditioning compared with controls¹⁹. Also long-term measurements show an aberrant response in FXS patients.

Objective measures, like the PPI and eye blink test, might be promising as more robust quantitative measurements. However, a major drawback of these tests is that they do not correlate with a functional behavioral outcome. Besides, these tests are quite expensive and difficult to execute¹³. Other functional measures that have recently received attention as potential new outcome measures in FXS, include neuroimaging techniques like functional near-infrared spectroscopy¹⁴⁶, Transcranial Magnetic Stimulation¹⁴⁷ and electroencephalogram¹⁴⁸ and more objective behavioral measures like the recently published Eye Contact Avoidance Scale¹⁴⁹. Clinical trials that investigate new outcome measures are currently recruiting patients, testing protein synthesis in brain (NCT00362843), imaging of glutamatergic transmission (NCT02719951) and Auditory Event Related Potential (NCT02920892).

5.3. Biochemical biomarkers in patients with FXS

Preclinical research on tissue or cells of FXS patients has led to the discovery of several potential biomarkers. Biochemical outcome measures have been used in clinical trials occasionally, e.g. ERK and MMP-9, a matrix metalloproteinase involved in synaptic plasticity, in blood^{105,150}. A recent paper quantified MMP-9 activity in peripheral blood of patients with FXS and showed a higher activity as compared with controls¹⁰⁵. One trial used brain derived neurotrophic factor (BDNF), a biomarker shown to be decreased in autistic individuals and *Fmr1* KO mice¹⁵¹. BDNF is not yet validated in FXS patients and in this study, the BDNF increase did not correlate with the clinical improvement.

Furthermore, it is not known whether BDNF function in brain is correlated to blood levels. Thus, more research is needed to evaluate the relevance and significance of this finding.

Lohith et. al. (2013) showed a higher mGluR5 receptor density in patients with FXS as compared with controls in post-mortem material (n=10 FXS patients, 4 FXS carriers and 17 matched controls)¹⁵². A significant difference in mGluR5 protein levels has also been reported in superior frontal cortex from post-mortem material of autistic children¹⁵³. Analyses of protein lysates of lymphocytes of 38 patients with FXS and 14 controls showed increased phosphorylation of the mammalian target of rapamycin (mTOR), p70 ribosomal subunit 6 kinase1 (S6K1) and of the serine/threonine protein kinase (Akt), all present in the mTOR pathway and downstream of mGluR5. Also increased phosphorylation of the cap binding protein eukaryotic initiation factor 4E (eIF4E) could be demonstrated, suggesting increased protein synthesis⁶². Increased protein synthesis and increased phosphoinositide 3-kinase (PI3K) activity was also demonstrated in lymphoblastoid cells from FXS patients¹⁵⁴.

Weng et. al. (2008) has studied the early-phase phosphorylation of ERK in lymphocytes from peripheral blood of FXS patients, compared with controls. Patients with FXS showed slower ERK activation compared with controls, showing a less efficient second messenger signaling¹⁵⁵. In contrast, an increased phosphorylation, and hence activation, of MEK1/2 and ERK was measured in cortex of post mortem brains from 4 patients with FXS (age range 10-86 years) as compared with 7 age matched controls⁶⁴. The increased ERK phosphorylation in blood is now regarded as a promising objective biomarker to measure effect in clinical trials targeting pathways involving the ERK signaling. It has been already used in clinical trials^{150,156}.

In contrast to these findings, a study by Qin *et al* (2013) demonstrated reduced protein synthesis in brains of 15 patients with FXS under sedation by propofol and 10 age matched controls, sedated and unsedated, using L-[1-(11)C] leucine positron emission tomography (PET) method¹⁵⁷. This PET method has been shown to be a reliable and reproducible method for measuring protein synthesis in human brain^{158,159}. This reduction is presumably due to the sedation effect, as was later demonstrated by comparing with sedated and not sedated *Fmr1* KO mice¹⁵⁷.

Also cholinergic dysfunction has been demonstrated, by measuring reduced choline levels in the right dorsolateral prefrontal cortex of 9 males with FRAX and 9 age-matched controls¹⁶⁰.

6. Clinical trials in fragile X syndrome

In FXS, the behavioral problems and intellectual disability cause a marked decrease in the patients and their families quality of life. Treatment of symptoms by a combination of psychopharmacological drugs, addressing hyperactivity, impulsivity, anxiety, aggression or mood disorders, is often applied, together with psychotherapy and behavioral therapy. These symptom based therapies have variable outcomes, and are

often insufficient. Besides, some drugs might counteract each other, enhance other symptoms or cause unwanted side-effects.

First steps in targeted treatment of FXS patients have been taken in the form of clinical trials with drugs targeting the mGluR5 pathway, NMDA and AMPA receptors, the GABAergic system, MMP-9 and cholinergic pathway. Table 2 summarizes published clinical trials so far, including the most important features, mechanism, outcome and side effects. Table 3 shows ongoing trials that are mentioned on www.clinicaltrials.gov. It is striking to note that in most cases, the smaller open label clinical trials have shown encouraging results, although often in a small subset of patients. However, the larger placebo controlled trials did not meet their endpoints, even when stratified to these patient groups, and all open label extension studies have been terminated prematurely. Only one study has attempted to influence the *FMR1* expression, by using valproic acid ¹⁶¹. Valproic acid is used for epilepsy, bipolar disorder and migraine. It has also shown effect in autistic patients and is off-label prescribed to FXS patients. Valproic acid has been shown to inhibit histone deacetylase activity, and treatment could lead to demethylation of the *FMR1* promoter, and thus, reactivation of the *FMR1* gene in patients with FXS. This open label trial with 10 patients with FXS, showed a significant decrease in hyperactivity, but no effect on *FMR1* mRNA levels in blood ¹⁶¹. Other conducted clinical trials can be further reviewed in Table 2.

Recently, a case report was published on treatment of a FXS patient with bumetanide, a diuretic NKCC1 chloride importer antagonist, which reduces intracellular chloride ¹⁶². This drug has previously shown to reduce autistic features in non-FXS children with autism spectrum disorder ¹⁴³. It showed no side effects and improvements in CARS, ADOS, ABC, RDEG, RRB, although significance is unknown. There are several pathways that have not been fully explored in FXS patients yet, like the endocannabinoid pathway, the DGK α pathway and the cholesterol biosynthesis. These may lead to promising drugs for future clinical trials.

Importantly, the different tests described in this overview, although widely used and validated, are mainly based on subjective measures. Also a very high placebo effect is noticed in clinical trials, probably caused by the hope for improvement of the patients, and rater-sensitive outcome measures. This might overestimate treatment effects in non-placebo controlled trials or conceal the effect in placebo controlled trials. Recently, a phase III clinical trial with STX209 (arbaclofen) was terminated abruptly because STX209 did not show an advantage over placebo treatment on the primary endpoint of social withdrawal, although parents were very enthusiastic about the drug ¹⁶³. Also it has been noticed that subgroups of patients might react differently to treatment, for example shown in Jacquemont *et al* (2011), where the mosaicism of methylation of the *FMR1* gene, seems to influence the outcome of the treatment ¹⁶⁴. In many clinical trials the treatment period might be too short and small changes might be overlooked due to the limitations of the tests used.

Paying attention to the trial design (placebo-controlled, randomized, placebo-lead-in period, intention to treat setting), choosing relevant outcome measures, but also the development and validation of reliable and robust outcome measures is required.

Table 2. Conducted clinical trials with targeted treatment for fragile X syndrome patients

Study	Drug, dosage, treatment regimen	Mechanism	Study Design: Phase, design	Participants: N sex average age in years (min-max)	Outcome measures	Main results	Side effects
mGluR5 antagonists							
Berry-Kravis et al. 2009 ¹⁴⁵	Fenobam 50/100/150 mg	mGluR5 negative modulator	Pilot OL	6 male 6 female	PPI	Significant improvement PPI Reported: calmed behavior, better eye contact	Mild sedation
Jacquemont et al. 2011 ¹⁶⁴	Single dose AFQ056/ Mavoglurant	Selective mGluR5 negative allosteric modulator	IIb R, DB, P, CO	23.9 (18.7-30.7) 30 male 25.5 (18-36)	ABC-C CGI-S/I, efficacy index VABS RBS-R SRS VAS PPVT KITAP	Overall significant improvement in RBS-R (stereotypy, restricted interests) <i>Subgroup</i> (100% methylated CpG sites): significant improvement ABC-C (lethargy, stereotypy, hyperactivity, inappropriate speech), CGI-I, CGI-efficacy, RBS-R (stereotypy, restricted interests), SRS and VAS	Mild, incidental. Headache 4 pt on AFQ056

Table 2. Conducted clinical trials with targeted treatment for fragile X syndrome patients (continued)

Berry-Kravis et al. 2016 ¹⁸¹	AFQ056/ mavoglurant	Selective mGluR5 negative allosteric modulator	III R, DB, P + OL extension	175 (11 females) P n=44, 25 mg bid n=44, 50 mg bid n=42, 100 mg bid n=45	ABC-C _{FX} total and subscales CGI-I RBS-R	No significant improvement, also when stratified to methylation status. Cognitive data difficult to interpret.	Most mild, comparable to placebo. Most psychiatric, gastroint- estinal, dizziness and insomnia
	TI 25, 50, 100 mg bid			24.2-26.9 (18- 45)	SRS	Open label extension study not published yet, terminated prematurely due to lack of efficacy.	
	TR total 12 wks				CNS VS		
Berry-Kravis et al. 2016 ¹⁸¹	AFQ056/ mavoglurant	Selective mGluR5 negative allosteric modulator	III R, DB, P + OL extension	139 (12 females) P n=39, 25 mg bid n=31, 50 mg bid n=27, 100 mg bid n=39 14.4-14.6 (12- 17)	ABC-C _{FX} total and subscales CGI-I RBS-R SRS TEA-Ch	No significant improvement, also when stratified to methylation status. Cognitive data difficult to interpret.	Most mild, comparable to placebo. Most psychiatric, gastroint-testinal, dizziness, headache, vomiting and insomnia
	TI 25, 50, 100 mg bid					Open label extension study not published yet, terminated prematurely due to lack of efficacy.	
	TR total 12 wks						
Unpublished NCT010- 15430 ¹⁸²	RO4917523 basimglurant	Selective mGluR5 antagonist	II R, DB, P	40	Safety and tolerability	Safe	Well tolerated, unspecified
	TI 0.1-1.5 mg/day in 6 wks			18-50 y			
	TR total 6 wks						

Table 2. Conducted clinical trials with targeted treatment for fragile X syndrome patients (continued)

Unpublished NCT01517698 ¹⁸² https://imfar.confex.com/imfar/2015/webprogram/Paper19054.html	RO4917523 basimglurant 0.5, 1.5 mg/day TR total 12 wks	Selective mGluR5 antagonist	IIa R, DB, P	185, 63 adolescents, 122 adults, 34 female P n=63, 0.5 mg n=60, 1.5 n=62 14-50 y	ABC CGI-S and I ADAMS RBANS VAS SRS VABS	No improvement	Mild: vomiting, nasopharyngitis, and headache
Unpublished NCT01750957 ¹⁸² https://imfar.confex.com/imfar/2015/webprogram/Paper18427.html	RO4917523 basimglurant 0.5, 1.5 mg/day TR total 12 wks	Selective mGluR5 antagonist	IIa R, DB, P	47 children P n=17, 0.5 mg n=15, 1.5 mg n=15 5-13 y	CGI-S and I ABC RBANS VAS	No improvement	Mild, aggressiveness
GABA _B receptor agonists							
Berry-Kravis et al. 2012 ¹⁴⁰	arbaclofen STX209 TI 2-20 mg/d TR <12 y: 20 mg/d >12 y: 30 mg/d Total 4 wk WO 1 wk	GABA _B agonist Decrease glutamate release, decrease mGluR5 activity	II R, DB, P, CO	55 male 8 female 6-11: n=24 12-17: n=22 18-40: n=17	CGI-S/I ABC-C VAS VABS SRS RBS-R ADHD-RS	Significant improvement VAS, ABC-social avoidance (post-hoc) Blinded treatment preference. Better response in subjects with ABC-lethargy/social withdrawal >8 No benefit above placebo. OL suspended for autism and FXS	1 discontinuation for irritability
Berry-Kravis et al. 2012 ¹⁶³	arbaclofen STX209 Fixed doses 5 mg bid, 10 mg bid, 10 mg tid TR total 8 weeks	GABA _B agonist Decrease glutamate release, decrease mGluR5 activity	III R, DB, P + OL extension	172 P n=44, 5 bid n=38, 10 bid n=39, 10 tid n=38 Unknown (12-50)	ABC-C _{FX} social avoidance, irritability, hyperactivity subscales, CGI-S, CGI-I, VABS	Most mild, headache, vomiting, nausea, irritability, agitation, anxiety, hyperactivity, decreased appetite and infections, also in placebo	

Table 2. Conducted clinical trials with targeted treatment for fragile X syndrome patients (continued)

Berry-Kravis et al. 2012 ¹⁶³	arbaclofen STX209	GABA _B agonist	III R, DB, P + OL extension	125	ABC-C _{FX} social avoidance, irritability, hyperactivity subscales, CGI-I, VABS	Slight benefit in irritability subscale. Rest no benefit above placebo. OL suspended for autism and FXS	Most mild, headache, vomiting, nausea, irritability, agitation, anxiety, hyperactivity, decreased appetite and infections, also in placebo
MMP9 antagonist							
Paribello et al. 2010 ¹⁴²	Minocycline	MMP-9 inhibitor	Pilot OL	18 male	ABC-C	Significant improvement ABC-C irritability, stereotypy, hyperactivity, inappropriate speech, VAS, CGI improvement in language, attention, social communication, anxiety.	Minor diarrhea, seroconversion to positive ANA
Utari et al. 2010 ¹⁸³	100-200 mg/d TI 2 wk	MMP-9 inhibitor	Ret	2 female	VAS	Questionnaire on Likert scale (language, academic abilities, attention, behavior, physical features, side effects) CGI-I	21 pt side effects, 1a gastro-intestinal, discoloring nails, 4 discontinued because behavior worsening, 2 other side effects
	TR total 8 wk			18 (13-32)	CGI		
	Various 25-200 mg/d >2 wks Mean 3.5 mnth (range 2-20 wk)			43 male 7 female 13.3 (0.3-25)			
Leigh et al. 2013 ¹⁴³	Minocycline	MMP-9 inhibitor	II R, DB, P, CO	47 male 8 female	VAS	Significant but modest improvement on CGI-I, VAS (anxiety/mood-related behaviors)	Mild, most not significant between treatment and placebo. Most gastro-intestinal.
	25 mg/d for weight <25 kg	MMP-9 inhibitor		9.01 (minocycline)	ABC-C-FX VABS		
	50 mg/d for weight 25-50 kg			9.40 (placebo) (3.5-16)	EVT-2		
	100 mg/d for weight >50 kg						
	3 mnth						5 pt tooth discoloration
	WO						

Table 2. Conducted clinical trials with targeted treatment for fragile X syndrome patients (continued)

Dziembowska et al. 2013 ¹⁰⁵	Minocycline 3 mnth each Dose not mentioned	MMP-9 inhibitor	II R, DB, P, CO	10 male	MMP-9 levels CGI-I	Reduction of MMP-9 levels to normal CGI-I improvement in 9 of 10 patients
Other targets						
Berry-Kravis et al. 2006 ¹⁸⁴	CX516 900 mg/d PL 1 wk TI 600 mg, 1 wk TR 900 mg, 3 wk	AMPA receptor- positive modulator	II, R, DB, P	38 male 11 female 27.9 (18-49)	VABS ADOS SNAP-IV ABC-C VAS CGI-S/I Cognitive test battery (TVPS, WJR, RBANS, PLS-4, GARS) ABC-C CGI VAS VABS Cognitive test battery (la PPVT-II, RBANS) ERK-activation	Minimal 12.5% allergic rash, 1 discontinuation
Berry-Kravis et al. 2008 ¹⁵⁰	Lithium 0.8-1.2 mEq/L TI 1-4 wk TR total 2 mnth	Reduces mGluR5 – dependent protein synthesis in dendrite	Pilot OL	15 male 11 (6-23)	Significant improvement ABC-C (total, hyperactivity, inappropriate speech), VAS (aggression, abnormal vocalization, self-abuse, work refusal, outbursts, over emotionality, anxiety, meltdowns, mood swings, tantrums, perseveration, crying), CGI, VABS and RBANS Enhanced ERK activation	Mild- moderate, no discontinuation, polyuria/ polydipsia, elevated TSH

Table 2. Conducted clinical trials with targeted treatment for fragile X syndrome patients (continued)

Erickson et al. 2009 185	Memantine TI: 5 mg/d TR: 15- 20 mg/d Mean 34.7 wks (range 8-104) Donepezil	Uncom- petitive NMDA receptor antagonist	Pilot OL	6 male 18.3 (13-22)	CGI-S/I ABC SRS ADHD-RS (language and social behavior) ABC	No significant improvement, 4 of 6 improved on CGI-I	Increased irritability 2 discontinued
Kesler et al. 2009 160	3 wk 5 mg/d 3 wk 10 mg/d	Acetyl- choline- sterase inhibitor	Pilot OL	6 male		Significant improvement in ABC (hyperactivity, irritability), CNT, CBCL/ABCL (attention)	Mild
Torrioli et al. 2010 161	Valproic acid TI 10-30 mg/ kg, 1 mnth TR 30 mg/ kg/d total 6 mnth	histone deacetylase inhibition (possible DNA demeth- ylation of FMR1)	Pilot OL	10 male 10.6 (7-16) 18.8 (14-44)	CPRS-R CTRS VABS CGI-S SNAP-IV K-SADSL PL	Significant improvement CPRS, decrease in hyperactivity FMR1 mRNA no significant change	Mild 2 discontinued due to worsening of behavior.
Erickson et al. 2011 156	Riluzole TI 50 mg/d, 1 wk TR 100 mg/d Total 6 wk	Inhibits glutamate release, enhances glutamate reuptake, potentiates GABA neurotrans- mission	Pilot OL	6 male 22.5 (19-24)	CGI-I CY-BOCS-PDD ABC SRS CGI-S ADHD-RS PPVT ERK activation	1 treatment responder (CGI-I and CY-BOCS-PDD) ERK activation: significant correction	Mild ALT and AST increase

Table 2. Conducted clinical trials with targeted treatment for fragile X syndrome patients (continued)

Erickson et al. 2013 ¹⁵¹	Acamprosate	NMDA antagonist GABAaR agonist (possibly mGluR5 antagonist)	Pilot OL	10 male 2 female 11.9 (6-17)	CGI-S/I ABC SRS CY-BOCS-PDD ADHD-RS PPVT CELF VABS BDNF	CGI-I 9 of 12 patients treatment responders Significant improvement on ABC (social withdrawal, hyperactivity, social avoidance), CGI-S, SRS, ADHD-RS and VABS Significant increase BDNF after treatment No significant improvement	Mild, most irritability and increased repetitive behavior
Sahu et al. 2012 ¹⁸⁶	Donepezil T1 2.5 mg/d, 4 wk TR 5 mg/d Total 12 wk Metadoxine MG01CI	Acetyl choline-sterase inhibitor	II R, DB, P	20 male 9.9 (donepezil) 8.3 (placebo) (6-15)	SBIQ CPRS CARS		Mild, incidental, including somnolence, decreased appetite
Unpublished NCT02126995 http://www.alcobra-pharma.com/releasedetail.cfm?ReleaseID=919218	T1 2 wks 700 mg/day, 2 wks 1400 mg/day TR total 6 wks	monoamine-independent modulator of GABA	II R, DB, P	62 (75% male) 24 (15-55)	ADHD RS-IV CGI VABS KITAP RBANS	No improvement over placebo Significant improvement in VABS Daily Living Skills Domain and KITAP Distractibility subscale	Similar to placebo

Table 2. Conducted clinical trials with targeted treatment for fragile X syndrome patients (continued)

Unpublished	NMZ-2566 trofinetide	synthetic analogue of neurotrophic peptide derived from IGF-1, role in inflammation and microglia	II R, DB, P	70 males	Fragile X syndrome Rating Scale Fragile X Domain-Specific Concerns CGI-I Caregiver Top 3 concerns ABC-C _{FX} ABC-C _{FX}	Improvement on clinician and caregiver assessments (not specified which significant)	Mild
NCT01894958 http://www.neurenpharma.com/IRM/PDF/1557/TrofinetidesuccessfulinPhase2trialinFragileX	35 or 70 mg bid TR total 28 days			P n=25, 35 mg bid n=24, 70 mg bid n=21 12-45			
	Lovastatin	Inhibitor Ras-ERK1/2	Pilot OL	15 (13 males)		Majority of subjects significant improvement in primary and secondary outcome measures	Mild: headache
	TI to 40 mg TR total 3 mo			6-31 y	CGI-S VABS		

Abbreviations:

Drug and Design: CO: Cross-over, d: day, DB: Double blind, mnth: month, OL: Open label, P: Placebo controlled, R: Randomized, Ret: retrospective, TI: titration, TR: treatment, wk: weeks, WO: wash-out, II: phase 2

Outcome measures: ABC-C: Aberrant Behavior Checklist – Community edition , ABC-C-FX: Aberrant Behavior Checklist – Fragile X edition, ADHD-RS: ADHD Rating Scale, ADOS: Autism Diagnostic Observation Scale , BDNF: brain derived neurotrophic factor, CARS: Childhood autism rating scale, CBCL/ABCL: Achenbach Child and Adult Behavior Checklists, CELF: Clinical Evaluation of Language Fundamentals, CGI-I: Clinical Global Impression-Improvement, CGI-S: Clinical Global Impression-Severity, CNS VS: Computerized Neurocognitive Assessment Software, CNT: Contingency Naming Task, CPRS: Conner’s parent rating scale, CTRS: Conner’s teacher rating scale, CY-BOCS-PDD: Children’s Yale-Brown Obsessive Compulsive Scale modified for PDD, ERK: extracellular-signal regulated kinase, EVT: Expressive Vocabulary Test, GARS: Gilliam Autism Rating Scale, KITAP: Test of Attentional Performance for Children, K-SADSL PL: Kiddie schedule for affective disorder and schizophrenia, HVL: Hopkins Verbal Learning Test, MMP-9: Matrix metalloproteinase 9, PLS-4: Preschool Language Scale–4, PPI: Prepulse inhibition, PPVT: Peabody Picture Vocabulary Test, RBANS: Repeatable Battery for Assessment of Neuropsychological status, RBS: Repetitive Behavior Scale, SBIQ: Stanford-Bine IQ, SNAP-IV: Swanson Nolan Pelham questionnaire parent rating scale, SRS: Social responsive scale, TVPS: Test of Visual—Perceptual Skills, VABS: Vineland Adaptive Behavior Scale , VAS: Visual Analogue Scale, WJR: Woodcock-Johnson Tests of Cognitive Ability

Table 3. Ongoing clinical trials with targeted drugs in fragile X syndrome patients

Drug / intervention	Mechanism	Design	Participants: N, sex	Outcome measures
			Age (years)	
Ganaxolone NCT01725152	GABA _A receptor positive modulator	II R, P, DB, CO	60, both sexes	ABC-C CGI-I PARS PPI Social Gaze (eye tracking) ERP
Acamprosate NCT01911455	NMDA antagonist GABAAR agonist (possibly mGluR5 antagonist)	II R, P, DB	6-17 y	VAS ADAMS ABC-C
Combination therapy: lovastatin and minocycline NCT02680379	Combination therapy: RAS-ERK1/2 inhibitor MMP9 inhibitor	Pilot R, P, DB	5-23 y 26, both sexes	CGI-I ABC-C CGI-I SRS ADAMS RBANS
Lovastatin and a Parent-Implemented Language Intervention NCT02642653 AFQ056 NCT02920892	Inhibitor Ras- ERK1/2 Selective mGluR5 negative allosteric modulator	R, P, DB	104, both sexes 10-17 y	Expressive Language Sample composite score CGI-S and I VAS Weighted Child Intentional Communication Score Biomarker - Auditory Event Related Potential Cognitive measures on language and adaptive functioning

*Table 3. continued (left) Abbreviations:**Design: CO: Cross-over, DB: Double blind, OL: Open label, P: Placebo controlled, R: Randomized, SD: single dose I: phase 1, II: phase 2, III: phase 3**Outcome measure: ABC-C: Aberrant Behavior Checklist – Community edition, ADAMS: Anxiety, depression, and Mood Scale, CGI-I: Clinical Global Impression-Improvement, CGI-S: Clinical Global Impression-Severity, ERP: Event-related brain potentials, fMRI: functional magnetic resonance imaging, KITAP: Test of Attentional Performance for Children, PARS: Pediatric Anxiety Rating Scale, PPI: Prepulse inhibition, RBANS: Repeatable Battery for Assessment of Neuropsychological status, SNAP-IV: Swanson Nolan Pelham questionnaire parent rating scale, SRS: social behavior scale, VAS: Visual Analogue Scale*

Additionally, results of clinical trials must be made publicly available, to prevent publication bias. Trials need to be registered in online databases like clinicaltrials.gov, but many of the clinical trials have not been published in a peer-reviewed journal, even years after completion of the trial, as can be appreciated in Table 2.

Moreover, since FXS is a complex disorder, in which several pathways seem to be deregulated, it is not expected that targeting only one pathway will ameliorate all symptoms. That means that probably the best form of treatment will consist of different drugs to tackle the whole spectrum of problems.

7. Conclusion

Compelling evidence indicates that the abnormal synaptic connectivity found in FXS is associated with a broad spectrum of clinical, behavioral and neuroanatomical abnormalities. The advanced knowledge about the molecular and cellular mechanisms of synaptic dysfunction has led to therapeutic strategies developed to reverse the intellectual and behavioral problems of patients with FXS. The generation of mouse models for FXS served as a pre-clinical starting point to understand the molecular mechanisms underlying FXS and paved the way for drug discovery research. This review describes how cellular and animal models have provided reliable outcome measures and putative biomarkers that will aid in the development and evaluation of therapeutic interventions in FXS. In addition, we discussed several clinical trials that have been conducted using a variety of existing and new drugs designed to correct intellectual and behavioral deficits observed in patients with FXS.

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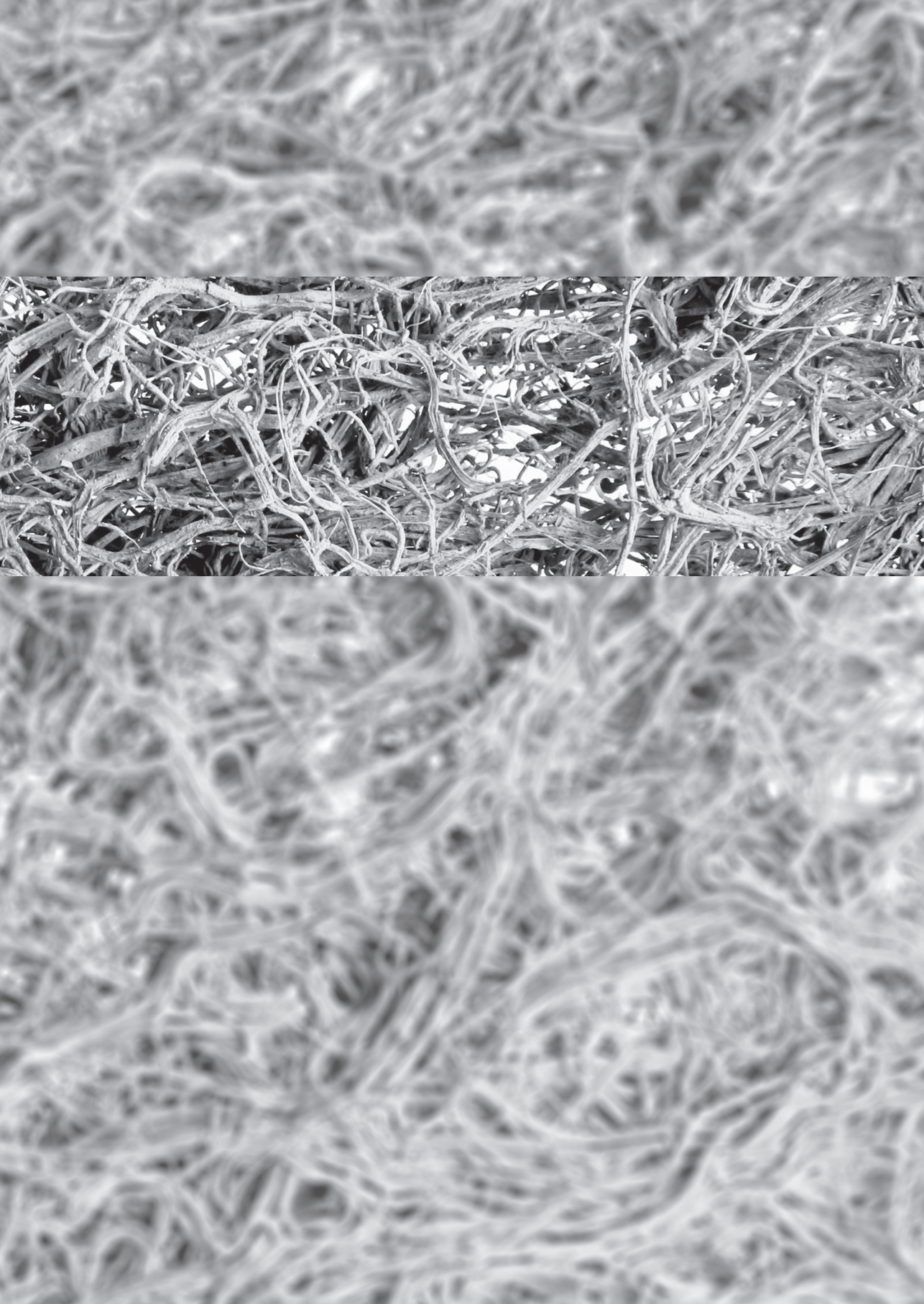
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CHAPTER 2

Combination therapy in Fragile X syndrome; targeting the
mGluR5 and GABA pathways simultaneously

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Abstract

Fragile X syndrome (FXS) is the most common monogenetic cause of intellectual disability and autism. The disorder is characterized by altered synaptic plasticity in the brain. Synaptic plasticity is tightly regulated by a complex balance of different synaptic pathways. In FXS, various synaptic pathways are disrupted, including the excitatory metabotropic glutamate receptor 5 (mGluR5) and the inhibitory γ -aminobutyric acid (GABA) pathways. Targeting each of these pathways has demonstrated beneficial effects in animal models, but not in patients with FXS. This lack of translation might be due to oversimplification of the disease when targeting only one affected pathway. We hypothesize that targeting more than one pathway simultaneously, a combination therapy, might improve treatment effects. We targeted the GABA-pathway separately with bumetanide and in combination with genetic mGluR5 reduction in the FXS mouse model. Our functional outcome measure was a robust social behavior paradigm: the automated tube test (ATT). We report that targeting the GABA pathway results in a partial correction of social behavior deficits, similar to the previously reported effect of targeting the mGluR5 pathway. Targeting both pathways simultaneously, however, did not result in a beneficial synergistic effect, but even in a slight worsening of the ATT phenotype. This implicates both pathways are interconnected and important for social behavior. To our knowledge, this is the first time chronic combination therapy has been tested in FXS. We believe that alternative strategies focused on combination therapy should be further explored, including targeting pathways in different cellular compartments or cell-types.

1. Introduction

Fragile X syndrome (FXS) is a common X-linked hereditary cause of intellectual disability and autism spectrum disorders (ASD). It occurs in about 1:7000 males and 1:11000 females ^{1,2}, leading to invalidating cognitive, behavioral and medical symptoms ³⁻⁶. A disease modifying therapy is currently lacking. The symptoms are caused by lack of FMRP, an RNA-binding protein that plays a critical role in the process which determines neuronal connectivity, called synaptic plasticity ⁷. In the *Fmr1* knock-out mouse (*Fmr1* KO) this synaptic plasticity has been shown to be disrupted, leading to neuronal dysfunction. Several pathways have been implicated to be involved in aberrant synaptic plasticity in FXS, revealing them as possible targets for therapy. The metabotropic glutamate receptor 5 (mGluR5) pathway and the γ -Aminobutyric acid (GABA) pathway are only two examples ⁸. Many studies have shown that these pathways can indeed be targeted in the *Fmr1* KO mouse, in some cases leading to improvement of several disease characteristics. This has been nicely reviewed in Braat *et al*, Gross *et al* and Scharf *et al* ⁸⁻¹¹. Interestingly, FMRP is not only present in the postsynaptic compartment, but is also expressed in the presynaptic compartment and other cell-types in the brain, although little is known about its function there ¹²⁻¹⁶.

The mGluR5-pathway was the first proposed and best studied involved pathway in the pathogenesis of FXS, leading in 2004 to the “mGluR5 theory” ¹⁷. Activation of mGluR5 leads to downstream local protein synthesis in the postsynaptic compartment, which is essential for synaptic plasticity. This local protein synthesis is controlled by FMRP and its absence results in exaggerated mGluR5-dependent protein synthesis and consequently aberrant synaptic plasticity. Several studies have shown that genetic reduction of mGluR5 restores FXS related phenotypes in *Fmr1* KO mice, including molecular, anatomical, electrophysiological and behavioral characteristics ^{9,18-24}. Also, pharmacological reduction of mGluR5 activity has repeatedly showed improvements of many phenotypes in the *Fmr1* KO mouse. These promising pre-clinical results have led to randomized, placebo controlled clinical trials in FXS patients with mGluR5 antagonists mavoglurant/AFQ056, fenobam ²⁵⁻²⁷ and basimglurant (NCT01015430 , NCT01517698 and NCT01750957, results are published online <https://imfar.confex.com/imfar/2015/webprogram/Paper19054.html>, <https://imfar.confex.com/imfar/2015/webprogram/Paper18427.html>). However, despite the evidence for effectiveness of mGluR5 antagonists from animal model studies, these clinical trials did not result in improvement of symptoms in FXS patients.

Another important pathway implicated in FXS, is the GABAergic pathway, the major inhibitory pathway in the adult brain. Both the GABA_A and GABA_B receptor have been implicated in FXS pathogenesis ²⁸. The function of the ionotropic GABA_A receptor, a synaptic and perisynaptic chloride channel, can be influenced with bumetanide, which reduces intracellular chloride concentration in most neuronal cell-types, including excitatory GABAergic neurons ²⁹. Also GABAergic drugs have been tested in clinical

trials with FXS patients³⁰. However, these have not led to an effective treatment for FXS patients (NCT02126995, NCT01725152, NCT01911455, NCT01282268, NCT00788073, <http://onlinelibrary.wiley.com/doi/10.1002/ana.24226/epdf>). For both the mGluR5 and the GABA pathway, the seemingly promising results in mice have not led to a successful therapy for patients yet^{31,32}. The search for a cure for FXS has been consistently hampered by the lack of robust outcome measures in mice. Also in clinical studies, objective and reliable endpoints to measure the improvement of relevant aspects of the disease phenotype are lacking^{33,34}. We recently reported a new robust social dominance paradigm for *Fmr1* KO mice, the automated tube test (ATT), which can be related to deficits in social behavior, a phenomenon often observed in FXS patients^{20,35}. We have demonstrated previously that both genetic and pharmacological reduction of mGluR5, leads to partial, but notably not full correction of the ATT phenotype²⁰. This partial correction indicates that targeting the mGluR5 pathway is not sufficient to fully restore deficits in this type of social behavior. However, current studies in mice as well as in humans have been consistently limited to targeting only one pathway at a time. Considering the partial correction observed and the multitude of affected pathways involved in aberrant synaptic plasticity in FXS, we hypothesized that targeting both excitatory and inhibitory pathways simultaneously as combination therapy, might be more beneficial in treating FXS than targeting a single pathway.

In the present study, we demonstrate that solely targeting the GABA pathway with bumetanide partially corrects the ATT phenotype, suggesting it might be beneficial in treatment strategies for FXS. However, targeting both the GABA pathway with bumetanide and the mGluR5 pathway with genetic reduction simultaneously shows no beneficial synergistic effect on social behavior in the ATT. This study is the first to present chronic combination therapy in the FXS mouse model, by targeting the excitatory and inhibitory synaptic pathways simultaneously.

2. Materials & Methods

2.1. Experimental animals and genotypes

The FXS mouse model we used is the *Fmr1* knock-out mice (*Fmr1* KO, *Fmr1* $-/-$), as created by Mientjes *et al* in 2006³⁶, and their wild-type littermates (WT, *Fmr1* $+/+$). The mice were back-crossed in C57BL/6J for more than ten generations. For the double transgenic experiments, in which mGluR5 was genetically reduced, male C57BL/6J mice that carry a heterozygous functional deletion of the *mGluR5* gene (*Grm5* $+/-$)³⁷ and were wild-type for the *Fmr1* gene (*Fmr1* $+/+$) were bred with females heterozygous or homozygous for the *Fmr1* gene (*Fmr1* $+/-$ and *Fmr1* $-/-$ respectively). Male offspring consisted of single transgenics; *Fmr1* KO *Grm5* $+/+$ (or simply *Fmr1* KO), *Fmr1* WT *Grm5* $+/+$ (normal WT littermates), *Fmr1* WT *Grm5* $+/-$, and double transgenic mice *Fmr1* KO *Grm5* $+/-$, as described in²⁰.

For all experiments, only male mice were used, considering the X-linked nature of FXS. Mice were housed individually after weaning between 3 and 4 weeks after birth, since this housing has been shown to result in the most robust phenotype and is necessary for the drinking water protocol²⁰. Mice were kept under normal laboratory conditions in IVC cages. Food and water was accessed ad libitum. The behavior testing was started between postnatal weeks 12-14, when mice reached young adulthood. The weight of the mice was measured. Genotyping was performed on toe-clip at the age of 1 week and repeated for validation of the genotype on toe or ear tissue obtained after ATT performance. All experiments were approved by the Local Dutch Animal Ethical Committee (DEC).

2.2 Behavior test: Automated tube test

The ATT was performed in the ATT apparatus (Benedictus, the Netherlands). The sliding doors of the setup and the tracking system were automated, resulting in a more standardized performance. It consists of a Plexiglas tube with a starting box on each side. The protocol is according to previous publications^{20,35}. In short:

Training: Prior to the training, mice were allocated a color code on the tail. Researchers are blind for genotype and treatment throughout the experiments. Mice were habituated to the ATT through a training protocol for 5 days, followed by 2 days of rest. At day 1, each mouse was placed in the left box (starting box) and was allowed to explore and walk to the right box (end box) for 180 seconds. This was repeated for the right to left side. From day 2-5 the exploration time was limited to 30 seconds per trial, repeating 3 times each side. Mice that did not leave the starting box within 5 seconds, received an air puff as a negative stimulant, to motivate them to access the tube.

Tournament: On every testing day, mice were first habituated with two training trials. During the tournament, matches consisted of placing a mouse in each side and opening the entrance doors simultaneously. Mice that did not access the tube within 5 seconds were motivated with an air puff. When both mice reached the center door, it opened. When one of the mice returned to its starting cage with all four paws, the match was finished and that mouse was assigned the loser. Consequently, the other mouse won the match. In each experiment two groups can participate. Matches between all mice from one group were performed against all mice of the other group on day 1, with an even distribution of side of the ATT and a random order, and time between matches. On day 2-4, all matches were repeated, every day a reversed starting side and a different order. The matches were added up and a percentage of matches won from the total number of matches was calculated per group per day.

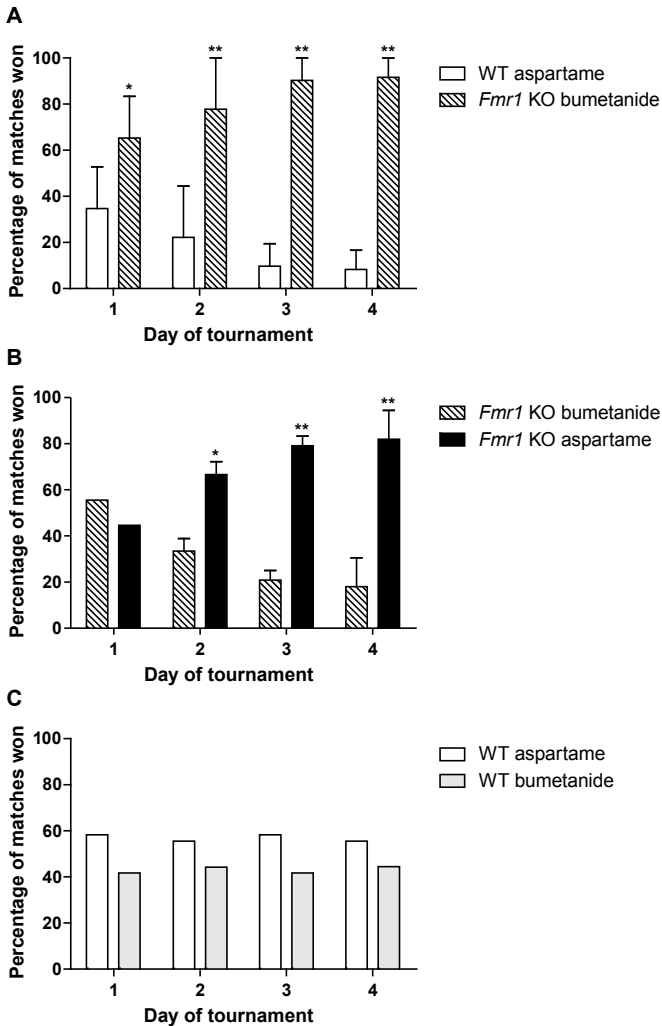


Figure 1: Percentage of matches won by *Fmr1* KO mice and WT littermates treated with bumetanide or aspartame drinking water. Bumetanide treatment results in a partial correction of the *Fmr1* KO phenotype and does not affect WT littermates. **(A)** *Fmr1* KO mice treated with bumetanide continue to show a strong phenotype compared to their WT littermates receiving aspartame drinking water ($p < 0.01$ on day 1 and $p < 0.001$ on day 2-4, $n = 12$ per group). **(B)** Strong reduction of ATT phenotype after bumetanide treatment: *Fmr1* KO receiving aspartame water win most matches against *Fmr1* KO treated with bumetanide after day 1 ($p < 0.01$ on day 2, $p < 0.001$ on day 3 and 4, $n = 12$ per group). **(C)** WT mice receiving bumetanide in their drinking water and WT receiving aspartame drinking water win equal amounts of matches ($p > 0.1$ for all days, $n = 6$ per group). Data shown as mean percentage $\pm$ sem. P-values: ** < 0.001 , * < 0.01 , ^ < 0.05

2.3. Chronic drug administration

Mice received bumetanide (Centrapharm) dissolved in drinking water in a concentration of 0.01 mg/ml, based on ³⁸, and kept in light-tight bottles. Aspartame was added to reduce the bitter drug taste. Control mice received aspartame drinking water. Mice were chronically treated from weaning at postnatal week 4 until the end of the experiment, postnatal week 13-16.

2.4. Statistical analysis

All ATT tests were performed according to a randomized block design with a minimum of two blocks, with exception of the control experiment described in Figure 1C. The outcome per mouse of each match was designated as winning or losing. The total number of wins per group was summed up and the percentage of matches won was calculated. A binomial distribution test was used to determine a significant difference between the match outcome and the null hypothesis: in an experiment, both groups are similar if approximately 50% of matches are won per group. To analyze the intrinsic validity of the test, the stability of the test outcome across the week was calculated. A stability > 70% compared to the previous day from day 3 compared to day 2, was considered stable. A high stability is important to exclude influence of external factors during the experiments.

3. Results

For all experiment, there was no difference in weight of the mice and number of assistant air puffs (data not shown). The stability was high for all experiments, i.e. the percentages of matches with a similar outcome compared to the previous day was above 70% from day 3 compared to day 2 (supplementary Figure S1).

3.1. Dominant phenotype in ATT in *Fmr1* KO mice could be partially restored by chronic treatment with bumetanide

Previously, we have shown that *Fmr1* KO mice display a robust dominant ATT phenotype compared to WT littermates, resulting in significantly increased percentage of matches won by *Fmr1* KO mice ²⁰. This phenotype is similar for mice receiving aspartame drinking water (supplementary Figure S2). To explore whether the *Fmr1* KO ATT phenotype can be reversed by chronic bumetanide treatment, we treated *Fmr1* KO mice with bumetanide in their drinking water from weaning until the end of experiment and tested them against their WT or *Fmr1* KO littermates receiving aspartame drinking water (Figure 1A and 1B). In the matches of *Fmr1* KO treated with bumetanide against WT Littermates

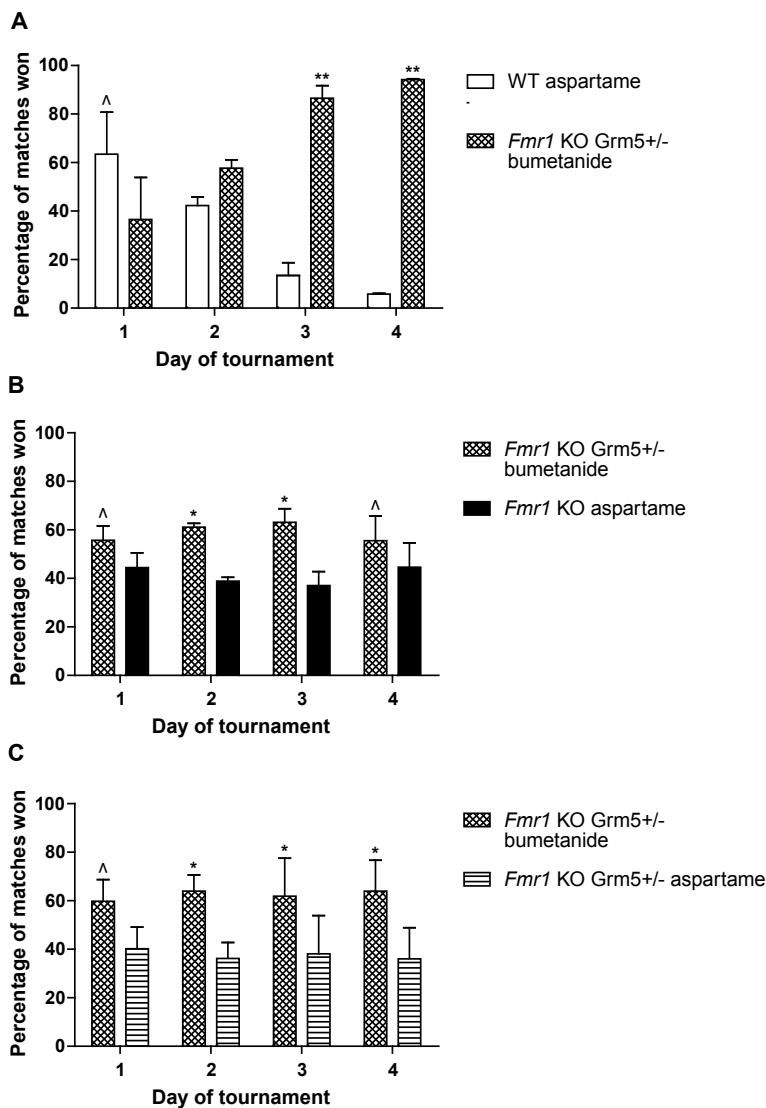


Figure 2: Percentage of matches won by double mutants *Fmr1* KO Grm5^{+/-} mice treated with aspartame or bumetanide and single mutant *Fmr1* KO or WT littermates treated with aspartame drinking water. Combination of genetic mGluR5 reduction and bumetanide (Figure 2: continued) treatment results in a slight worsening of the ATT phenotype compared to mGluR5 reduction alone. (A) *Fmr1* KO who are Grm5^{+/-} and treated with bumetanide lose most matches against WT receiving aspartame drinking water on day 1 ($p=0.02$) but win most matches on day 3 and 4 ($p<0.001$, $n=10$ mice per group). (B) *Fmr1* KO mice who are Grm5^{+/-} and treated with bumetanide win slightly more matches than

Fmr1 KO receiving aspartame drinking water ($p=0.04$ on day 1 and 4 and $p<0.01$ on day 2 and 3, $n=18$ mice per group). (C) *Fmr1* KO who are *Grm5*^{+/-} and treated with bumetanide win slightly more matches than *Fmr1* KO *Grm5*^{+/-} receiving aspartame drinking water ($p=0.01$ to $p=0.002$, $n=17$ mice per group). Data shown as mean percentage $\pm$ sem. P-values: ** <0.001 , * <0.01 , ^ <0.05

receiving aspartame drinking water, the *Fmr1* KO mice treated with bumetanide won the majority of the matches, starting with 65% on day 1 ($p<0.01$) and increasing to above 90% on day 3 and 4 ($p<0.001$, $n=12$ per group, Figure 1A). However, *Fmr1* KO that received aspartame drinking water, won most matches from *Fmr1* KO littermates treated with bumetanide (Figure 1B): 67% of matches on day 2 ($p<0.01$, $n=12$ per group) and increasing to 82% on day 4 ($p<0.001$). This points to a partial rescue of the *Fmr1* KO phenotype after bumetanide treatment. Tournaments between WT mice treated with bumetanide against WT littermates receiving aspartame drinking water, showed no difference between treated and untreated WT littermates (Figure 1C, $n=6$ per group). To summarize, bumetanide treatment reduces the ATT phenotype of *Fmr1* KO mice compared to their untreated *Fmr1* KO littermates receiving aspartame drinking water, but not compared to their WT littermates receiving aspartame drinking water. Importantly, bumetanide treatment does not affect WT animals.

3.2. Double treatment using oral bumetanide in combination with genetic reduction of mGluR5 does not result in an effective treatment

As was demonstrated by ²⁰, the ATT phenotype could be partially reversed after genetic reduction of mGluR5 in *Fmr1* KO mice (double mutants *Fmr1* KO *Grm5*^{+/-}) as well as by pharmacological reduction of the mGluR5 activity using an mGluR5 antagonist. To test if we could reach a higher response in *Fmr1* KO mice for the ATT phenotype using a combination therapy, we treated double mutants (*Fmr1* KO *Grm5*^{+/-}) with bumetanide and compared them to their WT or *Fmr1* KO littermates receiving aspartame drinking water. *Fmr1* KO *Grm5*^{+/-} mice treated with bumetanide lost most matches on day 1 (37% of matches won, $n=10$ mice per group, $p=0.02$, Figure 2A). On day 2 they started winning more matches, however, this difference was not significant. On day 3 and 4 *Fmr1* KO mice that were *Grm5*^{+/-} and treated with bumetanide, won almost all matches from their WT littermates receiving aspartame drinking water (>85% of matches, $p<0.001$, Figure 2A). *Fmr1* KO mice that were *Grm5*^{+/-} and treated with bumetanide won slightly, but significantly, more matches than *Fmr1* KO mice receiving aspartame drinking water (Figure 2B), winning between 55% and 63% of the matches ($p=0.04$ to $p<0.01$, $n=18$ mice per group).

To further investigate to what extent bumetanide changes the effect of genetic reduction of mGluR5 in terms of social behavior in the ATT, we performed tournaments between *Fmr1* KO mice that are *Grm5*^{+/-} and treated with bumetanide (i.e., combination therapy) against *Fmr1* KO that are *Grm5*^{+/-} and received aspartame

drinking water (i.e. single therapy). *Fmr1* KO mice that were *Grm5*^{+/-} and treated with bumetanide won slightly, but significantly, more matches than *Fmr1* KO mice that were *Grm5*^{+/-} and received aspartame drinking water, winning 58% to 62% of the matches ($p=0.01$ to $p=0.002$, $n=17$ mice per group, Figure 2C).

In summary, a delayed phenotype was seen in matches between *Fmr1* KO mice that were *Grm5*^{+/-} and received bumetanide drinking water, against WT littermates receiving aspartame drinking water. In contrast to what we observed for bumetanide treatment or genetic reduction of mGluR5 separately, combining both resulted in a small but significant worsening of the ATT phenotype in *Fmr1* KO mice compared to *Fmr1* KO mice receiving aspartame drinking water. Also comparing *Fmr1* KO mice that were *Grm5*^{+/-} and treated with bumetanide to their double mutant littermates that received aspartame drinking water, showed a small but significant worsening of the ATT phenotype when combining both genetic mGluR5 reduction and bumetanide treatment.

4. Discussion

In FXS, intellectual disability is often accompanied by autistic behavior and social deficits, leading to major disabilities. Aberrant social behavior in mouse models of FXS is one of the important features of FXS that is being evaluated when testing efficacy of potential pre-clinical therapeutic interventions. In this paper, we are the first to present the effect of chronic treatment with bumetanide, an indirect GABAergic enhancer, on social behavior in FXS. We demonstrated that chronic treatment of *Fmr1* KO mice with bumetanide partially reverses the FXS phenotype, using a robust social behavioral paradigm, the ATT. This indicates that treatment with bumetanide has a beneficial effect on social behavior in FXS patients. Previously, we have demonstrated a similar partial rescue in the ATT, with genetic as well as pharmacological reduction of mGluR5 activity²⁰. In the present study we tested if a combination therapy of oral bumetanide and genetic reduction of the mGluR5 in *Fmr1* KO mice would increase efficacy of treatment. We did not observe a synergistic effect of this combination therapy, but instead we observed a slight worsening of the ATT phenotype in *Fmr1* KO mice. Both the excitatory mGluR5 as well as the inhibitory GABAergic pathway, have been extensively studied in *Fmr1* KO mice and have been established to play a major role in the pathogenesis of FXS. For a review, we refer to Braat *et al* and Ligsay *et al*^{8,31}. Compelling evidence has demonstrated that aberrant synaptic plasticity in FXS is (partly) caused by an excitatory-inhibitory imbalance, due to malfunctioning of these pathways^{8,31}. In this study, we first targeted the GABAergic pathway using the commercially available diuretic bumetanide, an Na⁺-K⁺-2Cl⁻-co-transporter 1 (NKCC1) blocker, which reduces intracellular chloride levels in neurons, including specific excitatory GABAergic neurons. While the GABA_A receptor inhibits depolarization in adult neurons, its function in immature neurons during early development is excitatory, switching to inhibitory while the neurons mature. This important neurodevelopmental switch depends on the intracellular chloride levels,

regulated by the chloride importer NKCC1^{29,39} and has been shown to be delayed or absent in *Fmr1* KO mice^{38,40} and FXS derived human embryonic stem cells⁴¹. Reduction of chloride levels with bumetanide, forces the neuron to switch from immature to mature chloride concentrations and consequently also to mature GABAergic function²⁹. This has been demonstrated by bumetanide treatment of pregnant mice, which restored electrophysiological and behavioral phenotypes in their *Fmr1* KO offspring³⁸. Since we administered bumetanide after this developmental switch, the improvement we measure is encouraging in terms of treatment initiation later in life. However, the neurochemistry changes underlying remains to be elucidated.

A delayed developmental GABAergic switch from excitatory to inhibitory, has also been implicated in other disorders than FXS, including autism²⁹, epilepsy⁴², Parkinson's disease⁴³ and schizophrenia⁴⁴. Bumetanide has been tested in clinical trials with patients with autism. An open label clinical trial with bumetanide in 5 children with ASD and a randomized placebo controlled trial in 60 children with ASD, showed improvement in behavioral scores after bumetanide treatment^{45,46}. An open-label trial with bumetanide in adolescents with ASD showed improved emotional recognition, sensory perception, neuropsychological testing and activation of brain regions involved in facial perception (e.g. inferior occipital cortex and fusiform cortex), emotional processing (e.g. nucleus accumbens and amygdala), social processing (e.g. superior temporal cortex) and attentional processing (e.g. intraparietal sulcus and superior parietal lobule) using fMRI⁴⁷. In addition, combination of bumetanide treatment with educational treatment (n=28) was more efficient than educational treatment only in children with ASD (n=32)⁴⁸. A recent clinical trial with 80 pediatric patients showed a significant improvement of several behavioral scales⁴⁹. Since bumetanide treatment is promising in patients with autism, it is plausible that it might be beneficial to treat social behavior deficits in FXS patients as well. So far, only one case-report has been published on an open label bumetanide trial with one FXS patient. In this patient, bumetanide treatment showed improvement in several autism severity and behavioral scales⁵⁰.

Since only partial correction in the ATT was observed for bumetanide treatment or genetic mGluR5 reduction alone²⁰, we hypothesized that targeting both pathways simultaneously, a combination therapy, might lead to a complete correction. Interestingly, the partial rescue that we observed for both treatments separately is lost when they are combined, even leading to a slight worsening. This illustrates that in this case, combining two targets attenuates their therapeutic efficacy on FXS social behavior deficits. It has been previously suggested by⁵¹ that synaptic plasticity is a tightly regulated process. The authors demonstrated an optimal window for protein synthesis levels. Deviations to either side of this optimum result in decreased functioning of the synapse and aberrant synaptic plasticity. Considering this optimum, our results might be explained by either an opposing effect of bumetanide and mGluR5 reduction, or by overshooting the effect of one treatment when combined with the second. In both cases, this means that restoring this tightly regulated balance will need tremendous

fine-tuning. Unfortunately, it is poorly understood how the mGluR5 and GABAergic pathways are interconnected at the synapse and no biochemical read-out is available to test whether the right balance has been reached ^{52,53}. To complicate matters, the required balance might be significantly different in different brain regions or even synapse-specific within one neuron, since FMRP is not localized in every spine ^{54,55}. The translation of preclinical to clinical studies is challenging in many biomedical fields ⁵⁶. Also in the FXS field, major limitations have been encountered, including the lack of reliable and robust outcome measures ^{33,34}. Our study shows that the ATT is a robust social behavior paradigm for the FXS mouse model and that it can be used to test efficacy of drugs. The ATT is a readout for specific social behavior deficits, probably related to autistic behavior, although the exact brain regions involved are unknown. Previous studies have demonstrated social deficits in *Fmr1* KO mice, in different versions of the tube test ^{16,57,58}. Possibly, the robustness of the ATT might also be its drawback in drug-testing. The test outcome of each match is binomial, by default excluding subtle outcomes. Moreover, a winner and loser effect exists, stimulating a winner mouse to win more and a loser mouse to lose more during the tournament week ²⁰. Over time, the phenotype tends to become stronger, often almost black and white, leaving no space for subtle effects. For these reasons, albeit the robustness of the ATT and its usability in FXS pre-clinical studies, it is possible that a complete correction of this endpoint will not be feasible.

In this study, we only used one specific social behaviour paradigm, which does not inform us on the effect on other FXS behavioural and cognitive phenotypes. In addition, it is not known whether the glutamatergic-GABAergic balance is the most relevant for this specific ATT phenotype. Possibly, other pathways are more important and targeting those will result in a beneficial effect. Even though we did not find a beneficial synergistic effect in this case, it seems plausible that targeting only one pathway is not sufficient to ameliorate FXS completely. We are only starting to grasp the complex role of FMRP in synaptic plasticity. FMRP binds many post-synaptic mRNAs, all of which are involved in important neuronal synaptic pathways. Moreover, FMRP does not only have a postsynaptic function, but is also present in the presynaptic compartment and other cell types ^{12-15,57,59}. Recently, a missense mutation in *FMR1* has been identified in a patient, demonstrating a specific function of FMRP in the presynaptic compartment ⁵⁹. The patient only displayed ID and seizures, but not the behavioral problems associated with FXS, suggesting different pathways in different cellular compartments might be associated with specific FXS symptoms and phenotypes. Additionally, FMRP is present in other non-neuronal cell-types, where its function is even less understood ¹³⁻¹⁵. For example, compelling evidence demonstrates the role of astrocytes, not only in neuronal maintenance, but also in active control of synaptic function, leading to the new concept of the tripartite synapse ⁶⁰. FMRP is present in the astrocytes, and its absence has been demonstrated to hamper astrocyte function, opening a new field of possible therapeutic strategies.

Despite the multitude of involved pathways, practically all studies so far are focused on targeting one of the pathways separately. Since none of the identified drugs in pre-clinical studies have led to substantial improvement of the disease in humans in clinical trials ³¹, the question has been raised whether these observed pre-clinical treatment effects reflect a relevant and versatile treatment strategy. Possibly, all these studies oversimplify the disease by targeting only one pathway. Our study is one of the first studies to test a combination of targeted treatments in FXS and the first to test chronic treatment. Only two previous publications have studied combination therapy before. However, these studies used acute treatment and did not address social behavior. Lim *et al* observed a synergistic beneficial effect on synaptic plasticity and behavior when targeting serotonin and dopamine-pathways in *Fmr1* KO mice simultaneously ⁶¹. Pacey *et al* showed an additional synergistic effect of acute targeting of mGluR5 (MPEP) and GABAb (R-baclofen) in *Fmr1* KO mice on seizures, both in a lower dose than needed when administered separately ⁶². One case-report mentioned combination therapy with two drugs in combination with intensive educational treatment in 2 children with FXS, resulting in improvement of cognition and behavior ⁶³. Other research fields have a longer history of combining targeted treatments to improve therapy. For example, studying the complex genetics of cancer has led to the identification of key-oncogenic cellular pathways, enabling the use of a combination of targeted pharmacological treatments to selectively block and kill tumor cells ⁶⁴. However, these settings often have access to high throughput study models in cell culture and well-defined outcome measures, which are lacking in neurodevelopmental research. In recent years, combination therapy in neurodevelopmental syndromes has been proposed, for example in Rett syndrome ⁶⁵. In the near future, a clinical trial treating FXS patients with a combination of lovastatin and minocycline will start (NCT02680379). New pre-clinical studies are needed to further evaluate the role of FMRP in other cell-types and to investigate whether combination therapy is the key solution for FXS treatment, by targeting multiple pathways in different cellular compartments or cell-types.

In conclusion, we show that chronic treatment with bumetanide results in a beneficial effect on the ATT phenotype in *Fmr1* KO mice. This illustrates the potential of bumetanide as a new treatment for specific social deficits in patients with FXS. In addition, the present study is the first example to investigate chronic combination therapy in *Fmr1* KO mice. Although we could not demonstrate a synergistic beneficial effect in the present study by targeting both the excitatory and inhibitory pathways simultaneously, we believe it is essential to target the complexity of the pathophysiology of FXS with combination therapy. We presume that, in the near future, this approach will become more successful by targeting multiple pathways involved in FXS, using other drugs or doses, or by targeting pathways in different cellular compartments or cell-types.

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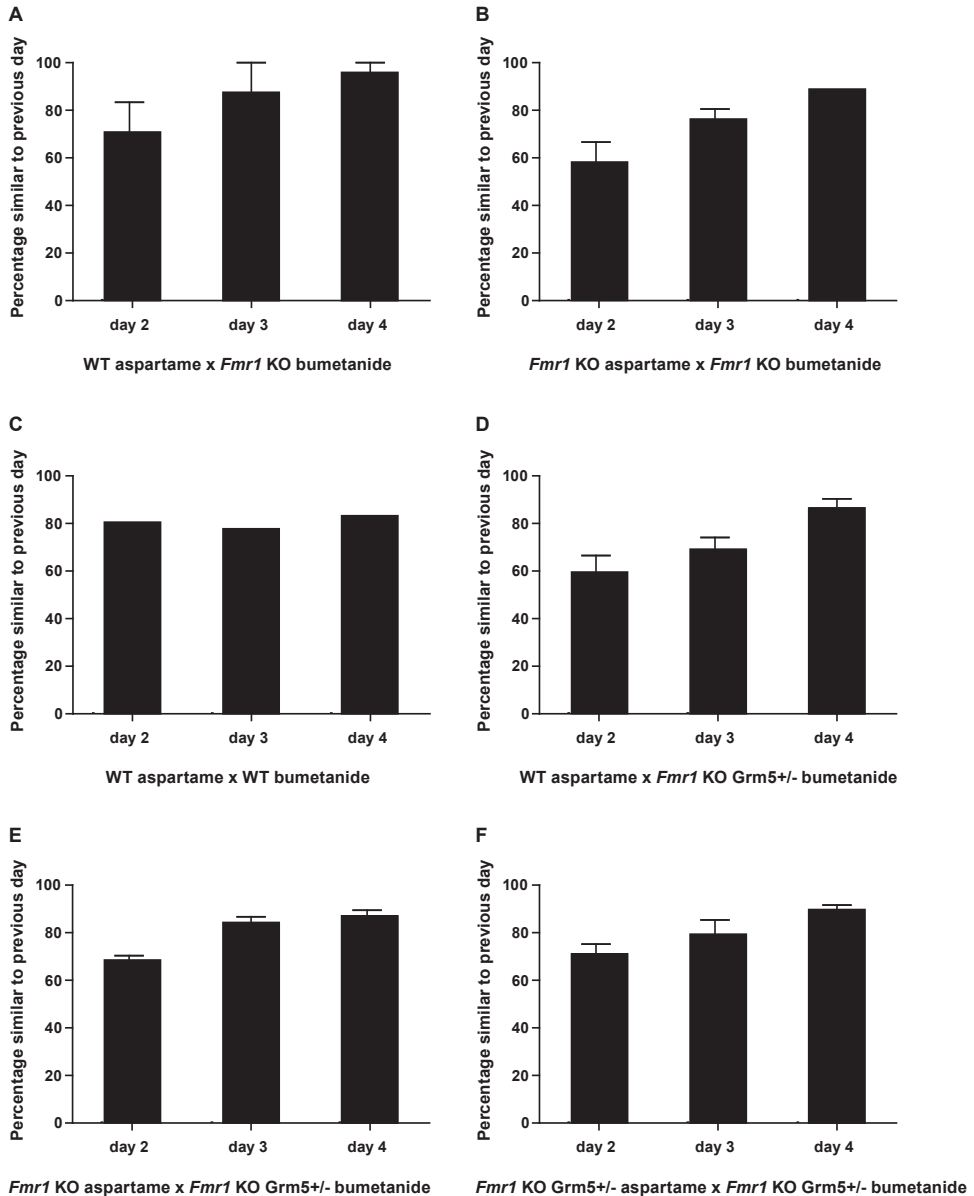
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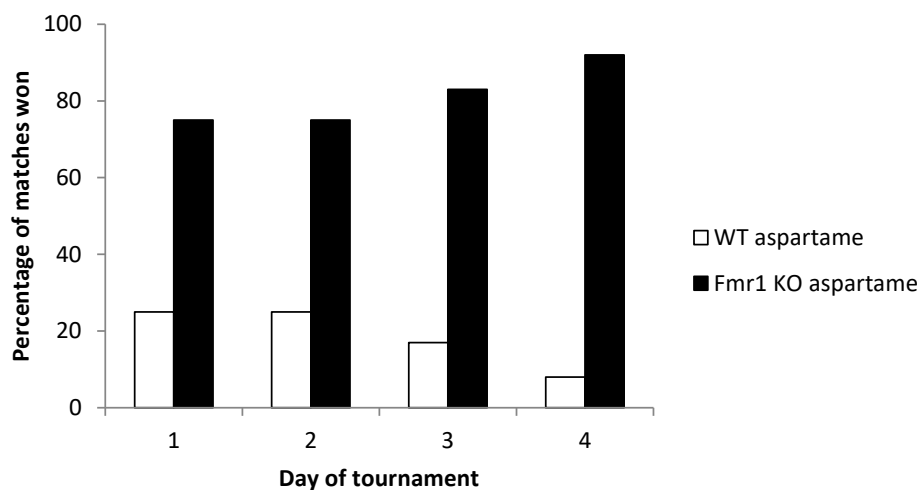
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Supplementary data

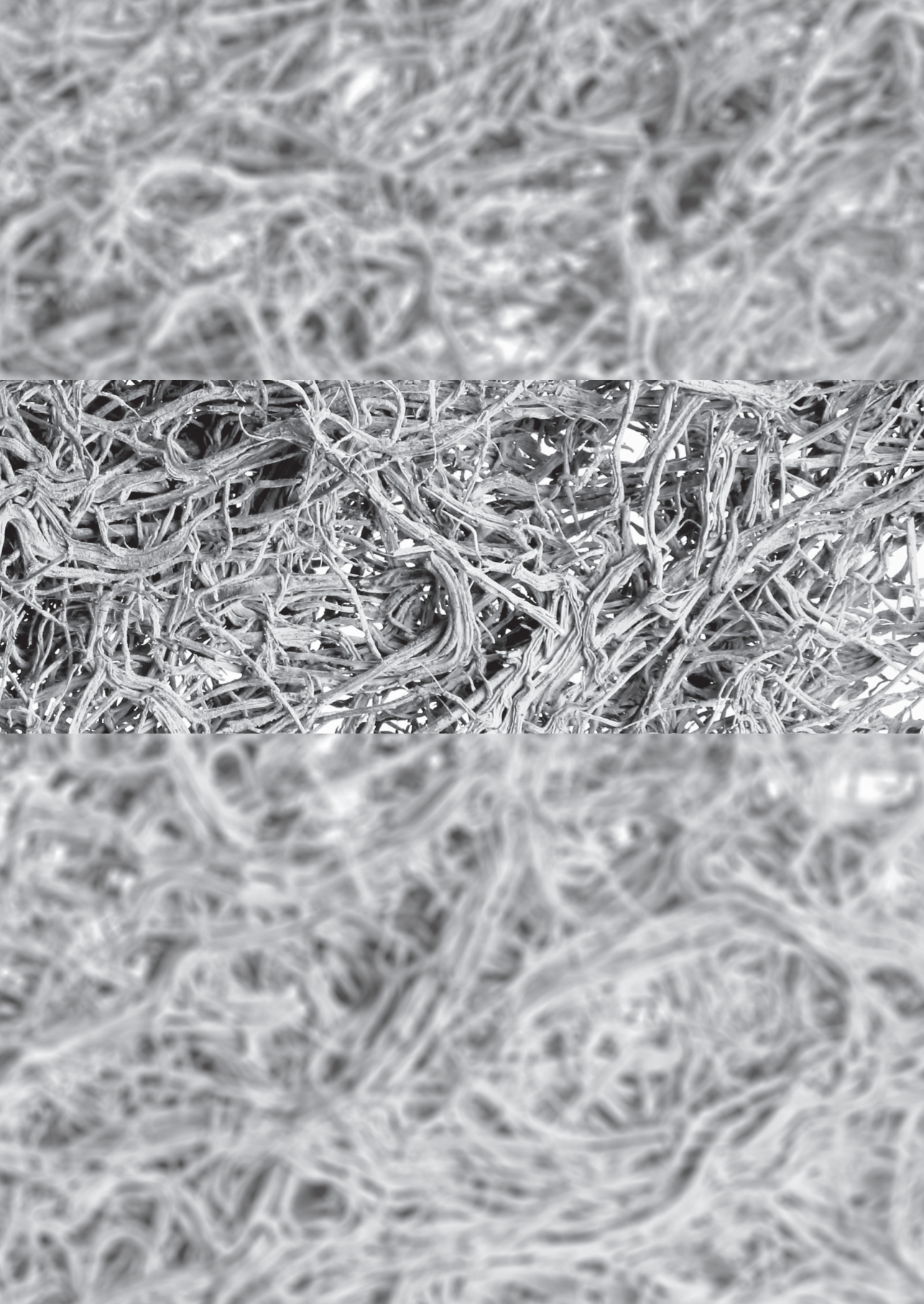


Supplementary Figure S1: Stability of wins expressed as percentage of matches that have a similar outcome compared to the previous day. (A) Tournaments between WT littermates receiving aspartame drinking water and *Fmr1* KO mice treated with bumetanide. (B) Tournaments between *Fmr1* KO mice receiving aspartame drinking water and *Fmr1* KO mice

(Figure S1: continued) treated with bumetanide. (C) Tournaments between WT littermates receiving aspartame drinking water and WT littermates treated with bumetanide. (D) Tournaments between WT littermates receiving aspartame drinking water and Fmr1 KO mice that are heterozygous for mGluR5 and treated with bumetanide. (E) Tournaments between Fmr1 KO mice receiving aspartame drinking water and Fmr1 KO mice that are heterozygous for mGluR5 and treated with bumetanide. (F) Tournaments between Fmr1 KO mice that are heterozygous for mGluR5 and receive aspartame drinking water and Fmr1 KO mice that are heterozygous for mGluR5 and treated with bumetanide.



Supplementary Figure S2: control experiment of WT treated with aspartame against Fmr1 KO treated with aspartame. Fmr1 KO mice receiving aspartame drinking water display a dominant phenotype over WT littermates receiving aspartame drinking water ($p=0.002 - <0.001$). $N = 6$ mice per group.



CHAPTER 3

Paradoxical effect of baclofen on social behavior in the FXS mouse model

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Submitted

1. Introduction

Fragile X syndrome (FXS) is a major X-linked cause of intellectual disability (ID) and autism spectrum disorders (ASD). Features include developmental delay with an average IQ of 40, deficits in executive functioning and a wide range of psychiatric problems like repetitive behavior, social deficits, hyperactivity, anxiety and irritability¹⁻³. In addition, FXS can be accompanied by medical problems, including epilepsy, hyperlaxity and cardiac disorders^{1,4}. Currently, there is no disease modifying therapy available. In 1991, the most common genetic cause of the disease was identified as a CGG repeat expansion in the 5′ untranslated region of the *FMR1* gene on Xq27.3, leading to hypermethylation of the promotor region and subsequent silencing of the gene⁵⁻⁷. Silencing of transcription of the *FMR1* gene results in absence of its protein product, the fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein that binds approximately 4% of the total brain mRNA. Many of these mRNAs are coding for proteins important in neuronal connectivity and synaptic plasticity, including PSD95, SAPAP1-3, α -CaMKII, Arc/Ar3.1, Shank1⁸⁻¹⁰. FMRP plays a significant role in transporting these specific mRNAs to the postsynaptic compartment and subsequent local translational control^{11,12}. In *Fmr1* knock-out (KO) mice, absence of FMRP has been shown to result in aberrant synaptic plasticity by disrupting the metabotropic glutamate receptor type 5 (mGluR5) pathway^{13,14}. The *Fmr1* KO mouse model displays many phenotypes similar to FXS patients, including ASD-like behaviors (social behavioral deficits, repetitive behavior), hyperactivity, anxiety and audiogenic seizures, although many of these phenotypes vary depending on the genetic background of the mouse, the age of the mice and the test protocol¹⁵⁻¹⁸.

As many FXS patients also manifest sleeping disorders, anxiety and seizures, which are related to the inhibitory neuronal function, involvement of the brain's major inhibitory γ -aminobutyric acid (GABA) pathway has been proposed as well¹⁹. Both the ionotropic GABA_A and the metabotropic GABA_B receptors have been implicated in FXS and ASD. Studies have demonstrated decreased expression of several GABA_A receptor subunits and proteins involved in the metabolism of the GABA neurotransmitter¹⁹⁻²². The GABA_B receptor acts postsynaptically by hyperpolarization of neurons. Presynaptically, GABA_B receptor activation leads to reduced glutamate release, resulting in reduced mGluR5 receptor activation and downstream protein synthesis. The combination of hyperactive glutamatergic signaling and the decrease of GABAergic signaling might lead to overall hyperexcitability in FXS, due to an imbalanced excitatory-inhibitory network function²³. While many studies have focused on targeting the mGluR5 pathway in the development of a treatment for FXS²⁴⁻²⁶, several studies have now attempted to improve FXS related symptoms by correcting this reduced GABAergic activity. These were performed using baclofen, R-baclofen or arbaclofen. Baclofen is a structural analog of GABA, acting as agonist of the GABA_B receptor. It consists of the potent R- and the less potent S-enantiomer, in equal amounts^{27,28}. Arbaclofen (STX209) is the more effective R-enantiomer of baclofen²⁹. The effect of arbaclofen has been tested in *Fmr1* KO mice^{22,30-33}. Acute

arbaclofen treatment corrected several *Fmr1* KO phenotypes, including AMPA receptor internalization, increased protein synthesis in hippocampal neurons, audiogenic seizure susceptibility, abnormal spine morphology features, and repetitive and hyperactive behavior in *Fmr1* KO mice³¹. Acute R-baclofen injection showed improvement in social deficits in a version of the three chamber sociability test and hyperactivity³². In addition, acute baclofen treatment improved sensory hypersensitivity, electrophysiology, social behavior and hyperactivity measures³³. The above mentioned results prompted the initiation of a randomized double-blind placebo controlled clinical trial with arbaclofen in 63 children and adults with FXS, resulting in improvement in post-hoc analysis of the most severely affected patients (n=27)³⁴. However, the subsequent larger phase 3 clinical trial with arbaclofen has been terminated prematurely, due to lack of efficacy³⁵.

The assumed involvement of GABA_B in social behavior and the fact that chronic treatment with baclofen has not been evaluated on social behavior in *Fmr1* KO mice yet, prompted us to evaluate chronic baclofen treatment in two social behavioral paradigms: the automated tube test and the three chamber sociability test. Unexpectedly, both tests show a paradoxical effect of chronic baclofen treatment on social behavior in *Fmr1* KO mice, worsening their phenotype. In addition, treatment of wild type (WT) mice with baclofen induced aberrant social behavior in these mice, which is similar to the FXS phenotype. Altogether, the latest results of the clinical trial and our current results indicate that baclofen might be questionable as targeted treatment for FXS.

2. Materials and methods

2.1. Animals

Fmr1 KO mice and their WT littermates were used, previously generated as described earlier³⁶. Only males were used, because of the X-linked nature of FXS and because females are known to have a different phenotype in one of the behavioral tests³⁷. Since housing influences the automated tube test phenotype³⁸, animals used in the automated tube test were housed individually after weaning, 3-4 weeks postpartum. For the three chamber sociability test, animals were group-housed with 3-4 males per cage. Stranger mice, that are used in the three chamber sociability test, were male age-matched adult animals of a C57BL/6JRj background (Elevage Janvier, Le-Genest-Saint-Isle, France). The age of test animals during experiment was between 12 and 16 weeks, when mice reached young adulthood. The mice were weighed and mice that lost more than 10% of their weight were excluded. Genotyping was performed on toe-clip at 1 week of age and repeated for validation of the genotype on toe or ear tissue obtained after the behavioral experiments. Animals were kept under standard laboratory conditions with food and water ad libitum and 12 hours light-dark cycles. Experiments were performed during the light phase at a fixed timeslot. All experiments were approved by the local animal welfare committee.

2.2. Behavioral tests

2.2.1. Automated tube test

The automated tube test, adapted from the original tube test³⁹, has been developed to measure hierarchy and social dominance and is often used in ASD research³⁷. We performed the test according to the protocol described by Van den Berg *et al* and De Esch *et al*^{37,38}. In short, we used the automated tube test apparatus from Benedictus b.v., a tube made of transparent Plexiglas with a starting box on each side. The experiment is preceded by 5 days of training and 2 days of rest. The experimenter was blinded for genotype and treatment. Mice were identified using a color code on the tail. Mice were placed in one of the side-boxes (starting box), and motivated to walk to the other side (end box). The first day, they had to reach the end box within 180 seconds, the next days within 30 seconds. On day 2 to 4, the training was repeated for 3 times on each side. If mice did not leave the starting box within 5 seconds, a negative stimulus, an air puff, was administered, until the tube was entered. After the training week, a tournament week started. Each day started with two training trials. During matches, the designated mice were placed in the starting boxes and the doors were opened. The center door opened when both mice were in its proximity. The mouse that entered its starting cage with all four paws was designated as the loser, while the other mouse was the winner. The group that wins most matches, is designated as dominant. All matches were repeated on day 2-4 of the tournament week in a different order and a reversed starting side.

2.2.2. Three chamber sociability test

The three chamber sociability test has been developed to measure social interaction and interest for an unfamiliar mouse compared to an object (empty cage), and is often used in research on neuropsychiatric diseases like ASD.^{40,41} The protocol is fully described in Gantois *et al* (2013)⁴², which is a modified version of a previously described setup⁴⁰. In short, the setup consists of a three chamber transparent Plexiglas cage, divided in a center, left and right chambers. The left and right chambers contain a wired cylindrical cage where a mouse previously unknown to the test mouse, the stranger mouse, can be placed. The set-up contains two cameras, connected to the ANY-maze™ Video Tracking System software (Stoelting Co., IL, USA). Prior to the experiment, animals were handled to accustom them to the experimenter and room for 2 weeks. On the day of the experiment, animals were acclimated to the room for at least 30 minutes. Just before the test, each mouse was habituated to the center chamber for 5 minutes, followed by 5 minutes of the sociability test with a stranger mouse placed in the wired cage in the left or right chamber. In the other chamber, an empty cage was placed. The doors were opened and the mouse could explore all three chambers. The side where the stranger mouse is placed, was evenly distributed between genotypes, treatments and time of day. The movements of the mice were tracked automatically, including time

spent in chamber. Time in direct contact with the stranger mouse or the empty cage, i.e. time spent sniffing, was manually scored by the experimenter. The experimenter was blinded for genotype and treatment during the experiment.

2.3. Locomotor activity

Locomotor activity was automatically measured using the ANY-maze_{TM} Video Tracking System software during the acclimatization and sociability phase of the three chamber sociability test.

2.4. Drug treatment

Mice were treated with baclofen (Bufa/Spruyt-Hillen B.V.) in their drinking water. Aspartame was added to reduce the bitter taste in a concentration of 0.1%. Control water contained aspartame only. Drinking water was refreshed three times a week. It has been previously shown that baclofen crosses the blood-brain barrier 43 and that 0.5 mg/ml of arbaclofen, a the R-baclofen enantiomer, reaches acceptable pharmacokinetics and treatment effects in *Fmr1* KO mice 31. Racemic baclofen consists of both the pharmacologically active R- and the inactive S-enantiomer, in equal amounts 27,28. For that reason a dose of 1 mg/ml baclofen was used. Based on the increased locomotor activity that was measured in this dose of baclofen, we later reduced the dose to 0.25 mg/ml. *Fmr1* KO mice and WT littermates were treated with baclofen or aspartame drinking water from weaning until the end of an experiment.

2.5. Statistical analysis

For the automated tube test, the outcome of each match per mouse was designated as winning or losing and the total number of wins per group was summed up and presented as the percentage of matches won from the total amount of matches. A binomial distribution test was used to determine a significant difference between the total match outcome and the null hypothesis: if both groups are similar, around half of matches are won by each group. To analyze the intrinsic validity of the test, the percentage of matches with a similar outcome to the previous day (i.e., the stability of the test outcome) was calculated (data not shown). A stability > 70% compared to the previous day was considered stable.

The three chamber sociability test data was analyzed with statistical software (SPSS for Windows, IBM, NY, USA). Overall effects were evaluated with Repeated Measures Analysis of Variance (RM-ANOVA), as previously described by Gantois *et al*⁴². Genotype

(*Fmr1* KO and WT littermates) and treatment (baclofen with aspartame and aspartame only) were included as between-subject factor, and stranger side (empty and stranger) was included as within-subject factor. Data are presented as mean $\pm$ standard error (SE). For the locomotion analysis, differences between concentrations were calculated with a 2-sided T-Test and overall difference was calculated with univariate ANOVA.

3. Results

3.1. Chronic baclofen treatment (1 mg/ml) worsens the social behavior phenotype in *Fmr1* KO mice and induces aberrant social behavior in WT littermates

3.1.1. Automated tube test

As a start we performed a control experiment to determine if aspartame in the drinking water affects the outcome of the ATT. These experiment show that *Fmr1* KO mice receiving control aspartame water win most of the matches over their WT littermates receiving control aspartame water (supplementary Figure S1). This is indicated as a dominant phenotype, which is similar to the previously published phenotype without aspartame drinking water ³⁸.

To test whether baclofen treatment affects the dominant *Fmr1* KO automated tube test phenotype, we treated *Fmr1* KO mice with 1 mg/ml baclofen and compared them to WT littermates receiving aspartame drinking water only. Each experiment contained two groups of 6 mice, resulting in a total of 36 matches per day. We show that *Fmr1* KO mice treated with 1 mg/ml baclofen win most matches from WT littermates receiving aspartame water (Figure 1A). They won 80% of the matches on day 1, which increases to over 90% on day 4 ($p < 0.001$). In addition, *Fmr1* KO mice treated with baclofen became dominant over *Fmr1* KO mice receiving aspartame drinking water (Figure 1B), winning more than 80% of the matches ($p < 0.001$). The control experiment of WT littermates receiving aspartame drinking water versus WT littermates treated with baclofen, shows that WT mice treated with baclofen (1 mg/ml) become dominant, by winning more than 90% of the matches ($p < 0.001$) (Figure 1C).

3.1.2. Three chamber sociability test

The time a mouse spent in direct contact with and sniffing the cage where the stranger mouse was placed, or the empty cage, was manually recorded by the experimenter and is depicted in Figure 1D. This measure is expressed as sniffing time, and is an indication for sociability^{15,40}. All groups sniffed the stranger mouse significantly more than the empty cage (two-way RM ANOVA with within-subject factor side of stranger, $p < 0.001$). *Fmr1* KO mice receiving aspartame drinking water show an increased sociability phenotype, i.e., sniff the stranger mouse more than WT littermates receiving aspartame drinking water (RM ANOVA with stranger side as within subject and genotype as between-subject factor, $p < 0.01$). WT mice treated with baclofen showed increased time sniffing of the stranger mouse compared to WT mice receiving aspartame water ($p < 0.01$). This increased sniffing time was similar to average time sniffing of *Fmr1* KO mice receiving aspartame water. The average time sniffing the stranger was similar for *Fmr1* KO with and without baclofen treatment (RM ANOVA stranger side * treatment, $p > 0.1$).

The total time a mouse spent exploring the chamber with the stranger mouse or the empty cage, was automatically recorded by the software. Time spent in chamber is depicted in supplementary Figure S2A. Two-way RM ANOVA showed a significant stranger side effect for all groups ($p < 0.001$), i.e., all groups spent more time in the stranger room than the room with empty cage. *Fmr1* KO mice receiving aspartame drinking water spent more time in the stranger chamber than WT littermates receiving aspartame drinking water, however, this difference was not significant (two-way RM ANOVA stranger side * genotype $p = 0.075$). There was no difference in the time spent in the stranger chamber for *Fmr1* KO mice receiving aspartame and *Fmr1* KO mice receiving baclofen water (two-way RM ANOVA $p > 0.1$ for both concentrations). WT littermates receiving baclofen spent more time in the stranger room than WT littermates receiving aspartame water, however, this difference was not significant. 13 *Fmr1* KO mice and 13 WT littermates receiving aspartame drinking water, and 12 *Fmr1* KO mice and 11 WT littermates receiving baclofen drinking water, were used.

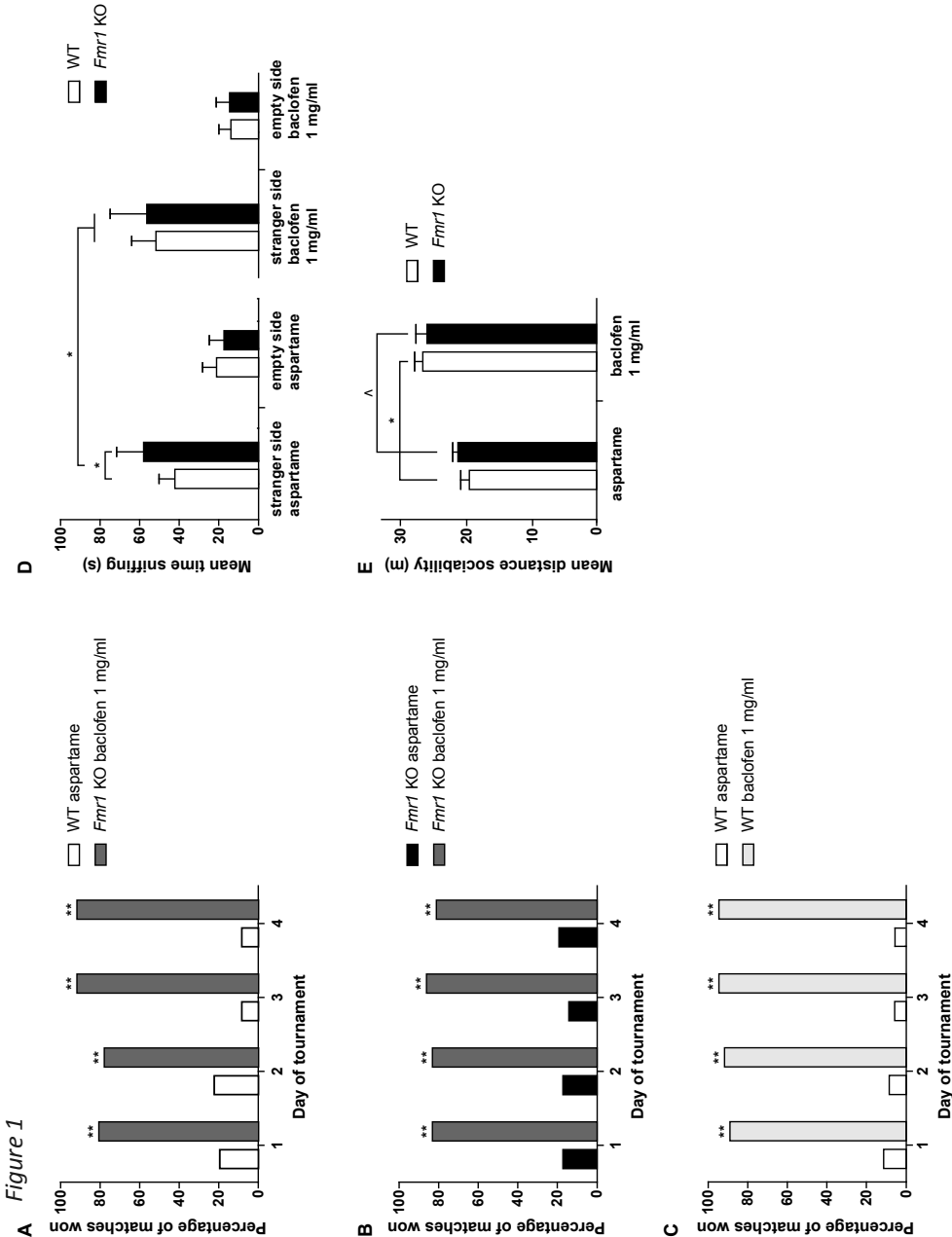


Figure 1: The effect of 1mg/ml baclofen treatment on the behavior of Fmr1 KO mice and wildtype littermates in the automated tube test and the three chamber sociability test. (A-C) Results of the automated tube test, expressed in percentage of matches won. (A) Fmr1 KO mice treated with baclofen win most matches against WT littermates receiving aspartame drinking water ($p < 0.001$, $n = 6$ per group). (B) Fmr1 KO mice treated with 1 mg/ml baclofen water win most matches against Fmr1 KO mice receiving aspartame water ($p < 0.001$, $n = 6$ per group). (C) WT littermates treated with 1 mg/ml baclofen win most matches against WT littermates receiving aspartame water ($p < 0.001$, $n = 6$ per group). (D) Sociability behavior, in the three chamber sociability test, expressed as average time spent sniffing the stranger mouse, compared to the time spent sniffing the empty cage. All groups sniff the stranger mouse more than the empty cage ($p < 0.001$). Fmr1 KO mice receiving aspartame water sniff the stranger mouse significantly more than WT littermates receiving aspartame water ($p < 0.01$). WT littermates receiving baclofen water sniff the stranger mouse more than WT littermates receiving aspartame water ($p < 0.01$), making baclofen-treated WT littermates comparable to Fmr1 KO mice receiving aspartame or baclofen. Data are expressed as mean $\pm$ SE; WT aspartame $n = 13$, Fmr1 KO aspartame $n = 13$ WT baclofen $n = 11$, Fmr1 KO baclofen $n = 12$. (E) Locomotor activity during sociability phase of the three chamber sociability test. WT and Fmr1 KO receiving baclofen drinking water show increased locomotor activity compared to WT and Fmr1 KO mice receiving aspartame water (2-sided independent samples T-test, $p = 0.001$ and $p = 0.034$, respectively) Data presented as mean $\pm$ SE. P-values: $^{\wedge} < 0.05$, $ < 0.01$, $** < 0.001$*

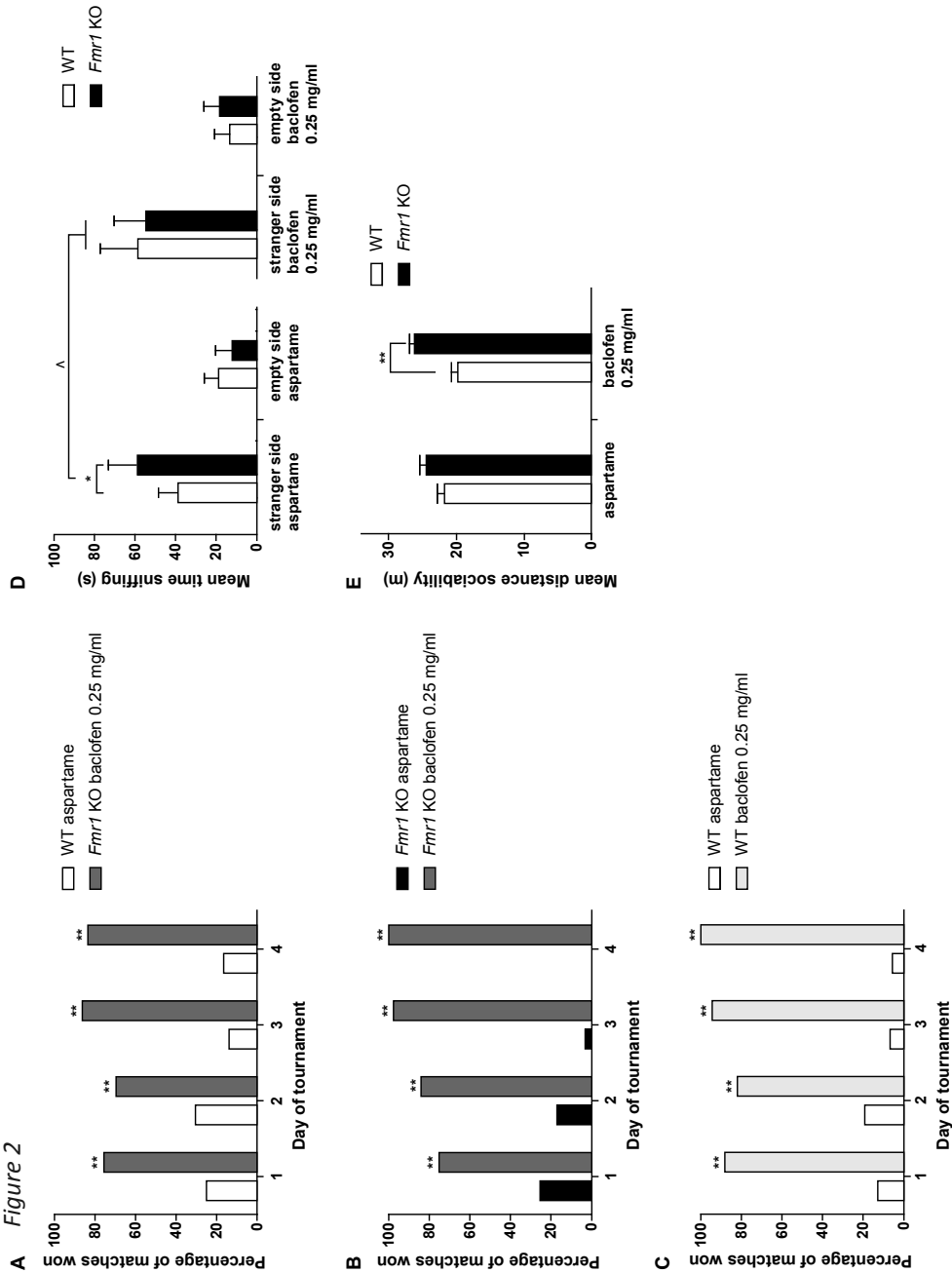


Figure 2: The effect of 0.25 mg/ml baclofen treatment on the behavior of Fmr1 KO mice and WT littermates in the automated tube test and the three chamber sociability test. (A-C) Results of the automated tube test expressed in percentage of matches won. (A) Fmr1 KO mice treated with 0.25 mg/ml baclofen win most matches against WT littermates receiving aspartame drinking water ($p < 0.001$, $n = 6$ per group). (B) Fmr1 KO mice treated with 0.25 mg/ml baclofen water win most matches from Fmr1 KO mice receiving aspartame water ($p = 0.002$ to $p < 0.001$, $n = 6$ per group). (C) WT littermates treated with 0.25 mg/ml baclofen win most matches from WT littermates receiving aspartame water ($p = 0.01$ to $p < 0.001$, $n = 4$ per group). (D) Three chamber sociability test. Sociability behavior, expressed as average time spent sniffing the stranger mouse, compared to the time spent sniffing the empty cage. WT aspartame $n = 10$, Fmr1 KO aspartame $n = 10$, WT baclofen $n = 10$, Fmr1 KO baclofen $n = 10$. All groups sniff the stranger mouse more than the empty cage ($p < 0.001$). Fmr1 KO mice receiving aspartame water sniff the stranger mouse significantly more than WT littermates receiving aspartame water ($p = 0.004$). WT littermates receiving baclofen water sniff the stranger mouse more than WT littermates receiving aspartame water ($p = 0.011$), making baclofen-treated WT mice comparable to Fmr1 KO mice receiving aspartame or Fmr1 KO mice receiving baclofen. Data are expressed as mean $\pm$ SE. (E) Locomotor activity during sociability phase of the three-chamber sociability test. Fmr1 KO mice receiving baclofen have increased locomotor activity compared to WT littermates receiving baclofen (2-sided independent samples T-test, $p < 0.0001$). Data presented as mean $\pm$ SE. P-values: $^{\wedge} < 0.05$, $^ < 0.01$, $^{**} < 0.001$*

3.2. Chronic baclofen treatment (1 mg/ml) increases locomotor activity

The question addressed in this paper was whether baclofen can reduce abnormal social behavior of *Fmr1* KO mice. Abnormal behavior in mouse models could be confounded with many environmental or intrinsic factors, for example, drug induced hyperactivity⁴⁴. Because baclofen treatment unexpectedly led to a worsening of the social behavior phenotype in *Fmr1* KO mice in the automated tube test and an induced phenotype in WT littermates in both behavioral tests, we analyzed the locomotor activity which is automatically recorded by the software during the acclimatization and the sociability phase of the three chamber sociability test. The mean distance travelled during the sociability phase is depicted in Figure 1E. Supplementary Figure S3A shows the mean distance during the acclimatization phase. During the sociability phase, both *Fmr1* KO mice and WT littermates treated with 1 mg/ml baclofen showed a significantly increased locomotor activity compared to littermates receiving aspartame water (2-sided independent samples T-test $p=0.003$ and 0.004). The same effect was observed during the acclimatization phase ($p=0.001$ and $p=0.034$).

3.3 A lower concentration of baclofen (0.25 mg/ml) shows similar results, without an increase of locomotor activity.

Because an increased locomotor activity was observed in mice treated with 1 mg/ml baclofen, which could result from overdose, we reduced the treatment dose to 0.25 mg/ml baclofen and repeated both behavioral tests.

3.3.1. Automated tube test

Fmr1 KO mice and WT littermates were treated with 0.25 mg/ml baclofen or aspartame drinking water. This resulted in a similar effect as was seen for 1 mg/ml: *Fmr1* KO mice treated with baclofen win 75% of the matches on day 1, which increases to 100% on day 4 ($p=0.002$ to $p<0.001$) in matches against *Fmr1* KO mice receiving aspartame water (Figure 2B). WT littermates treated with baclofen win 80-100% of matches against WT littermates receiving aspartame drinking water ($p=0.01$ to $p<0.001$) (Figure 2C). Baclofen treatment did not affect the phenotype in matches between *Fmr1* KO mice receiving baclofen and WT littermates receiving aspartame (Figure 2A). The experiments contained two groups of 6 mice each, except for the control experiment of WT littermates receiving aspartame drinking water versus WT littermates receiving baclofen drinking water, which contained 4 mice per group.

3.3.2. Three chamber sociability test

Figure 2D depicts the time sniffing for all four groups. All mice sniffed the stranger mouse significantly more than the empty cage ($p < 0.001$). *Fmr1* KO mice receiving aspartame drinking water sniff the stranger mouse more than WT littermates receiving aspartame drinking water (two-way RM ANOVA $p < 0.01$). WT mice treated with baclofen showed increased time sniffing of the stranger mouse compared to WT mice receiving aspartame water ($p = 0.01$), resulting in an induced phenotype in WT mice. There was no difference in the time spent sniffing the stranger between *Fmr1* KO mice receiving aspartame drinking water or baclofen drinking water. For each group and genotype, 10 animals were used.

The mean time spent in chamber is depicted in supplementary Figure S2B. Two-way RM ANOVA showed a significant stranger side effect for all groups ($p < 0.001$), all groups spent more time in the stranger room than the room with the empty cage. *Fmr1* KO mice receiving aspartame drinking water spent more time in the stranger room than WT littermates receiving aspartame drinking water, however, this difference was not significant (two-way RM ANOVA stranger side * genotype, $p = 0.06$). WT littermates receiving aspartame water spent significantly less time in the stranger room than WT littermates treated with baclofen (two-way RM ANOVA stranger side * treatment $p = 0.035$). There was no difference in the time spent in chamber for *Fmr1* KO mice receiving aspartame water and *Fmr1* KO mice receiving baclofen water (two-way RM ANOVA $p > 0.1$).

3.3.3. Locomotor activity is not increased at a lower dose

The locomotor activity of *Fmr1* KO mice and WT littermates was not increased in mice treated with 0.25 mg/ml baclofen compared with mice treated with aspartame drinking water during the sociability phase (Figure 2E) and the acclimatization phase (supplementary Figure S3B). However, in mice treated with 0.25 mg/ml baclofen, a small but significant difference between *Fmr1* KO mice and WT littermates was measured during the sociability and acclimatization phase (Figures 1E and S1B), showing higher locomotor activity in *Fmr1* KO mice than WT littermates (2-sided independent samples T-test, $p = 0.005$ during acclimatization and $p < 0.001$ during sociability phase).

4. Discussion

FXS is often characterized by social behavior deficits and autism spectrum features, that can significantly impair the patient's social and adaptive abilities. The main goal of our study was to test whether we can correct abnormal social behavior in the FXS mouse model, using baclofen, a GABA_B agonist. Since FXS is a life-long disease, and

drug treatment is not expected to permanently reverse the features, probably any drug treatment would need to be chronic. For that reason, we investigated chronic baclofen treatment, instead of the often used acute treatment. To measure its efficacy, we used two social behavioral paradigms: the automated tube test and the three chamber sociability test. Our results show no improvement of the FXS phenotype after chronic baclofen treatment in both social behavioral tests. As a matter of fact, baclofen seems to increase the dominant phenotype of *Fmr1* KO mice in the automated tube test and even induce aberrant behavior in WT littermates in both behavioral tests. We observed this worsening of the phenotype with two doses of baclofen. The highest dose, 1 mg/ml, was based on pharmacokinetic studies comparing acute intraperitoneal and chronic oral administration of the R-baclofen specific arbaclofen in *Fmr1* KO mice. In this study, the largest effect was measured on locomotor activity, repetitive behavior and convulsions with 6 mg/kg intra-peritoneal injection, which corresponds to 0.5 mg/ml arbaclofen in drinking water³¹. The only data that was described for chronic treatment was a corrected spine phenotype after 12 days of 0.5 mg/ml arbaclofen in drinking water³¹. We corrected for the presence of the less potent S-baclofen in baclofen, by doubling the dose to 1 mg/ml and treated our mice for more than seven weeks. In contrast to their findings for acute treatment, we observed increased locomotor activity after chronic baclofen treatment, possibly reflecting hyperactivity. This finding led us to hypothesize that the worsening of the social behavior phenotype in the behavioral tests might have been confounded with drug induced hyperactivity. It is known from literature that drug induced hyperactivity can influence social behavior, for example, in the BTBR mouse model of autism⁴⁴. For that reason, we repeated the behavioral experiments with a lower dose of 0.25 mg/ml baclofen, where locomotor activity was not increased. However, still a worsening of the social behavioral phenotype and a deleterious effect on WT littermates was observed, independent of the locomotor activity.

Hyperactivity is an important hallmark of FXS in patients. Increased locomotor activity in the open field test has been described for *Fmr1* KO mice^{16,18,32,45-48}. When analyzing the locomotor activity of the three chamber sociability test, we did not observe an increased locomotor activity in untreated *Fmr1* KO mice compared with their WT littermates. However, when treated with 0.25 mg/ml baclofen, we observed a small but significant hyperactive phenotype in the *Fmr1* KO mice, compared with their WT littermates. We did not see this phenotype in untreated mice or mice treated with 1 mg/ml baclofen. In literature, various groups report different findings for locomotor activity in the three chamber sociability test. Some papers report no differences between *Fmr1* KO mice and WT littermates^{32,42}, while others show increased locomotor activity in *Fmr1* KO mice⁴⁸. The three chamber sociability test is obviously not validated as an open field test, and it is not investigated to what extent this locomotor activity is dependent on anxiety, hyperactivity and other behavioral characteristics. The phenotype we observed in 0.25 mg/ml, but not in 1 mg/ml, could account for increased anxiety or hyperactivity in *Fmr1* KO mice, provoked by baclofen treatment in a dose that does not sedate the animals as a side effect. It is also possible that a higher dose of baclofen influences

both WT and *Fmr1* KO mice, resulting in a ceiling effect without further increasing the locomotor activity, while the lower dose slightly affects *Fmr1* KO mice, without affecting WT littermates.

Both baclofen and arbaclofen have been tested in *Fmr1* KO mice and have been shown to reduce FXS related phenotypes, like audiogenic seizures, abnormal spine morphology, synaptic protein synthesis, AMPA receptor internalization, repetitive behavior and hyperactivity^{22,31-33}. Our results are in a striking contrast to these previously reported papers. This difference could be due to our use of chronic treatment. The only study that used chronic arbaclofen treatment, although administered only for 2 weeks in drinking water, did not assess behavior but only spine morphology³¹. This study and other studies have shown a reduction of hyperactivity with acute arbaclofen treatment^{31,33}. Another difference with some of the studies is our use of racemic baclofen instead of R-baclofen or arbaclofen²⁸. Unfortunately, it is difficult to assess the optimal dose, since there is no relevant functional biomarker or endophenotype to screen for desirable levels of baclofen in the brain. Furthermore, our results are limited to the social behavioral phenotype, in which the relevance of the GABA_B system is unknown.

In addition to FXS, the GABA_B pathway has also been implicated in several other neurodevelopmental disorders, including Rett syndrome, Down syndrome and autism⁴⁹. The effects of R-baclofen and arbaclofen have also been studied in mouse models for autism. R-baclofen has been shown to reverse social deficits and repetitive behavior in two autism mouse models²⁷. In addition, arbaclofen has been evaluated for patients with ASD. As was the case in the FXS clinical trial, the larger phase 2 randomized placebo controlled clinical trial with 150 participants with ASD showed no improvement in the primary outcome measure. However, it did demonstrate improvement of some specific outcome measures in post-hoc analyses^{50,51}. It is currently not known why the promising results in preclinical studies could not be replicated in patients with FXS or ASD. Our results do not support efficacy of baclofen, possibly revealing drawbacks of baclofen treatment that might be related to the lack of efficacy observed in patients.

In this study, we used two social behavioral tests linked to autism and aberrant social behavior in mice, one of the core features of FXS. Both tests have been shown to be useful in detecting efficacy of targeted treatment, indicating their potential in pre-clinical screening for therapy. The three chamber sociability test is a modified test from Nadler *et al*^{40,41}, developed to measure social interaction and interest for an unfamiliar mouse compared to an object (empty cage), in neuropsychiatric diseases like ASD. Gantois *et al*⁴² showed a phenotype similar to ours in the three chamber sociability test: increased sociability in *Fmr1* KO mice, compared with WT littermates. The time spent sniffing the stranger mouse is the most important measure for sociability, involving direct and active social approach and interaction^{15,40}. The time in chamber is correlated to the time spent sniffing and could be used as an indirect measurement of social interaction^{40,42,52}. In our experiments, the time spent in the stranger chamber

only approached significance, indicating sniffing time is probably a more reliable measurement of sociability in *Fmr1* KO mice in our set up. Recent publications have also demonstrated enhanced sociability in *Fmr1* KO mice, expressed as time sniffing the stranger mouse^{17,42,46,53} as well as time spent in the stranger chamber^{18,42,54}. However, other studies have demonstrated decreased sociability in *Fmr1* KO mice, which seems more in line with the classic ASD features^{15,33,55}. The differences between these studies could be related to differences in genetic background and experimental set-up. We modified the three chamber sociability test from Gantois *et al*⁴², by recording only the first five minutes of the sociability phase, since most of the social approach takes place in this time interval and at that time, the *Fmr1* KO phenotype has been shown to be the most obvious^{40,42}. Also, we only used the sociability part, since that has been described to be the most sensitive and relevant to autism-like symptoms⁵². The second part, preference for social novelty, is more relevant to test social recognition and memory⁵² and is a less consistent phenotype^{15,18,56}. Our second behavioral test, the automated tube test, is modified from the paradigm developed by Lindzey *et al*³⁹ to test the effect of different genetic backgrounds on social dominance. *Fmr1* KO mice display a robust dominant phenotype over WT littermates, which can be partially reversed by genetic or pharmacological reduction of the mGluR5 activity³⁸. Also in the tube test conflicting results have been described by different groups^{46,57}, possibly dependent on differences in genetic background, experimental setup and especially the training protocol and housing conditions³⁸. In both the automated tube test and the three chamber sociability test, the FXS phenotype might be explained by an enhanced sociability or disinhibition in social behavior. This fits the observation that FXS patients often do seek social contact and are more empathetic and disinhibited, unlike the social withdrawal often seen in idiopathic ASD⁵⁸⁻⁶⁰.

Since baclofen is a GABA_B agonist, we expected sedation to be a major side effect, hence we were surprised to find an increased locomotor activity in mice treated with baclofen. Increased locomotor activity could be related to different behaviors in mice like hyperactivity and anxiety⁶¹ and might be a major confounder on any measurement of social deficits⁴⁴. Thus, it is important to address the cause of this paradoxical effect of baclofen. Similar paradoxical effects caused by baclofen have been described earlier in patients with psychiatric diseases or spasticity, as well as in mice (outbred CFW and ICR mouse). Symptoms of elevated mood disorders were observed, like agitation, insomnia, mania, disinhibition, aggression or seizures⁶²⁻⁶⁴. Genetic background and pathway characteristics probably play a role in the response to baclofen because variations in the GABAergic pathway functioning in different brain regions can ultimately lead to an undesired combined effect. For other GABAergic drugs like benzodiazepines, it has been previously hypothesized that variation in pathway characteristics in the patient's brain, leads to differences in the drugs response⁶⁵⁻⁶⁷. Benzodiazepines are known to often cause paradoxical effects in patients with specific neurodevelopmental disorders, especially autism, where the GABAergic pathway has been implicated^{65,68}. In addition, data regarding tolerance induction after chronic administration of baclofen has been

reported, although it seems that certain cellular targets of baclofen are more prone to this tolerance induction than others⁶⁹. The precise mechanism of tolerance induction is not known since only few studies have focused on chronic systemic baclofen treatment and results in literature are conflicting. One proposed mechanism is downregulation of the GABA_B receptor binding sites or phosphorylation of downstream signaling regulators^{30,69,70}. In addition, evidence has been published on cross-talk between GABA_B receptor and mGluR5 receptor after chronic treatment³⁰. Notably, treating *Fmr1* KO mice acutely with arbaclofen, corrects mGluR5 dependent phenotypes like AMPA-receptor internalization and protein synthesis, underlining the interdependence of both pathways³¹. Besides the above mentioned variations, also unstable R-baclofen concentrations due to the short half-life of the drug are known to contribute to a paradoxical effect²⁹. Because we treated the mice ad libitum with baclofen drinking water, we cannot exclude fluctuations in the blood concentrations. Nevertheless, without quantifying this behavior, mice treated with baclofen in both concentrations did seem to be more agitated or “jumpy”. However, no aggressive behavior was observed. With the lower concentration of baclofen, no increased locomotor activity was measured, but this does not exclude other elevated mood disorder effects on the mice. Probably, aggression, anxiety, mania, or any other behavioral changes, however subtle, could have confounded the social behavior of the mice. We do not know whether our results are due to this paradoxical effect of baclofen and if they are relevant for patients with FXS or other neurodevelopmental disorders. If they are, they predict highly undesirable side effects that may not outweigh any possible improvement. Altogether, this implicates that chronic treatment with baclofen might have a different effect per subject, depending on genetic variation, pathway-specific differences, and different effects of baclofen per brain region. Moreover, the effect could be dose-dependent, dependent on duration of treatment, and be affected by individual differences in metabolism. It would be interesting to unravel these variations and to be able to predict the effect of baclofen in a personalized patient care setting.

5. Conclusions

In conclusion, treatment of mice with baclofen, a GABAB agonist, resulted in a worsening of the social phenotype in *Fmr1* KO mice and even induction of aberrant social behavior in WT littermates. This deleterious effect might be due to hyperactivity, a paradoxical elevated mood effect, or agitation. Our results on chronic baclofen treatment are in sharp contrast with earlier publications on acute treatment of baclofen or arbaclofen in FXS, that have shown benefit on multiple FXS phenotypes. Since baclofen treatment worsens the FXS social behavior phenotype of *Fmr1* KO mice in the automated tube test and induces aberrant social behavior in WT littermates in two social behavioral tests, the question can be raised whether baclofen is a ubiquitously effective treatment for FXS, or might also worsen FXS related features in patients. It is unknown whether a similar unexpected effect, or paradoxical effect could be relevant in patients with FXS, possibly even in part explaining the disappointing results of the arbaclofen clinical trials. Future research should address whether this effect of chronic baclofen treatment is consistent for other FXS related phenotypes and whether it is specific for certain mice strains or patient groups. If baclofen would appear to have a beneficial effect in a subgroup of patients and a deleterious effect in others, the use of baclofen would require identification of predictive factors and a personalized approach.

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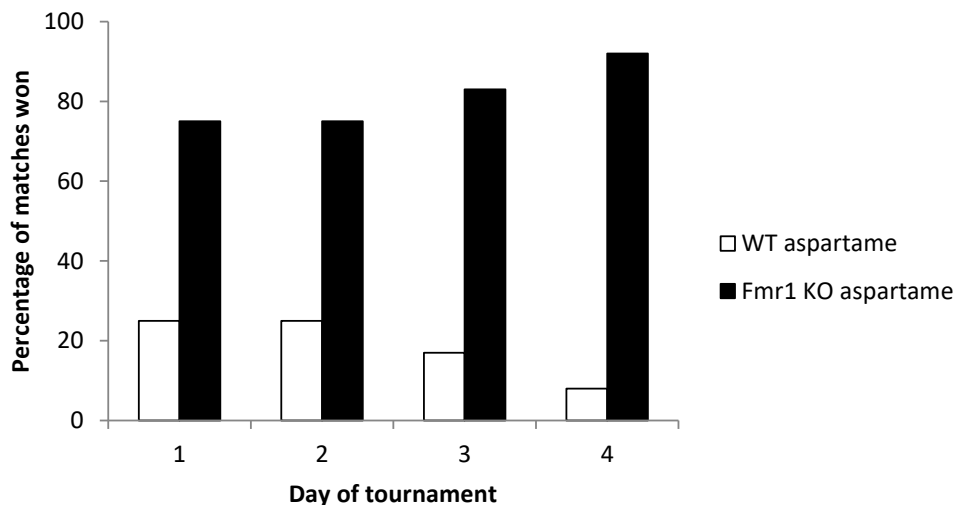
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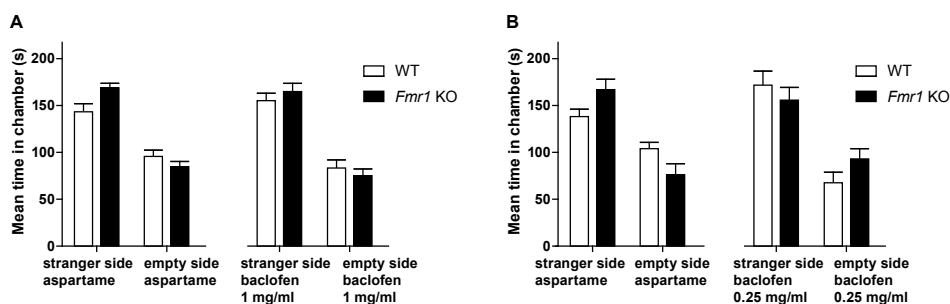
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Supplementary data

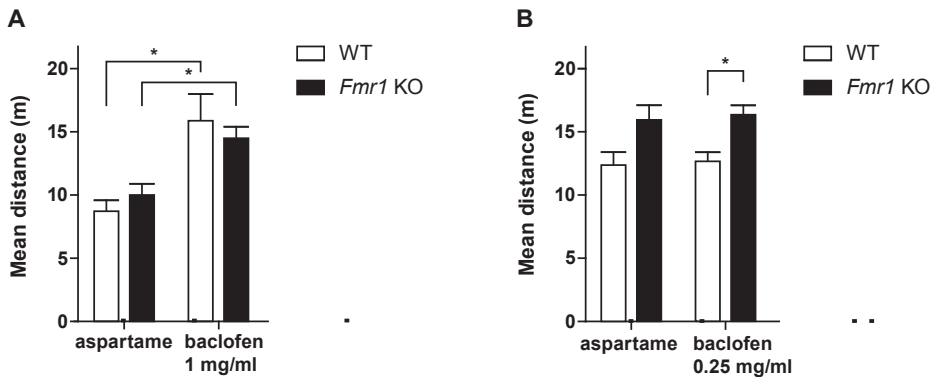


Supplementary Figure S1. control experiment WT littermates treated with aspartame against Fmr1 KO mice treated with aspartame. Fmr1 KO mice receiving aspartame drinking water display a dominant phenotype over WT littermates receiving aspartame drinking water ($p=0.002$ - <0.001). $N = 6$ mice per group.

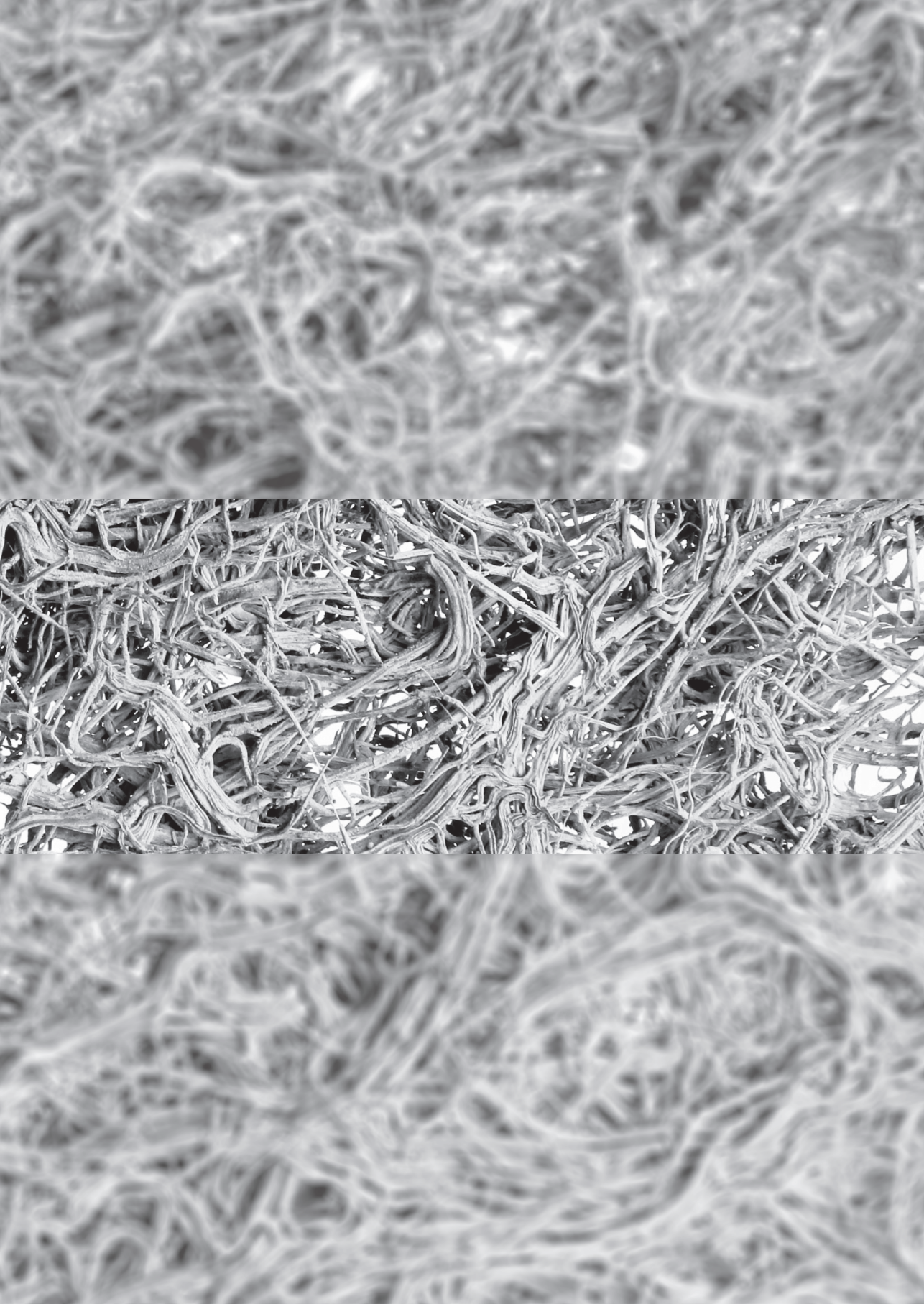


Supplementary Figure S2: Time spent in chamber with the stranger mouse compared to the empty cage, in the three chamber sociability test. WT littermates and Fmr1 KO mice treated with 1 mg/ml baclofen, 0.25 mg/ml baclofen or aspartame drinking water. (A) WT and Fmr1 KO mice treated with 1 mg/ml baclofen compared to aspartame drinking water. All groups spent significantly more time in the stranger room than the empty room ($p<0.001$). Fmr1 KO mice receiving aspartame water did not spend significantly more time in the stranger room, than WT littermates receiving aspartame water ($p=0.075$). Baclofen treatment did not show an effect on Fmr1 KO mice behavior ($p>0.1$). Overall RM ANOVA shows no significance on stranger side * genotype * treatment ($p>0.1$). (B) WT and Fmr1 KO mice treated with 0.25 mg/ml baclofen compared to aspartame drinking water. All groups spent significantly more time in the stranger room than the empty room ($p<0.001$). Fmr1 KO mice receiving aspartame water did not spend significantly more time

(Figure S2: continued) in the stranger room, than WT littermates receiving aspartame water ($p=0.06$). WT mice treated with baclofen spent significantly more time in the stranger room, compared to WT littermates receiving aspartame water ($p=0.035$). Baclofen treatment did not have an effect on *Fmr1* KO mice behavior ($p>0.1$). Overall RM ANOVA showed significant treatment effect ($p=0.045$). Data are expressed as mean $\pm$ SE. Number of mice in 1 mg/ml experiment WT aspartame $n=13$, *Fmr1* KO aspartame $n=13$ WT baclofen $n=11$. Number of mice in 0.25 mg/ml experiment $n=10$ per group. P-values: $^{\wedge}<0.05$, $^*<0.01$, $^{**}<0.001$



Supplementary Figure S3: Locomotor activity during the acclimatization phase of the three chamber sociability test, of WT and *Fmr1* KO mice receiving baclofen or aspartame drinking water. Expressed as mean distance in meters (m) travelled in 5 minutes during the acclimatization phase of the three chamber sociability test. (A) Locomotor activity, expressed as mean distance in meters (m) travelled in 5 minutes during the acclimatization phase of the three chamber sociability test in mice treated with 1 mg/ml baclofen or aspartame drinking water. WT and *Fmr1* KO mice treated with baclofen show increased locomotion compared to mice receiving aspartame water (2-sided independent samples T-test $p=0.003$ and 0.004 respectively). (B) Locomotor activity, expressed as mean distance in meters (m) travelled in 5 minutes, during the acclimatization phase of the three chamber sociability test in mice treated with 0.25 mg/ml baclofen or aspartame drinking water. WT littermates and *Fmr1* KO mice treated with baclofen have similar locomotion activity compared to littermates receiving aspartame water. *Fmr1* KO mice show higher locomotor activity than WT littermates when treated with baclofen (2-sided independent samples T-test, $p=0.005$). This is not the case for *Fmr1* KO mice and WT littermates receiving aspartame drinking water ($p>0.1$). Data presented as mean $\pm$ SE. Number of mice in 1 mg/ml experiment WT aspartame $n=13$, *Fmr1* KO aspartame $n=13$ WT baclofen $n=11$. Number of mice in 0.25 mg/ml experiment $n=10$ per group. P-values: $^{\wedge}<0.05$, $^*<0.01$, $^{**}<0.001$



CHAPTER 4

Targeting the RhoGTPase pathway rescues synapse maturation and memory in a mouse model of fragile X syndrome

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Submitted

Abstract

Fragile X syndrome (FXS) is caused by a CGG expansion in the 5'untranslated region and transcriptional inactivation of the fragile X mental retardation 1 (*Fmr1*) gene. This leads to dendritic abnormalities with increased density of long, thin immature spines, which are thought to underlie the intellectual disability phenotype of FXS patients and *Fmr1* knock out mice. Compelling evidence suggests that these morphological changes involve disturbances of actin cytoskeleton dynamics and RhoGTPase signaling. Here, we use cytotoxic necrotizing factor 1 in *Fmr1* knock out mice to explore the potential of RhoGTPase pathway stimulation as a putative therapeutic strategy for FXS. Cytotoxic necrotizing factor 1 is known to induce rearrangement of the cytoskeleton through lasting enzymatic activation of the Rho-family of small GTPases. We found that *in vivo* administration of cytotoxic necrotizing factor 1 normalizes the phosphorylation state of the actin filament modulator cofilin in *Fmr1* knock out cortical tissue. Application of cytotoxic necrotizing factor 1 to primary hippocampal neuronal cultures reverses the increased filopodia formation observed in knock out neurons. Furthermore, intraventricular infusion of cytotoxic necrotizing factor 1 rescued hippocampal- and cortical-dependent cognitive functions in *Fmr1* knock out animals with no effect on anxiety or locomotor behavior parameters. The beneficial effect of CNF1 appears to be mediated through a rescue of memory acquisitions in the *Fmr1* knock out mice. These data demonstrate the potential therapeutic effect of permanent activation of the small RhoGTPases pathway in FXS.

1. Introduction

Fragile X syndrome (FXS, OMIM #300624) is the leading monogenetic cause of intellectual disability disorder ¹. FXS affects mainly males (1: 7000) ² and is caused by a CGG repeat expansion mutation (>200 repeats) in the 5' untranslated region of the fragile X mental retardation 1 gene (*FMR1*) ³. The mutation results in the silencing of *FMR1* transcription and loss of the fragile X mental retardation protein (FMRP) ⁴. Only a few cases are due to point mutations in the coding sequence of the *FMR1* gene (reviewed in Suhl *et al* ⁵). The disorder is characterized by behavioral and cognitive impairments, often including mild to severe levels of intellectual disability, autistic-like features and impaired social skills (reviewed in Garber *et al* ⁶). Anxiety and hyperactivity can also be present amongst other emotional disturbances.

Morphological changes in the shape and density of dendritic spines of FXS neurons represent a hallmark of the disorder and are considered as the cellular basis of the observed intellectual disability and the behavioral phenotype. Both FXS patients and *Fmr1* knock out (KO) mice show long, thin and tortuous spines in pyramidal neurons across different cortical regions, hippocampus, olfactory bulb, amygdala and cerebellum (reviewed in Bostrom *et al* and He *et al* ^{7,8}).

Loss of FMRP in FXS appears to impair synapse formation and synaptic plasticity by disturbing local expression of several target RNAs in the postsynaptic compartment. FMRP binds specific RNAs and microRNAs, but also homodimerizes and interacts with several nuclear and cytoplasmic proteins ⁹ to control RNA transport, mRNA stability and local protein synthesis. Loss of FMRP has been suggested to interfere with actin cytoskeleton organization ¹⁰⁻¹², which is critically regulated by small GTPases of the Rho family during synaptic plasticity ¹³. In fact, in FXS RhoGTPase signaling was reported to be abnormal ¹⁴⁻¹⁶. For example, Rac1 is associated and necessary for normal spine development and long-term synaptic plasticity ¹⁴. In *Fmr1* KO mice, the levels and activation state of Rac1 were found to be elevated ^{14,17}. Depletion of FMRP in FXS patients and *Fmr1* KO mouse models possibly prevents RhoGTPases from properly carrying out their functions.

Cytotoxic necrotizing factor 1 (CNF1) is an *Escherichia coli* toxin that catalyzes deamination of glutamine 63 of Rho, leading to its constitutive activation and the reorganization of actin stress fibers ^{18,19}.

Through a permanent activation of RhoGTPases including Rac1, CNF1 can thus effectively reorganize cytoskeletal structures in neuronal cells ^{13,20,21}. In fact, various studies in rodents showed that CNF1 acts as a cognitive enhancer ²¹⁻²³ and restores cognitive function in mouse models of different intellectual disability disorders, such as Rett syndrome ^{24,25} and Alzheimer's disease ^{26,27}. It also corrects motor impairment in a model of Parkinson's disease ²⁸.

In this study, we used CNF1 toxin to modulate actin cytoskeleton dynamics in the *Fmr1* KO mouse model of FXS. We were able to rescue both cognitive dysfunction and the underlying neuronal morphological impairments of *Fmr1* KO mice, without improvement of emotional and locomotor phenotypes. Our study shows that manipulating the RhoGTPase pathway might be an effective therapy in FXS patients, particularly for cognitive improvement.

2. Material and Methods

2.1. CNF1 preparation and activity

CNF1 was obtained from *E. coli* pISS392 strain (kindly provided by V. Falbo, Istituto Superiore di Sanità, Rome, Italy) and purified as described previously²⁹. CNF1 activity was confirmed by assessing F-actin organization in Human epithelial type-2 (HEp-2) cells. HEp-2 cells were grown in minimal Eagle's medium supplemented with 10 % fetal calf serum (Flow Laboratories, Rockville, MD), 5 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded at a density of 2×10^4 cells/cm² and 24 h later exposed to 100 pM CNF1 for 48 h.

For F-actin visualization, cells were fixed in 3.7 % paraformaldehyde in PBS (pH 7.4) for 10 minutes and permeabilized in 0.25 % Triton X-100 in PBS for 5 min. Cells were stained with fluorescein-conjugate phalloidin (Sigma-Aldrich, St Louis, MO) used at the final concentration of 0.5 µg/ml for 30 minutes at 37 °C. Nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich) for 5 minutes at room temperature. Following extensive washing in PBS, cells were mounted on glass coverslips and analyzed with a fluorescence microscope (Olympus BX51).

2.2 Primary hippocampal neuronal cultures

We used *Fmr1* knock-out (>10x backcrossed in C57/Bl6, from now referred to as *Fmr1*^{Bl6} KO) and their respective C57/Bl6 wild-type (WT^{Bl6}) littermate controls³⁰ for primary cultures of hippocampal neurons as described previously³¹. In short, *Fmr1*^{Bl6} KO mice and WT^{Bl6} littermates were sacrificed at embryonic day (E) 17. After isolation of the hippocampi, the cells were dissociated by a 15 minutes trypsin-EDTA treatment followed by a mechanical dissociation step. Finally, 100.000 neurons were plated on poly-L-lysine (100 µg/ml, Sigma-Aldrich) and laminin (50 µg/ml, Sigma-Aldrich) coated glass coverslips and cultured in neurobasal medium (Gibco, Netherlands), containing 1% penicillin/streptomycin, 1% Glutamax and 2% B27. After 19 days *in vitro* (DIV), the medium was changed with supplemented neuronal medium containing either 100 pM CNF1 or fresh supplemented neuronal medium only. Simultaneously, the neuronal cells were transfected with a mCherry construct under the control of a chicken β-actin promoter using Lipofectamine 2000 (Invitrogen, Netherlands). One day after

transfection, cells were fixed in 4% formaldehyde, washed in PBS and, after a Hoechst 33342 (Invitrogen) staining step, mounted in Mowiol mounting solution.

2.3. Quantification of spines

Images of β -actin mCherry transfected neurons were acquired with a 63x objective using a Zeiss LSM510 confocal microscope (Zeiss, Germany). The acquired images were analyzed using Image J³². The investigator was blind for genotype and treatment conditions. At DIV20, preferably two (otherwise one or three) well viewable distal secondary dendritic segments of 30–140 μ m per neuron were chosen for total protrusion morphometric analysis. The length and the width of each protrusion were measured. The length was defined as the distance from the base to the tip of the protrusion; the width was defined as the maximum distance perpendicular to the long axis of the protrusion. In order to make an objective distinction between spines and filopodia, we calculated the ratio between the width and the length for each protrusion. Protrusions with a ratio above or equal to 0.5 were considered as spines and conversely, protrusions with a ratio below 0.5 were considered as filopodia³³. Statistical analysis was performed using 2-way ANOVA.

2.4 Western blot of cofilin and phospho-cofilin

Cortical tissue from adult FVB.129P2-Pde6b⁺Tyr^{c-ch} *Fmr1*^{tm1Cgr/J} (from now referred to as *Fmr1*^{FVB} KO) and FVB.129P2-Pde6b⁺Tyr^{c-ch}/AntJ control (WT^{FVB}) (Jackson Laboratory, Bar Harbor, ME, USA) males was obtained, snap frozen in liquid nitrogen and kept at -80 °C. For protein extraction, tissue was homogenized in lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.25mM MgCl₂, 0.5% NP40 with RNase (5U/mL final) and protease inhibitor cocktails. After homogenization, the mixture was centrifuged at 14.000 g for 10 minutes at 4 °C. The supernatant containing the proteins was transferred to a new tube and total protein concentration in the supernatant was determined by Bradford method.

Western blot was performed as previously described¹⁰. Briefly, 10 μ g of total protein were denatured at 95 °C for 5 minutes and loaded onto a 20% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio Rad). Membranes were incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween and 2% non-fat dry milk) to prevent unspecific binding. Membranes were probed with the primary antibodies rabbit polyclonal anti-phospho-cofilin (1:1000, Cell Signaling, 77G2), rabbit polyclonal anti-cofilin (1:1000, Cell Signaling, D3F9) and mouse monoclonal anti- β -Actin (1:10000, #A5441, clone AC-15, Sigma-Aldrich), overnight at 4°C, with gentle shaking. After washing 3x for 15 minutes with blocking buffer, membranes were probed with diluted secondary antibodies at room temperature and then incubated with peroxidase-

conjugated goat anti-rabbit or goat anti-mouse antibodies. Immunoreactive bands were visualized with Western blot chemiluminescence reagents (Western Lightning, standard sensitivity or Immobilon Western, Millipore) and quantified with Image J software.

2.5 Toxin administration, stereotaxic surgical procedures, animal model, and behavioral tests

In vivo experiments were performed in two independent laboratories (Dierssen lab / Stork lab) with slight differences according to locally optimized procedures and experimental permissions, as described below.

2.5.1. Dierssen's laboratory

Adult male *Fmr1*^{FVB} KO and WT^{FVB} mice (10-16 weeks of age) were used. CNF1 was diluted to a final working concentration of 0.6 fmol/Kg with sterile 20 mM Tris-HCl buffer pH 7.5. Control injections were done with the vehicle solution (20 mM Tris-HCl buffer pH 7.5). After general anesthesia (mixture of 50 mg/Kg ketamine/10 mg/Kg medetomidine, intraperitoneally) a 25G needle attached to a 5 μ L Hamilton micro syringe was placed in the right lateral ventricle (in relation to lambda: antero-posterior -0.3 mm, lateral +1 mm, dorso-ventral -2 mm)³⁴. Injection of 1 μ L of the test solution was done at a rate of 0.25 μ L/minutes (Quintessential Stereotaxic Injector, Stoelting, USA). Five minutes after the injection, the needle was removed and the surgical wound was sutured with histoacryl (Braun, #1050060, Germany). Anesthesia effects were reversed using atipamezole (2.5 mg/Kg) and analgesia was achieved with buprenorphine (0.15 mg/Kg). After recovery from the operation, mice were housed individually under a 12-hour light/dark cycle and their health status was continuously monitored for the following 10 days, after which behavioral experiments started.

Elevated plus maze. The maze consisted of two opposing closed arms (in cm, 30 length x 6 wide x 15 height) and two opposing open arms (in cm, 30 length x 6 wide) extending from a common central region (6 x 6 cm) to form a "plus" shape made of black methacrylate (Panlab, Spain). The arms were elevated 40 cm above the floor. In a single 5 minutes trial, animals were placed in the center of the maze and the time spent, distance travelled and number of entries in the open and closed arms were recorded (WT-vehicle, n = 4; WT-CNF1, n = 5; *Fmr1* KO-vehicle, n = 4; *Fmr1* KO-CNF1, n = 4). Data were automatically collected by computerized video tracking system (Biobserve, Bonn, Germany).

Spontaneous alternation. The test was performed as previously described³⁵. The maze consisted of 3 arms (in cm, 32 length x 6 weight x 15 height) set at a 120° angle in a Y-shaped apparatus. All arms converged in an equilateral triangular central area. The

maze was located in the middle of a room containing no extra-maze cues, enclosed by 20 cm high black walls. Movement of animals was monitored with a camera placed above the maze and analyzed with a video tracking system (SMART V2.5, Panlab Harvard Apparatus). Mice were habituated to the experimental room (30 min) and then placed at the end of one of the arms and allowed to explore freely the maze for 8 minutes (WT-vehicle, $n = 12$; WT-CNF1, $n = 10$; *Fmr1* KO-vehicle, $n = 16$; *Fmr1* KO-CNF1, $n = 14$). The start arm was counterbalanced within experimental groups. Entries to each arm were scored when all four paws of the animal were placed inside an arm. Spontaneous alternation, expressed as a ratio, refers to the proportion of arm choices differing from the previous two [Alternation ratio = (number of alternations/total number of entries) - 1]. In this test, the animals must memorize the arm entered immediately prior to the arm they are currently occupying and the percentage of alternation is used as an index of short-term memory.

Novel object recognition. Object recognition memory was assayed on the Y-maze used in the spontaneous alternation. The test was performed as previously described³⁶. Briefly, on day 1, mice were habituated for 10 minutes to the Y-maze. On day 2, mice were put back in the Y-maze for 10 minutes where 2 identical objects were introduced at the end of each arm, and the time that the mice spent exploring each object was recorded. On day 3, mice were again placed in the Y-maze for 10 min, one of the familiar objects was replaced with a novel object, and the total time spent exploring each of the two objects (novel and familiar) was recorded (WT-vehicle, $n = 10$; WT-CNF1, $n = 13$; *Fmr1* KO-vehicle, $n = 13$; *Fmr1* KO-CNF1, $n = 14$). The exploration of the objects is considered as any investigative behavior (head orientation, or sniffing occurring) or deliberate contact that occurred with each object in a distance ≤ 2 cm. The animals that did not explore the objects within 10 minutes were excluded from the experiment. Memory was operationally defined by the discrimination index for the novel object (DI) as the proportion of time animals spent investigating the novel object minus the proportion spent investigating the familiar one in the testing period [Discrimination Index, $DI = (\text{novel object exploration time} / \text{total exploration time}) - (\text{familiar object exploration time} / \text{total exploration time})$]. The new object position was counterbalanced between subjects.

Social interaction. The test was performed in an apparatus that consisted of a squared arena with a white Plexiglas chamber (in cm, 70 length x 70 wide x 30 height), under 250-lux light conditions. All animals were transferred to the experimental room 20 minutes before starting the test. During the habituation phase, activity was first recorded for 10 minutes in a single trial. A second habituation phase (5 min) was performed with the 2 stimulus cages present, which were kept empty, to assess basal levels of exploration. To test for social preference a stimulus mouse (stranger 1) is confined inside one cylinder, the second cylinder is left empty, and placed either on the left or right side of the open field (counterbalanced) (WT-vehicle, $n = 10$; WT-CNF1, $n = 3$; *Fmr1* KO-vehicle, $n = 11$; *Fmr1* KO-CNF1, $n = 7$). One hour after the social preference test, animals were tested

for social recognition in a 5 minutes session. During this stage, a second stimulus mouse (stranger 2) is confined to the previously empty cylinder (WT-vehicle, $n = 10$; WT-CNF1, $n = 4$; *Fmr1* KO-vehicle, $n = 12$; *Fmr1* KO-CNF1, $n = 5$). After the stimulus-animal had been in the cylinder for 5 minutes, the subject animal was introduced in the middle of the arena and its behavior was monitored automatically using a video tracking system (SMART V2.5, Panlab Harvard Apparatus). A zone around the cylinders within 14 cm from its walls was defined as social approach zone. Exploration was defined as the orientation of the nose towards the cylinder at a distance of ≤ 2 cm. The exploration time for the familiar or the new subject was recorded. Sociability was measured as the time spent close to each cylinder (stranger 1 vs. empty). Preference for social novelty was defined by the discrimination index for the novel subject [$DI = (\text{stranger 2 exploration time} - \text{stranger 1 exploration time})/\text{total exploration time}$].

2.5.2. Stork's laboratory

Male 16-20 week old *Fmr1*^{FVB} KO and their WT littermates were used. After general anesthesia (50 mg/Kg pentobarbital) a 31G needle attached to a 10 μ L Hamilton micro syringe was placed in the right lateral ventricle (in relation to lambda: antero-posterior -0.3 mm, lateral +1 mm, dorso-ventral -2 mm)³⁴. Injection of 2 μ L of the test solution (0.6 fmol/Kg) was done at a rate of 0.25 μ L/minutes (World Precision Instruments, Berlin, Germany). Ten minutes after the injection, the needle was removed and the surgical wound was sutured. Mice were housed individually under a 12-hour light/dark inverted cycle and their health status was continuously monitored for the following 10 days, until experiments started.

Open field. To determine changes in exploration, general locomotion, and anxiety-like behavior mice were tested in a 20 minutes session in a squared open field arena just before, and 10 days after CNF1 or vehicle infusion. The arena consisted of an open plastic dark box (in cm, 50 length x 50 wide x 35 height). Animals were placed in the center of the arena. Total distance travelled, as well as the time, distance, and entries to the center (25 x 25 cm), corners, and remaining area of the arena were recorded using a video track system (ANY-maze, Stoelting, Wood Dale, IL) (WT-vehicle, $n = 9$; WT-CNF1, $n = 11$; *Fmr1* KO-vehicle, $n = 4$; *Fmr1* KO-CNF1, $n = 6$).

Fear conditioning. The training apparatus (TSE, Bad Homburg, Germany) comprised of a 36cm x 21cm x 21cm light-blue acrylic glass arena with a grid floor for delivery of electric foot shocks. It was enclosed in an isolation cubicle containing a speaker, a ventilation fan providing fresh air and background noise of 70 dB sound-pressure level (SPL). On the first day of the experiment, animals were exposed twice to the training apparatus and a set of 6 acoustic stimuli (2.5 kHz sinus tone, 85dB SPL, 10 s with 20 s inter-stimulus interval (ISI) serving as the unpaired conditioned stimulus (CS-). On the following day, after 2 minutes of habituation to the apparatus, animals were fear

conditioned with 3 conditioned stimuli (CS+; 10 kHz sinus tone, 85 dB SPL, 10 s with 20 s ISIs), each co-terminated with an unconditional stimulus (US; 1 s scrambled foot shock, 0.4 mA). Contextual fear memory was tested 24 hours post training by exposing animals for 6 minutes to the training box. Cued fear memory retrieval was tested 48 hours post training in a neutral context, consisting of a standard housing cage with sawdust filling, placed in the isolation cubicle. After 2 minutes of exposure to the new context alone, animals were confronted with a set of 4 CS-, followed by a set of 4 CS+, each separated by 20 s ISI. The percentage of freezing - complete immobilization except for respiratory movements, more than 1 s - was monitored online, using a photo beam detection system and used as a measure of fear memory (WT-vehicle, $n = 13$; WT-CNF1, $n = 15$; *Fmr1* KO-vehicle, $n = 10$; *Fmr1* KO-CNF1, $n = 11$).

2.5.3. Housing and ethical considerations

Animals were housed in standard laboratory cages with water and food available ad libitum in controlled environmental conditions of humidity (60%) and temperature ($22 \pm 2^\circ\text{C}$). Experimental procedures were performed in accordance with the guidelines of animal procedures and approved by the local ethical committees (in Barcelona the PRBB CEEA MDS-08-1059, MDS-11-1362 and in Germany the Landesverwaltungsamt Sachsen-Anhalt permission 42502-2-1009, UNI MD). Experimental procedures met the guidelines of the local Catalan law (5/1995 and Decrees 214/97, 32/2007), the German regulations for animal experiments, and the European regulations (2010/63/EU directive).

3. Results

3.1. CNF1 activity

Before being used in primary hippocampal neurons and animals, the activity of CNF1 was confirmed in HEp-2 cells. As expected, exposure to 100 pM CNF1 for 48 h induced a profound reorganization of the actin cytoskeleton, with the formation of stress fibers and membrane ruffles (Figure 1A, B). Moreover, CNF1 improved cell spreading, with a consequent remarkable increase in the cell size and with the appearance of a multinucleated phenotype (Figure 1B).

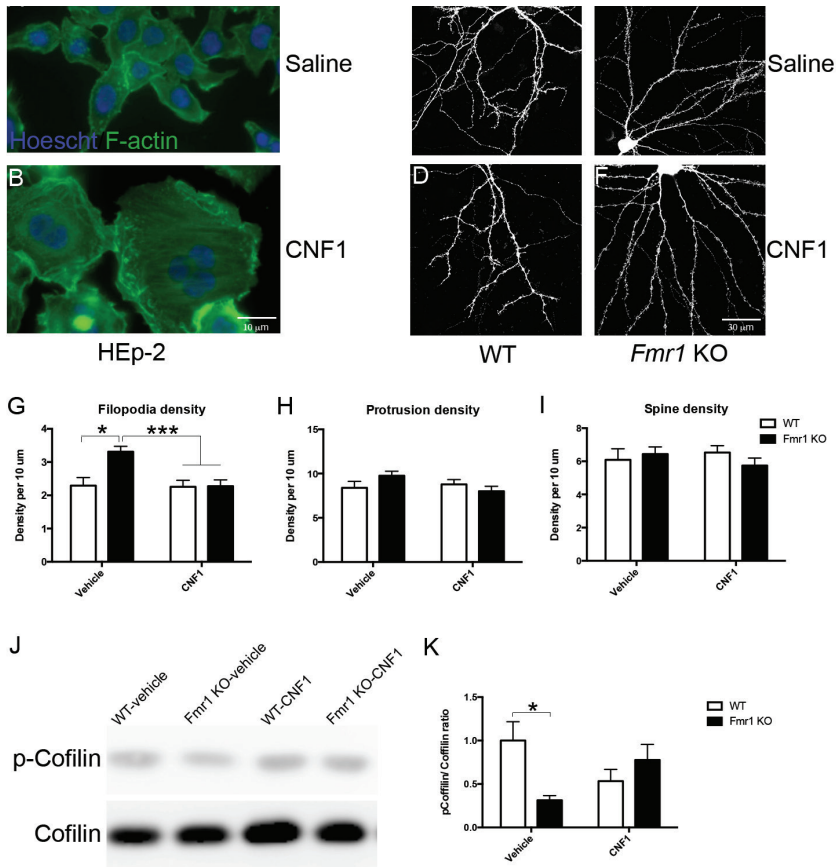


Figure 1: CNF1 administration rescues morphological and molecular features of *Fmr1* KO model. (A-B) Fluorescence photomicrographs showing F-actin organization in HEp-2 cells treated for 48 h with (A) saline or (B) CNF1. CNF1 induces a remarkable reorganization of F-actin into stress fibers (head arrows) and membrane ruffles (asterisks), and the formation of giant multinucleated cells. Scale bar, 10 μ m. (C-F) Photomicrographs showing hippocampal neurons at DIV20 from WT^{BL6} and *Fmr1*^{BL6} KO with and without CNF1 treatment, used for spine morphometric analysis. Scale bar, 30 μ m. Quantification of the density of (G) filopodia, (H) protrusion and (I) spines in distal secondary dendrites demonstrates a selective increase of filopodia in *Fmr1*^{BL6} KO cells that is reverted by the CNF1 treatment. (J) Representative Western blot pictures of cofilin, phospho-cofilin and actin housekeeping protein in cortical tissue of *Fmr1*^{FBV} KO animals and respective WT^{FBV} controls. (K) Quantification of phospho-cofilin/cofilin ratio confirms a reduced phosphorylation level that is normalized by CNF1 treatment. Values are means + SEM. * $p < 0.05$; *** $p < 0.001$.

3.2. CNF1 rescues immature dendritic spine phenotype of *Fmr1* KO primary hippocampal neurons

Primary hippocampal neuron cultures of WT^{BL6}-CNF1 (n = 30), *Fmr1*^{BL6} KO-CNf1 (n = 25), WT^{BL6}-saline (n = 13) and *Fmr1*^{BL6} KO-saline (n = 32) were analyzed at DIV20. The number of filopodia, spines, and total number of protrusions was expressed as number per dendritic segment of 10 μ m. We observed a statistically significant difference in filopodia density among groups (Figure 1C-G; genotype x treatment interaction, $F_{(1, 96)} = 5.792$ p = 0.0180). Bonferroni's posthoc comparisons revealed that the density of filopodia was higher in *Fmr1*^{BL6} KO-vehicle as compared with WT^{BL6}-vehicle neurons (p = 0.0116) and that treatment with CNF1 rescued *Fmr1*^{BL6} KO filopodia density to WT^{BL6} levels (Figure 1G; *Fmr1*^{BL6} KO-vehicle vs. *Fmr1*^{BL6} KO-CNf1, p = 0.0008; *Fmr1*^{BL6} KO-vehicle vs. WT^{BL6}-CNf1, p = 0.0003; *Fmr1*^{BL6} KO-CNf1 vs. WT^{BL6}-CNf1, p > 0.5). CNF1 treatment has no effect on filopodia density of WT neurons (p > 0.5). Moreover, we did not see any statistically significant differences in total protrusion density or spine density between groups (Figure 1H, I).

3.3. CNF1 infusion modulates phospho-cofilin/cofilin ratio in *Fmr1*^{FVB} KO animals to control levels

Cofilin is a constitutively active protein that damages actin filaments. Phosphorylation of cofilin at serine 3 causes inactivation, thereby allowing survival of elongating filaments. We quantified the levels of phospho-cofilin and total cofilin in the cortex of WT^{FVB} and *Fmr1*^{FVB} KO animals infused either with CNF1 or vehicle (Figure 1J). We observed a statistically significant genotype x treatment interaction in the ratio of phospho-cofilin/cofilin (Figure 1K; genotype x treatment effect, $F_{(1, 39)} = 8.504$ p = 0.0058). Bonferroni's posthoc comparisons revealed that *Fmr1*^{FVB} KO-vehicle animals show lower phospho-cofilin/cofilin ratio than WT^{FVB}-vehicle animals (p = 0.0225), an effect that was rescued after CNF1 treatment (p > 0.5). Moreover, although no differences between groups were observed in the phospho-cofilin levels, we observed a significant genotype x treatment interaction for total cofilin levels (data not shown; $F_{(1, 41)} = 7.152$ p = 0.0107). Posthoc tests revealed that CNF1 treatment increased the levels of cofilin in WT^{FVB}, but not in *Fmr1*^{FVB} KO animals (p = 0.0263).

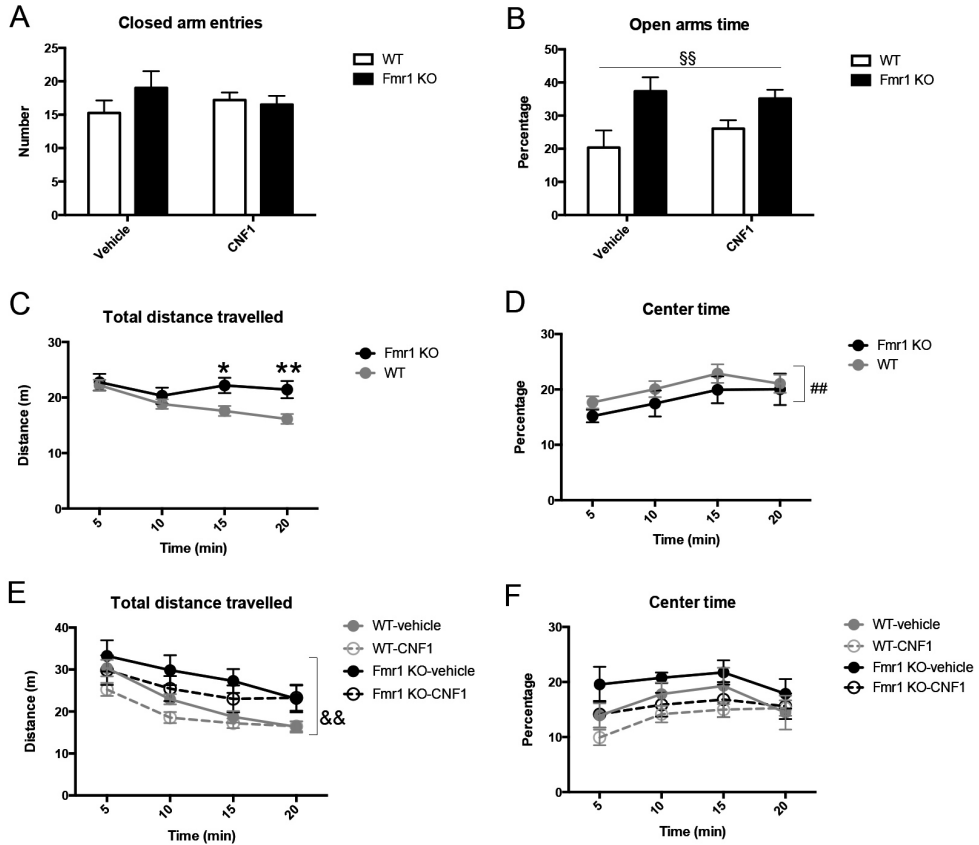


Figure 2: CNF1 treatment has no effect in either emotional or motor dysfunction of Fmr1 KO mice. (A) In the elevated plus maze no differences are observed between groups in the number of closed arms entries. **(B)** However, Fmr1^{FVB} KO animals spend more time in the open arms of the maze as compared with WT^{FVB} controls, irrespectively of CNF1 treatment. **(C)** In the open field, Fmr1^{FVB} KO animals show a lack of activity habituation and resulting hyperactivity compared to WT^{FVB} littermates. **(D)** By contrast, both genotypes similarly increase the center exploration in the course of the session, without genotype differences. When retested ten days after either CNF1 or vehicle administration, both **(E)** vehicle- and CNF1-treated Fmr1^{FVB} KO animals display hyperactivity compared with the respective WT^{FVB} controls. **(F)** However, no genotype or treatment effect is evident concerning the center exploration. Values are means + SEM. * $p < 0.05$; *** $p < 0.001$; genotype effect, $^{\$}$ $p < 0.01$; time effect, $^{\#}$ $p < 0.01$; time x group effect, $^{\&\&}$ $p < 0.01$.

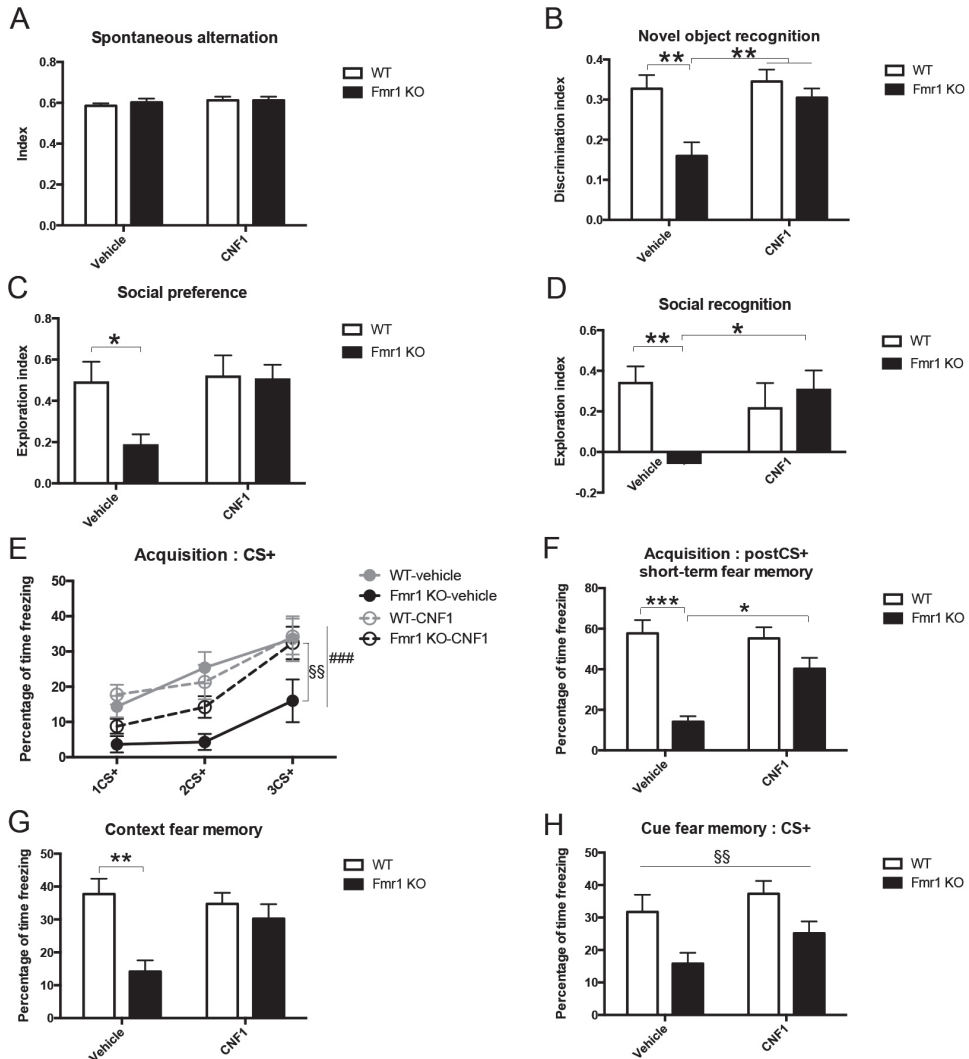


Figure 3: CNF1 administration rescues cognitive impairments in *Fmr1* KO animals. (A) No difference is observed in spontaneous alternation between *Fmr1*^{FVB} KO and *WT*^{FVB} treatment groups. (B) *Fmr1*^{FVB} KO animals show impairment in novel object discrimination index as compared with *WT*^{FVB} animals. This deficit is ameliorated upon CNF1 administration. In the social interaction test (C) a reduced interaction with a conspecific is evident in *Fmr1*^{FVB} KO-vehicle, as compared with *WT*^{FVB}-vehicle animals, that is fully recovered after CNF1 treatment. (D) Vehicle treated *Fmr1*^{FVB} KO mice furthermore show a profound impairment of social recognition memory that is rescued after CNF1 treatment to *WT*^{FVB} control levels. (E) In the course of fear memory learning *Fmr1*^{FVB} KO and *WT*^{FVB} mice increase their freezing behavior following CS+

(Figure 3: continued) presentations. Throughout the training session, *Fmr1*^{FVB} KO-vehicle animals show a reduction in the percentage of freezing time as compared with *WT*^{FVB}-vehicle animals. Levels are normalized by CNF1 treatment. (F) Short-term and (G) long-term context fear memory are impaired in *Fmr1*^{FVB} KO-vehicle animals and CNF1 administration rescues this deficit to control levels. (H) Auditory cued fear memory is reduced in *Fmr1*^{FVB} KO animals as compared with *WT*^{FVB} littermates regardless of the CNF1 treatment. Values are means + SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; treatment effect, ### $p < 0.001$; genotype effect, §§ $p < 0.01$; time x group effect, && $p < 0.01$.

3.4. CNF1 treatment has no effect on emotional and motor dysfunction exhibited by *Fmr1* KO mice

Fmr1^{FVB} KO and *WT*^{FVB} control animals (both vehicle and CNF1-treated groups) were tested for general anxiety in the elevated plus maze paradigm. After a 5 minutes session, no differences were observed in the total number of closed arm entries among groups (Figure 2A, $p > 0.5$), showing that general activity is not affected neither by genotype or by CNF1 treatment. However, *Fmr1*^{FVB} KO animals showed reduced anxiety levels, as revealed by the significantly higher percentage of time spent in the open arms as compared with *WT*^{FVB} animals (Figure 2B, genotype effect, $F_{(1, 13)} = 12.53$ $p = 0.0036$).

Spontaneous locomotor activity and exploration were measured in the open field apparatus before CNF1 treatment and re-tested in the same apparatus 10 days after treatment. Before treatment, *Fmr1*^{FVB} KO and *WT*^{FVB} animals exhibited a different locomotor activity pattern along the session (Figure 2C; genotype x time interaction $F_{(3, 84)} = 4.955$, $p = 0.0032$), in which *Fmr1*^{FVB} KO animals did not habituate to the open field and showing hyperactivity (Figure 2C; Bonferroni's posthoc; 15 min, $p = 0.0254$; 20 min, $p = 0.0072$). The percentage of time spent in the center of the apparatus in relation to the remaining area increases with time (Figure 2D; time effect, $F_{(3, 84)} = 4.924$ $p = 0.0034$), but no differences between genotypes were observed ($F_{(1, 28)} = 1.247$ $p = 0.2735$).

Animals were treated with either CNF1 or vehicle and were re-tested in the open field 10 days later. Repeated measures 2-way ANOVA revealed a statistically significant time x group interaction in the distance travelled (Figure 2E; $F_{(9, 78)} = 3.500$, $p = 0.0011$) that nevertheless did not survive posthoc comparisons. In addition, we observed a main effect of group ($F_{(3, 26)} = 3.731$ $p = 0.0235$) and a main effect of time ($F_{(3, 78)} = 88.90$ $p < 0.0001$) for the total distance travelled, indicating that both genotypes are adapting to the apparatus (although at different absolute values) with no effect of CNF1 treatment. Also, no changes were observed in the percentage of time spent in the center between vehicle and CNF1-treated *WT*^{FVB} and *Fmr1*^{FVB} KO animals (Figure 2F; $p > 0.5$).

3.5. CNF1 treatment rescues cognitive impairments in *Fmr1* KO animals

Fmr1^{FVB} KO and WT^{FVB} animals were tested in the Y-maze and the spontaneous alternation index was calculated. The total number of arm entries did not differ between groups (data not shown, $p > 0.5$), indicating that locomotor activity and motivation for exploration are not altered. Also, no differences were found in the spontaneous alternation index between treated and untreated WT^{FVB} and *Fmr1*^{FVB} KO animals (Figure 3A, $p > 0.5$), suggesting that hippocampal-dependent short-term memory is not altered between genotypes or affected by CNF1 treatment.

Novel object recognition (NOR) memory was tested within a 24 h retention period. We found a significant genotype x treatment interaction when comparing the NOR discrimination index between the groups (Figure 3B; $F_{(1, 46)} = 4.430$ $p = 0.0408$). Bonferroni's posthoc comparisons revealed that untreated *Fmr1*^{FVB} KO animals have an impaired NOR discrimination index as compared to untreated WT^{FVB} controls ($p = 0.0029$) and that CNF1 treatment rescues the impairment of *Fmr1*^{FVB} KO animals to WT^{FVB} values (*Fmr1*^{FVB} KO-vehicle vs. *Fmr1*^{FVB} KO-CNF1, $p = 0.0052$; *Fmr1*^{FVB} KO-vehicle vs. WT^{FVB}-CNF1, $p = 0.0003$; *Fmr1*^{FVB} KO-CNF1 vs. WT^{FVB}-CNF1, $p > 0.5$). CNF1 treatment has no effect in the NOR discrimination index of WT^{FVB} animals (WT^{FVB}-vehicle vs. WT^{FVB}-CNF1, $p = 0.074$).

In the social interaction test we first assessed social preference by measuring the time of exploration of each test animal towards a stranger animal versus that of an object and calculated an exploration index. Using a 2-way-ANOVA we observed a trend to a significant genotype x treatment interaction effect (Figure 3C, $F_{(1, 26)} = 2.686$ $p = 0.1133$). Posthoc comparisons showed that *Fmr1*^{FVB} KO-vehicle animals have a social preference impairment as compared with WT^{FVB}-vehicle controls (Bonferroni's posthoc, *Fmr1*^{FVB} KO-vehicle vs. WT^{FVB}-vehicle, $p = 0.0244$), not observed upon CNF1 treatment (Bonferroni's posthoc, *Fmr1*^{FVB} KO-CNF1 vs. WT^{FVB}-CNF1, $p > 0.5$). Next, a novel strange animal was introduced in the previously empty cylinder and social recognition was evaluated. A statistically significant genotype x treatment interaction was observed in the discrimination index (Figure 3D, $F_{(1, 27)} = 7.432$ $p = 0.0111$). Posthoc comparisons revealed that *Fmr1*^{FVB} KO are impaired in social recognition memory as compared with their WT control animals (Bonferroni's posthoc, *Fmr1*^{FVB} KO-vehicle vs. WT^{FVB}-vehicle, $p = 0.0017$). Moreover, CNF1 treatment rescues social recognition impairment of *Fmr1*^{FVB} KO animals to control levels (Bonferroni's posthoc, *Fmr1*^{FVB} KO-vehicle vs. *Fmr1*^{FVB} KO-CNF1, $p = 0.0112$; *Fmr1*^{FVB} KO-CNF1 vs. WT^{FVB}-CNF1, $p > 0.5$).

Fmr1^{FVB} KO and WT^{FVB} animals were trained in an auditory fear-conditioning paradigm and tested for emotional context and cue fear memory 24 h and 48 h after training. Mice were first presented to unpaired CS- in the conditioning box. Here, no differences were observed in the percentage of freezing levels to the CS- between groups (data not shown; $p > 0.5$). On the next day, animals were trained to associate an auditory

cue (CS+) with an unconditioned stimulus, a mild foot shock. The percentage of freezing levels increased in all tested groups with consecutive presentations of the CS+/US (Figure 3E; CS+ effect; $F_{(2, 90)} = 28.39$ $p < 0.0001$). We also observed a significant group effect (Figure 3E; $F_{(3, 45)} = 4.987$ $p = 0.0045$), which revealed that *Fmr1*^{FVB} KO-vehicle froze significantly less than WT^{FVB}-vehicle animals (Bonferroni's posthoc; 2CS+, $p = 0.0074$; 3CS+, $p = 0.0399$), an impairment that was rescued in *Fmr1*^{FVB} KO-CNF1 treated compared to WT^{FVB}-CNF1 animals ($p > 0.5$). We measured freezing levels in the 2 minutes after all CS+/US presentations and used as an indication of short-term fear memory. Analysis revealed a significant genotype x treatment effect (Figure 3F; $F_{(1, 45)} = 6.540$ $p = 0.0140$) in that *Fmr1*^{FVB} KO-vehicle animals show a reduced percentage of freezing time as compared to WT^{FVB}-vehicle animals (Bonferroni's posthoc; $p < 0.0001$) and CNF1 treatment rescued this impairment (Bonferroni's posthoc; *Fmr1*^{FVB} KO-vehicle vs. *Fmr1*^{FVB} KO-CNF1, $p = 0.0204$; *Fmr1*^{FVB} KO-CNF1 vs. WT^{FVB}-CNF1, $p > 0.5$).

Context fear memory was tested 24 h after CS+/US pairing in the same training box. *Fmr1*^{FVB} KO-vehicle animals showed significantly less percentage of freezing levels as compared to WT^{FVB}-vehicle animals, an effect that is rescued by CNF1 treatment (Figure 3G; genotype x treatment effect, $F_{(1, 45)} = 5.475$ $p = 0.0238$; Bonferroni's posthoc, *Fmr1*^{FVB} KO-vehicle vs. WT^{FVB}-vehicle, $p = 0.0015$; *Fmr1*^{FVB} KO-CNF1 vs. WT^{FVB}-CNF1, $p > 0.5$). Finally, mice were tested in a new environment for cue fear memory. The percentage of freezing levels of all groups did not differ in the first 2 minutes of the test before the presentation of the CS- nor during the presentation of the CS- (data not shown; $p > 0.5$) suggesting that no fear generalization has developed. When the CS+ was presented, treated and untreated *Fmr1*^{FVB} KO animals show a reduced percentage of freezing time as compared to WT^{FVB} controls (Figure 3H; genotype effect, $F_{(1, 45)} = 10.56$ $p = 0.0022$).

4. Discussion

Intellectual disability and cognitive impairments are main features of FXS patients¹. In *Fmr1* KO mouse models these features are reflected by a poor performance of mutants in several cognitive tasks that reflect learning and memory deficits (reviewed in Kazdoba *et al*³⁷). In this study we demonstrate that targeting the RhoGTPase pathway with the E.coli toxin CNF1 ameliorates this *Fmr1* KO phenotype. We demonstrate that CNF1 re-balances phospho-cofilin/ cofilin ratios in the mutants and restores immature spine morphology in *Fmr1* KO neurons indicating that it acts by recovering actin dynamics and synaptic functions in these cells. We then show that brain infusion of CNF1 toxin in *Fmr1* KO animals leads to a specific rescue of cognitive impairments in the novel object recognition, fear conditioning and contextual memory, and social recognition tasks. By contrast, no rescue was observed on locomotor hyperactivity or increased anxiety displayed by the *Fmr1* KO mice, indicating a selective recovery of cognitive functions. Intellectual disability and the underlying dendritic abnormalities are core features of FXS patients, both of which are modeled in mouse models of the disorder. Dendritic spines are protrusions along the dendrites that contain the majority of the excitatory

postsynaptic compartments. Spines are dynamic in morphology and strength, depending on the activity of the involved synapse, a process called synaptic plasticity^{38,39}. Many neurodevelopmental disorders are accompanied by morphological and functional anomalies of the spines, aberrant synaptic plasticity, leading to impairments in cognition and behavior^{40,41}. In postmortem material of FXS patients, long dendritic spines with increased spine density have been described⁴²⁻⁴⁴. This phenotype matches the morphology of spines during maturation and has been regarded as an immature phenotype. One hallmark of an immature spine phenotype is the increased number of immature, long dendritic spines with small heads, which are termed filopodia³³. Likewise, in the *Fmr1* KO mouse, a similar phenotype has been described in different brain areas, including the hippocampus and cortex^{31,40,45-54}. In addition, many studies have shown that targeting pathways involved in FXS can rescue these morphological anomalies⁵⁵. Here, we studied the dendritic spine morphology in primary hippocampal neuronal cultures from E17-18 pups. We cultured these for 21 days, when neurons *in vitro* are known to interconnect and form spines⁵⁶. We show a significant increased density of filopodia in the *Fmr1* KO neurons compared to their WT littermates, without differences in the density of the mature spines. This corresponds to the immature phenotype that has been previously described for FXS^{31,40,45,48}. However, different studies have reported variable and often contradictory results on spine morphology in FXS. These differences might be due to differences in mouse strains, age of mice, or time-point of neuronal culture during measurements. Also, studies differ in their use of *in vitro* versus *ex vivo* tissue and tissue derived from different brain areas. In addition, different staining or visualization techniques, different imaging techniques and different ways of quantification of spines have been published (reviewed in He *et al*⁸). All these differences in protocols and techniques make it difficult to compare published results. We tried to make the distinction between mature spines and immature filopodia objectively, by using the ratio of spine head/length ratio, instead of visual assignment³³. After treatment of the neurons with CNF1, we observed a correction of the increased filopodia density to WT levels. Cerri and colleagues¹³ have previously shown that CNF1 can affect spine density and length, together with reactivation of functional plasticity in adult rat visual cortex. This illustrates the importance of the RhoGTPase and actin dynamics in the regulation of synaptic plasticity in FXS. Also, our results underline the possibility of enhancing plasticity as a treatment for FXS. Although it is unknown in what way the rescue of the spine phenotype could be translated to clinical and/or behavioral improvement in FXS patients⁵⁷, our rescue of the immature spine phenotype suggests that targeting the RhoGTPase pathway might be considered as a therapeutic option for FXS.

In fact, we could demonstrate a general improvement of cognitive function in *Fmr1* KO mice upon intracerebroventricular infusion of CNF1. CNF1 toxin has been effective in improving cognitive function in mice in different cognitive behavioral tasks such as novel object recognition²², object in place²³, fear conditioning and spatial learning²¹. In our experiments we were able to specifically rescue the performance deficit of *Fmr1*

KO mice in a novel object recognition task and to increase the relative exploration of the novel object during the memory test session through CNF1 application. This effect appears to be related to the improvement of object specific memory, since no alteration in other parameters such as exploratory activity could be observed in this test. Our observations are in line with previously reported impairment of *Fmr1* KO mice in a novel object recognition memory task^{58,59} and suggest that CNF1 can improve the function of the perirhinal cortex and/ or hippocampus, which contribute to object recognition and object memorization in this task, respectively (reviewed in Antunes *et al*⁶⁰).

Further, we could demonstrate that treatment with CNF1 significantly ameliorates the social recognition memory deficits of *Fmr1* KO mice. Impairments in social recognition have previously been reported in *Fmr1* KO animals⁶¹ and are thought to represent an important aspect of the social interaction deficits that are considered a core symptom in FXS¹. They are based on complex cognitive processes whose neural substrates comprehend a wide range of brain regions, including medial amygdala, the medial preoptic area, the piriform cortex, olfactory bulb and the anterior cingulate cortex (reviewed in Van der Kooij *et al*⁶²). *Fmr1* KO animals are also known to show reduced non-social anxiety^{58,61} and hyperactivity¹⁷. Both features were also observed in our study, but not rescued by CNF1 treatment.

Finally, we found that deficits in *Fmr1* KO animals in contextual fear memory were also diminished upon CNF1 treatment. In fear conditioning, an initially aversive neutral stimulus or context is rapidly associated with an aversive stimulus and generates a conditioned fear response upon re-exposure, in rodents typically expressed as freezing behavior⁶³. While the amygdala mediates simple aversive stimulus association, the hippocampus is required for the acquisition and expression of fear memories to the training context (reviewed in Orsini *et al* and Sierra-Mercado *et al*^{64,65}). In line with previous findings (reviewed in Kazdoba *et al*³⁷), *Fmr1* KO animals showed a reduction in freezing levels during the acquisition of conditioned fear and during contextual fear memory retrieval, both of which were rescued to WT levels upon CNF1 treatment. By contrast, deficits of *Fmr1* KO animals in auditory cued fear memory were not rescued by CNF1 treatment, indicating a selective enhancement of hippocampus-dependent, but not amygdala-dependent learning in this task. This is in line with a general effect of CNF1 on cortical brain regions concerned with cognitive functions as also observed in the object recognition and social recognition paradigms. Moreover, the improvement of learning performance in the fear conditioning tasks suggests that CNF1 may have acted by improving synaptic efficacy at the time of training rather than actin-dependent memory consolidation processes.

To test whether the beneficial effect of CNF1 treatment is related to a recovery of actin dynamics in *Fmr1* KO mice, we examined cortical levels of cofilin and phospho-cofilin. Cofilin is a regulator of actin dynamics and is crucial for spine morphology, synaptic plasticity and learning (reviewed in Rust *et al*⁶⁶). Elevated levels of synaptic phosphorylated (inactive) cofilin 1 are associated with spine enlargement upon LTP induction or during learning (reviewed in Rust *et al*⁶⁶). An elevated cofilin activity as indicated by the observed reduction in phospho-cofilin/cofilin ratio in *Fmr1* KO mice is

therefore in agreement with the immature spine profile observed in untreated *Fmr1* KO neurons and their recovery by CNF1. In the past we showed that the reduced level of phospho-cofilin in the absence of FMRP was due to an elevated expression of PP2Ac that is higher in FXS ^{10,67,68}. We hypothesized that the abnormal actin remodeling we observed in FXS cell lines was due to an abnormal turnover of actin polymerization/depolymerization ¹⁰. Indeed, the more recent results showing that the low level of active cofilin is associated with an elevated level of Rac ^{10,17} and a decreased level of RhoA ⁶⁹ can support this hypothesis. FMRP negatively regulates Rac1 by binding to its RNA, and in turn, Rac1 positively regulates FMRP through inhibition of CYFIP, a cytoplasmic FMRP interacting partner ^{12,16,70}.

However, since no altered expression of PP2Ac in the presence of CNF1 was observed (not shown) we should conclude that CNF1 acts on the ratio of Rac1 and RhoA activation, balancing the level of these molecules versus PP2Ac and thus normalizing actin dynamics in *Fmr1* KO neurons.

5. Conclusion

CNF1, acting on cortical actin dynamics, ameliorates cognitive impairments in a mouse model of FXS but not disturbances in basic emotional or social behaviors. Recovery of spine morphology in hippocampal neurons and improvement of memory acquisition suggest an improvement of synaptic functions during learning to mediate these beneficial effects.

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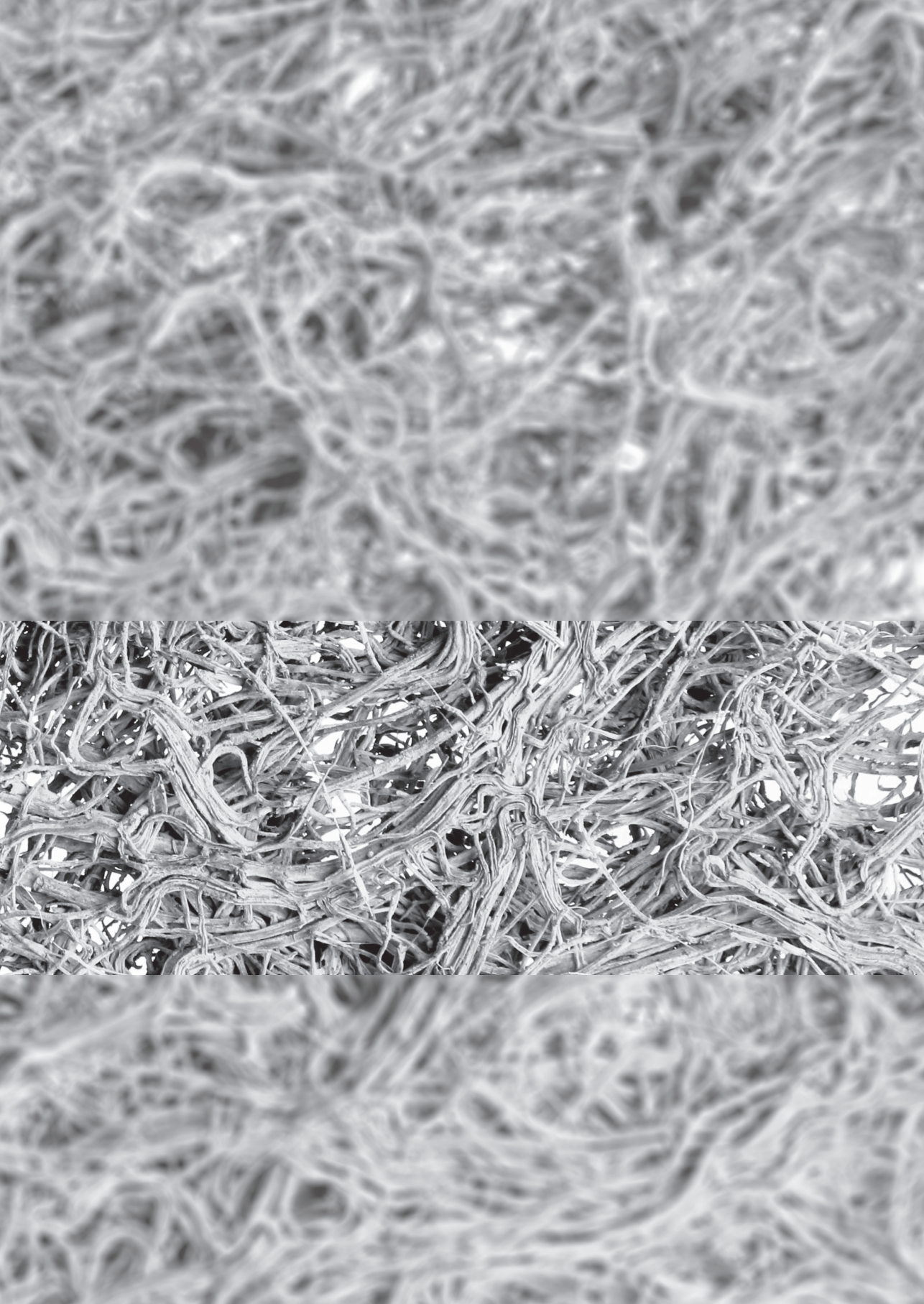
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CHAPTER 5

Targeting mGluR5 does not improve behavioral outcome measures in patients with fragile X syndrome: two randomized placebo-controlled trials with mavoglurant

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This study was part of a multicenter study, also performed in our center. The data, figures and tables are used from, based on or adapted from “Berry-Kravis E *et al*, Mavoglurant in fragile X syndrome: Results of two randomized, double-blind, placebo-controlled trials. *Sci Transl Med*. 2016 Jan 13;8(321):321ra5.” The data is property of Novartis Parma AG. Permission for its use was granted. Reprinted with permission from AAAS.

Abstract

Fragile X syndrome (FXS), an X-linked neurodevelopmental syndrome, is the most common inherited cause of intellectual disability and autism spectrum disorders. It leads to a wide range of psychiatric, behavioral, and physical problems. Currently, there is no disease modifying therapy. Various animal model studies have suggested aberrant mGluR5-dependent synaptic plasticity is involved in the pathogenesis of FXS. Downregulation of the aberrant mGluR5 pathway activity results in amelioration of several features, including the mGluR5 antagonist mavoglurant. Also, a phase 2 randomized, cross-over, placebo-controlled clinical trial using mavoglurant, showed improvement of symptoms in a subset of patients. Here we present the results of two multicenter, randomized, double-blind, placebo-controlled clinical trials with mavoglurant in adolescent (12-18 years, n=139) and adult (>18 years, n=175) patients with FXS. Participants received 25, 50, or 100 mg mavoglurant twice daily for 12 weeks, following a placebo run-in period. Stratification based on methylation status was applied. None of the strata showed a beneficial effect of mavoglurant on the primary outcome measure, ABC-CFX, and several other behavior checklists. We believe that future studies should be focused on developing more objective and suitable outcome measures for FXS, and probably start treatment at in childhood and for a longer period. In addition, the complexity of the FXS pathogenesis might require a combinational therapy to achieve relevant improvement.

1. Introduction

Fragile X syndrome (FXS) is one of the most frequent and well-studied monogenetic neurodevelopmental disorders with a prevalence of about 1:1:7000 in males and 1:11000 in females ^{1,2}. FXS is characterized by developmental delay and intellectual disability with an average IQ of 40 and a wide range of neuropsychiatric problems, including autism spectrum disorders, attention deficit hyperactivity disorder, mood disorders, and anxiety. Medical problems, like seizures, hypotonia, hyperlaxity, and recurrent otitis media are common ^{3,4}. Treatment is only symptom-based and not curative. There are currently no disease-modifying drugs available.

FXS is an X-linked disorder, in most cases caused by transcriptional silencing of the *FMR1* gene ⁵. This silencing is due to a CGG repeat expansion in the 5'-untranslated region of the gene and subsequent hypermethylation of the promotor region early in development ⁶. Thus, patients with FXS have severely reduced levels of the *FMR1*-protein (FMRP), which plays a major role in important neuronal functions, including synaptic plasticity. Probably its most explored function is binding and transport of specific mRNAs to the postsynaptic compartment and subsequent negative regulation of local protein synthesis, upon activation of the metabotropic glutamate receptor type 5 (mGluR5) ⁷. Absence of FMRP in the fragile X mouse model (*Fmr1* KO mouse) leads to exaggerated mGluR5-dependent protein synthesis and consequently aberrant synaptic plasticity. Initially, genetic and pharmacological reduction of mGluR5 restored several of the observed phenotypes in *Fmr1* KO mice ⁸ and led to the proposed pathophysiological mechanism in FXS: "the mGluR5 theory" ⁹. Since then, many preclinical studies have confirmed that both genetic reduction of mGluR5 and selective mGluR5 antagonists rescue FXS phenotypes in *Fmr1* KO mice and other FXS animal models (reviewed in ⁷). Thus far, several mGluR5 antagonists have been tested, including mavoglurant, a new and non-competitive mGluR5 antagonist ¹⁰.

These preclinical findings led to the first placebo-controlled, randomized, cross-over design clinical (phase 2) trial with mavoglurant in 30 males with FXS ¹¹. Patients were treated for 28 days with mavoglurant followed by 28 days with placebo or vice versa. Although no significant improvement was measured compared with placebo, post-hoc analysis on a subset of patients, with a completely methylated *FMR1* promotor and complete absence of *FMR1* mRNA in blood, suggested improvement, measured with the Aberrant Behavior Checklist scale (ABC-C) ¹², Clinical Global Impression – Improvement scale (CGI-I) ¹³ and Repetitive Behavior Scale (RBS) ^{14,15}.

These results prompted the initiation of the current large, international, double-blind placebo-controlled clinical trial in adult and adolescent patients with FXS. Patients were treated with mavoglurant, with a dose of 25, 50 or 100 mg twice daily (bis in die, bid) or with placebo for 12 weeks. Following the previous study results, patients were divided in two strata, dependent on whether they showed completely methylated or partially methylated *FMR1* promotor in blood. No significant improvement could be

demonstrated in any of the used scales in any of the patient groups, compared with placebo. Here we outline the results and discuss possible explanations for the negative results and future prospectives.

2. Materials and methods

2.1 Patients

Two clinical trials were performed with male and female patients with FXS. In the adolescent trial, patients were aged 12-17 years and in the adult trial 18-45 years. All patients had a confirmed genetic diagnosis of FXS (CGG repeat expansion > 200 in the *FMR1* gene and methylation of the promotor region). For all patients, informed consent was obtained from a parent, legal guardian, or representative.

Inclusion criteria were CGI-S score of ≥ 4 , ABC-C score of >20 and IQ score of 2 SDs below IQ test median at the screening visit. A caregiver who spent at least 6 hours per day with the patient was necessary, in order to keep track of and administer medication, and to assess questionnaires. The caregiver who completed the ABC-C was trained using a video. Female patients had to use a reliable form of contraception, before being included.

Important exclusion criteria were unacceptable values of laboratory tests (according to protocol), abnormal ECG results or a history of ECG abnormalities, medical history of psychiatric disorders (schizophrenia, bipolar disease, psychosis, and repeated hallucinations), uncontrolled epilepsy in the past 2 years, and cancer within the past 5 years other than localized skin cancer. Also the use of more than two psychoactive drugs, the use of CYP3A4 inhibitors/inducers, and the use of glutamatergic agents within 6 weeks prior to randomization were considered as exclusion criteria.

2.2. Study design

Both studies tested mavoglurant in a phase 2b, randomized, double-blind, placebo-controlled and parallel group setting in patients with FXS (NCT01253629 and NCT01357239). 31 centers in 10 countries participated in the adult trial and 38 centers in 16 countries in the adolescent trial, between November 2010 and January 2014 (supplementary Table 1).

Figure 1 depicts the study design. All patients started with 4 weeks of single-blind placebo run-in period, followed by a 12 week double-blind period. Subsequently, patients were randomized to one of the four treatment arms using Interactive Response Technology. The 4 treatment arms consisted of 3 different doses of mavoglurant: 25, 50, and 100 mg bid and placebo (Figure 1). Mavoglurant doses started with 25 mg bid and were up-titrated until the designated target dose.

If patients did not tolerate the dose, they were excluded and discontinued the trial. Upon

completion or discontinuation due to intolerability of the assigned dose, patients could enroll in the open-label extension study (NCT01348087 or NCT01433354). These results will be published separately. The groups were stratified for gender and geographical region. Region consisted of either the United States, Canada, and Australia, where a large proportion of patients are being treated with psychoactive drugs, or Europe, where less psychoactive drugs are being prescribed. The stratification for region was only used to check balanced distribution of patients among both regions. Furthermore, stratification for methylation status of the *FMR1* gene (DNA sample collected at the screening visit) was performed and patients were divided in complete methylation or partial methylation of the *FMR1* gene promotor. Difficulties in recruiting adolescent patients led to a later modification of the protocol and subsequent patients were randomized into either 100 mg mavoglurant bid or placebo. Thus, the 25 and 50 mg doses were not analyzed in the primary endpoint.

2.3. Assessments and outcome measures

2.3.1. Behavioral tests

Primary and key secondary outcome measures: The primary outcome measure in both studies was changes in total score of ABC-CFX^{12,16} in the completely methylated stratum. The key secondary outcome measure was changes in total score of ABC-CFX in the partially methylated stratum.

Other secondary outcome measures: Several other secondary outcome measures were used for both groups: CGI-I scale¹³, specific subscales of ABC-CFX, RBS-R^{14,15} and at specific sites also Social Responsive Scale (SRS)-A in adults or SRS in adolescents¹⁷. The used tests and timing are described more extensively by Berry-Kravis *et al*¹⁸. In short, ABC-C, RBS-R and SRS are caregiver-rated symptom checklists, assessing problems in behavior, repetitive behavior, and autistic impairment. The ABC-CFX is a modified version of the ABC-C, specifically adapted for FXS patients¹⁶. CGI-S scale is assessed by a clinician and expresses severity of illness, while the CGI-I expresses improvement¹³.

2.3.2. Cognitive tests

Computerized Neurocognitive Test Vital Signs (CNS VS) cognitive battery in adults¹⁹ and several subtests of the Test of Everyday Attention for Children (TEA-Ch) in adolescents²⁰ were used, assessing working memory and attention and inhibition, respectively. The specific tests are discussed by Berry-Kravis *et al*¹⁸.

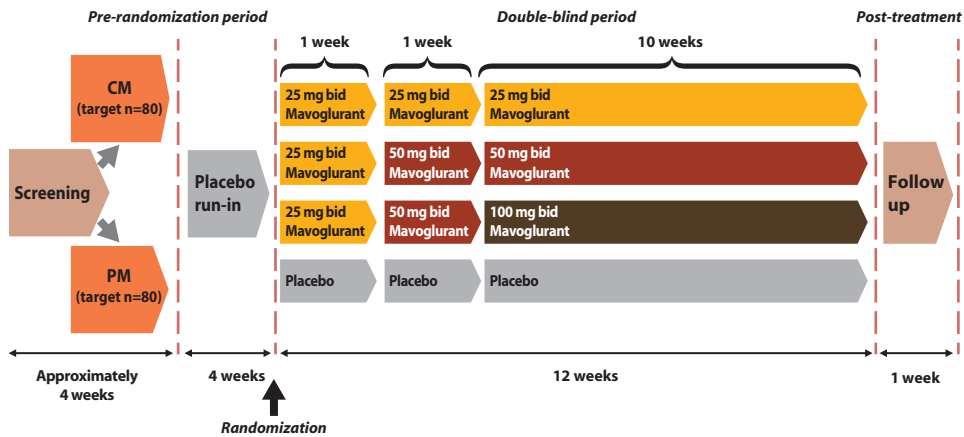


Figure 1: Study design. CM: complete methylation PM: partial methylation bid: twice daily

2.3.3. Safety measures

In addition, safety was assessed, using recording adverse events (AEs), medical physical examination, ECG and laboratory tests (hematology, blood chemistry and urine tests). The Neuropsychiatric inventory questionnaire (NPI-Q) was used to assess neuropsychiatric events during treatment ²¹.

2.4. Statistical analysis

Power calculation and statistics are described extensively in Berry-Kravis *et al* ¹⁸. In short, difference in effect between mavoglurant and placebo was calculated after 12 weeks of treatment, based on a mixed-model for repeated measures. Fixed effects are region, treatment, week, and interaction of treatment by week. ABC-CFX baseline score is covariate with unstructured covariance structure. Each treatment arm was compared with placebo after 12 weeks of treatment and presented with two-sided 95% confidence interval (CI) and p value. Type I error rate was controlled for by a multiplicity adjustment to the primary and key secondary outcome and the required level of significance was smaller than 0.05. All data were analyzed in two strata and combined, all according to initial randomization.

Table 1. Demographics and other baseline characteristics, by treatment and age.

Baseline characteristic	Placebo	Mavoglurant 25 mg bid	Mavoglurant 50 mg bid	Mavoglurant 100 mg bid
Adults	N=44	N=44	N=42	N=45
Adolescent	N=42	N=31	N=27	N=39
Age (years)				
Adults	26.2 (7.21)	26.9 (6.76)	26.7 (6.90)	24.2 (6.07)
Adolescents	14.4 (1.85)	14.4 (1.70)	14.6 (1.58)	14.6 (1.77)
Sex, male n (%)				
Adults	41 (93.2)	41 (93.2)	40 (95.2)	42 (93.3)
Adolescents	39 (92.9)	26 (83.9)	24 (88.9)	35 (89.7)
Race, Caucasian n (%)				
Adults	43 (97.7)	41 (93.2)	40 (95.2)	44 (97.8)
Adolescents	39 (92.9)	27 (87.1)	26 (96.3)	37 (94.9)
Region, European n (%)				
Adults	19 (43.2)	14 (31.8)	21 (50.0)	20 (44.4)
Adolescents	26 (61.9)	17 (54.8)	15 (55.6)	24 (61.5)
Baseline weight (kg)				
Adults	78.37 (18.63) (15.99)	83.39 (15.99)	83.47 (14.15)	77.35 (19.44)
Adolescents	83.47 (14.15) 77.35 (19.44) 60.96 (18.09)	60.11 (16.21)	65.62 (18.37)	60.75 (15.44)
Baseline height (cm)				
Adults	174.3 (8.69)	173.2 (8.88)	176.4 (5.89)	175.2 (7.58)
Adolescents	165.5 (10.50)	164.1 (11.54)	168.3 (9.58)	166.1 (10.74)
Baseline ABC-C _{FX} total score ^[a]				
Adults	44.6 (25.85)	40.9 (23.44)	48.4 (29.11)	38.3 (23.04)
Adolescents	46.8 (28.25)	56.7 (24.03)	48.9 (26.05)	50.2 (26.03)
Baseline ABC-C total score ^[b]				
Adults	46.5 (27.14)	42.6 (24.32)	50.6 (30.05)	40.0 (23.98)
Adolescents	48.3 (29.4)	58.5 (25.0)	50.7 (26.9)	52.1 (27.23)
Baseline CGI-S score, n (%)				
Adults				
3 (Mildly ill)	0	1 (2.3)	0	1 (2.2)
4 (Moderately ill)	14 (31.8)	17 (38.6)	18 (42.9)	18 (40.0)
5 (Markedly ill)	18 (40.9)	20 (45.5)	15 (35.7)	19 (42.2)
6 (Severely ill)	11 (25.0)	6 (13.6)	8 (19.0)	6 (13.3)
7 (Among the most extremely ill) patients)	1 (2.3)	0	1 (2.4)	1 (2.2)
Adolescents				
2 (Borderline mentally ill)	1 (2.4)	0	0	1 (2.6)
3 (Mildly ill)	0	0	0	1 (2.6)
4 (Moderately ill)	14 (33.3)	13 (41.9)	10 (37.0)	13 (33.3)
5 (Markedly ill)	17 (40.5)	9 (29.0)	10 (37.0)	17 (43.6)
6 (Severely ill)	9 (21.4)	8 (25.8)	6 (22.2)	7 (17.9)
7 (Among the most extremely ill) patients)	1 (2.4)	1 (3.2)	1 (3.7)	0
Patient IQ score in percentiles ^[c]				
Adults	0.06 (0.156)	0.03 (0.075)	0.09 (0.193)	0.11 (0.307)
Adolescents	0.02 (0.045)	0.02 (0.034)	0.03 (0.043)	0.05 (0.166)

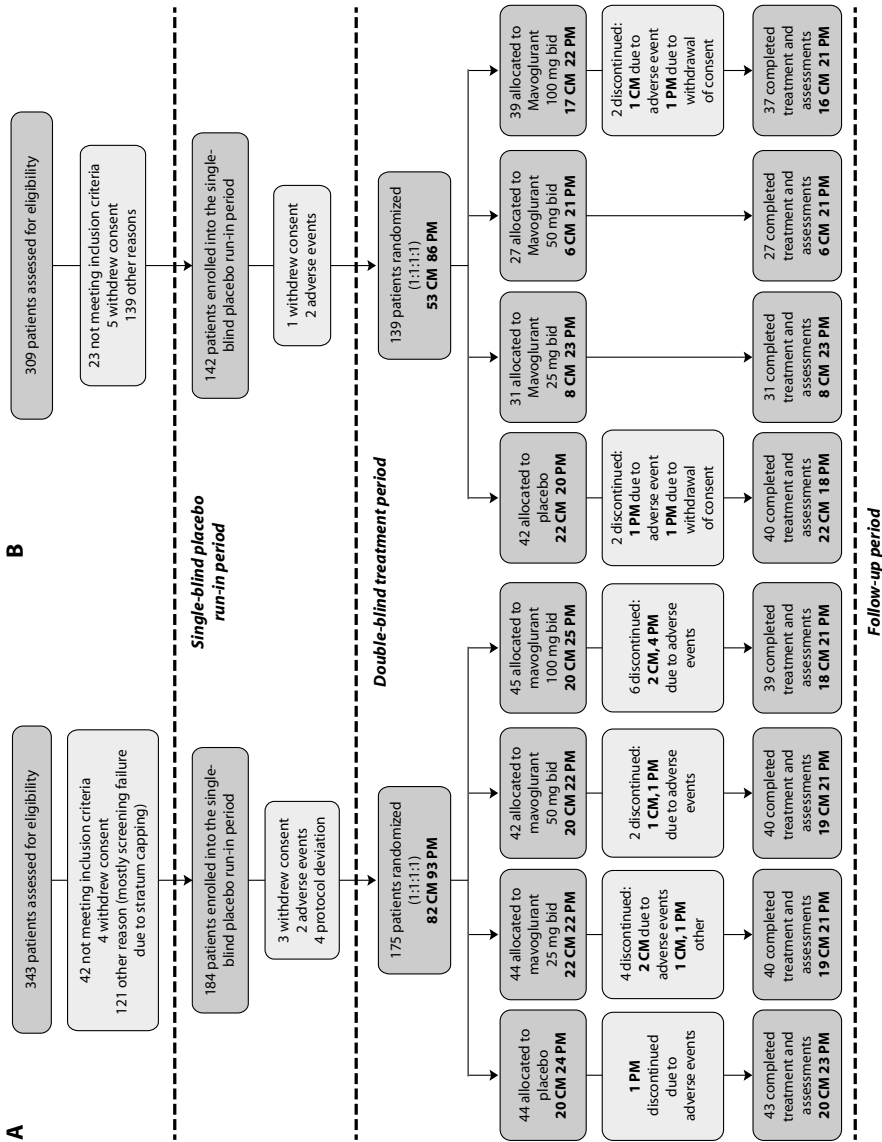


Figure 2: Consort diagram of the patients that were assessed, enrolled and randomized in both trials. (A) Adult trial. (B) Adolescent trial. CM: complete methylation PM: partial methylation bid: twice daily

3. Results

3.1. Patients

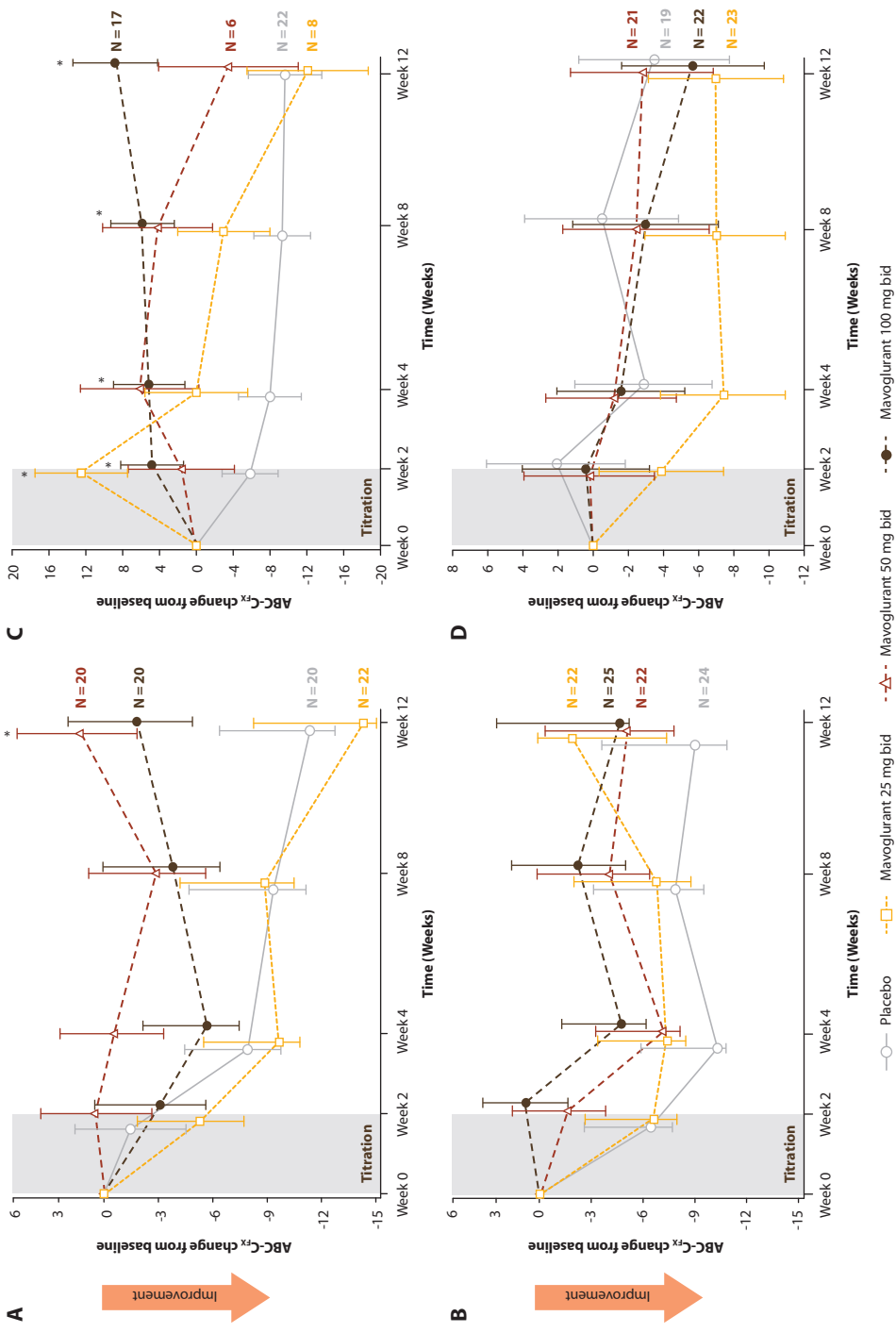
Figure 2 shows the flow and inclusion of patients. 175 adults (Figure 2A) and 139 adolescents (Figure 2B) were randomized into the four treatment arms, of which 162 and 135 completed the trial, respectively. 13 patients discontinued due to AEs. Due to slow enrollment in the adolescent study a protocol amendment excluded the 25 and 50 mg bid treatment arms for patients that were included afterwards. Since patients with partial methylation were more abundant than patients with complete methylation (ratio 2:1), slightly more patients with partial methylation were included and more patients had to be screened to fill the complete methylation stratum. Table 1 represents the demographic and background characteristics, which were comparable for all groups, especially for mean age and race. Since FXS is an X-linked disorder, more males are affected, which explains the higher number of males included (83%). Baseline measures were overall comparable, besides the ABC-C and ABC-CFX, which were higher in the adult 50 mg bid stratum and the adolescent 25 mg bid stratum. Almost all participants were Caucasian.

Data is presented as mean (SD). Percentage (%) is calculated based on the patients in the randomized set within the specified stratum. Bid: twice daily. ^[a] Based on the 55 item scale. ^[b] Based on the full 58 item scale. ^[c] Percentile was calculated using a standard normal distribution.

3.2. Primary and key secondary outcome measure

After 12 weeks of treatment, the ABC-CFX total score did not improve significantly in patients with FXS and a completely methylated *FMR1* gene promotor (primary outcome measure) and in patients with partially methylated *FMR1* gene promotor (key secondary outcome measure), compared to the placebo group. This was the case for all of the strata, in both studies (Figure 3). The results are discussed more extensively in Berry-Kravis *et al*¹⁸. In short, during the placebo run-in period, improvements of ABC-CFX were measured in both studies in all strata, including placebo (Figure S1). Also afterwards, an improvement was measured in all strata, however, none of the treatment arms showed a significant improvement compared with placebo. A small but significant worsening of ABC-CFX score from baseline compared with placebo was even measured in the adult 50 mg bid stratum ($p=0.018$) and adolescent 100 mg bid stratum ($p=0.004$). The results for the completely and partially methylated groups were comparable. In the partially methylated strata, all arms showed improvement, the largest improvement being in the placebo arm (adult study) and 25 mg bid (adolescent study). All strata were not significantly different from the placebo group.

Figure 3



*Figure 3: ABC-CFX mean change from baseline (week 0) until completion of trial (week 12), not including the placebo run-in period, of the adult and adolescent trial, for both the completely and partially methylated strata, treated with placebo, 25, 50 or 100 mg bid mavoglurant. (A) Adult population with completely methylated FMR1 promotor. 50 mg bid, week 12: $P=0.018$ (B) Adult population with partially methylated FMR1 promotor. 100 mg bid, week 2: $P=0.022$, week 4: $P=0.013$; week 8: $P=0.002$; week 12: $P=0.004$; 25 mg bid, week 2: $P=0.003$. (D) Adolescent population with partially methylated FMR1 promotor. Data presented as least squares (LS) means ($\pm$ SEM). * statistical significance at 5% level*

3.3. Other secondary outcome measures

3.3.1. Behavioral tests

None of the treatment groups showed a significant improvement in any of the behavioral scales (CGI-I, ABC-CFX subscales and RBS-R), compared with placebo, from baseline to 12 weeks of treatment (Tables 2-5).

In two subscales of ABC-CFX in the adult trial, a significant difference was measured, in favor of placebo group (Table 2). Also, stratification for methylation status did not reveal a significant improvement for both completely and partially methylated groups and showed even small but significant worsening on several subscales in the adolescent completely methylated group (supplementary data of Berry-Kravis 2016¹⁸). For the CGI-I, RBS-R and SRS, no significant difference was measured after 12 weeks of treatment in any of the strata compared with placebo (Tables 3, 4, and 5).

3.3.2. Cognitive tests

The results of the cognitive outcome measures (CNS VS and TEA-Ch) are summarized in Tables 6 and 7. Many patients were not able to perform these tests, possibly as a result of their intellectual and behavioral impairments. As a consequence, a small number of patients per stratum remained, yielding inconclusive results.

3.3.3. Effect of age and methylation

A younger age did not predict a better outcome on the ABC-CFX, tested in a post hoc analysis pooling both studies and considering age as a continuous variable (Figure 4). Also the level of methylation did not correlate with the efficacy of the treatment (Figure 5).

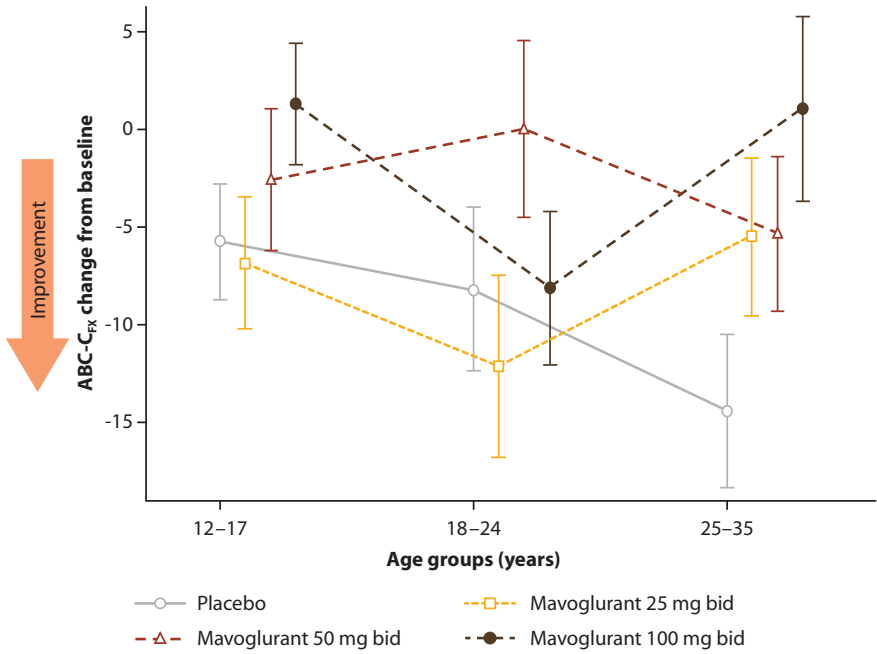


Figure 4: ABC-CFx total scores correlated to age from baseline to week 12. Data is presented as LS means ($\pm$ SEM).

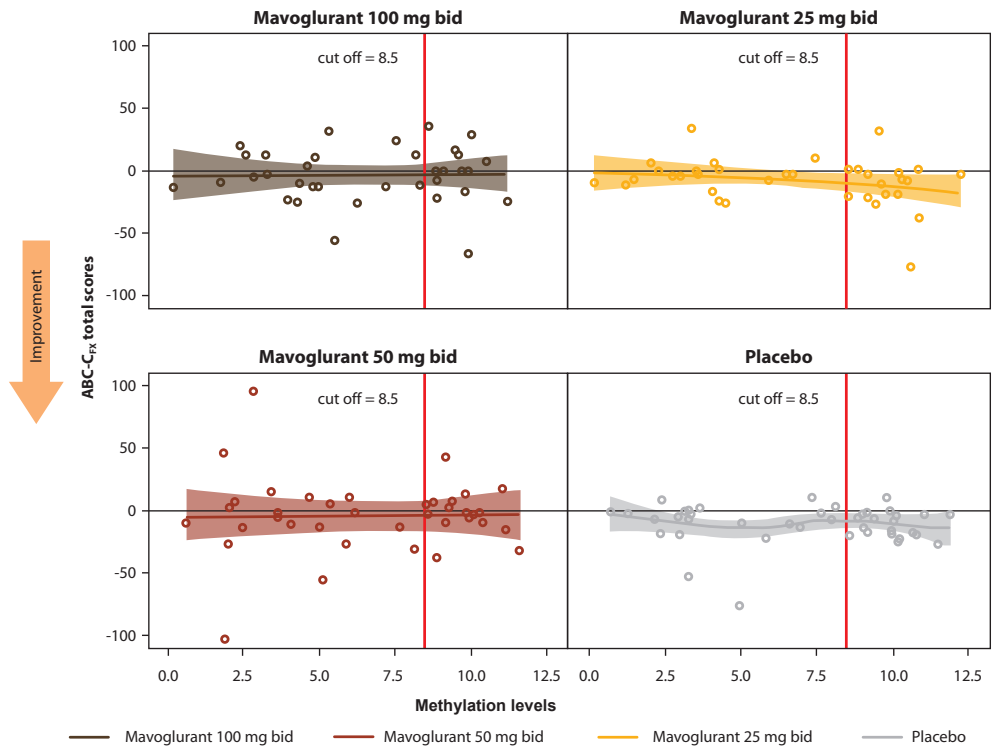


Figure 5: ABC-CFX changes of total scores in comparison to baseline (X-axis) in relation to methylation levels in blood (Y-axis) from adult and adolescent patients together. The methylation analysis was performed during screening. A methylation level of ≥ 8.5 is considered FXS with completely methylated FMR1 promotor. Methylation level < 8.5 is considered partially methylated FMR1 promotor. Bid: twice daily

Table 2. Change from baseline to week 12 for ABC-C_{FX} subscale scores in all patients.

ABC-C _{FX} subscale	Placebo		Mavoglurant 25 mg bid		Mavoglurant 50 mg bid		Mavoglurant 100 mg bid	
	Adult	Adolescent	Adult	Adolescent	Adult	Adolescent	Adult	Adolescent
Irritability	N=44 -3.2 (1.13)	N=41 -1.2 (1.22)	N=44 -2.8 (1.22)	N=31 -1.9 (1.41)	N=42 1.0 (1.18)	N=27 -0.6 (1.52)	N=45 -0.7 (1.21)	N=39 1.5 (1.28)
Lethargy/withdrawal	-2.2 (0.72)	-1.3 (0.71)	p=0.781 -1.7 (0.77)	p=0.687 -0.9 (0.83)	p=0.010* -0.9 (0.75)	p=0.785 -1.0 (0.89)	p=0.121 -0.9 (0.76)	p=0.137 0.5 (0.75)
Stereotypic behavior	-1.1 (0.43)	-0.8 (0.45)	p=0.627 -1.1 (0.46)	P=0.660 -1.6 (0.52)	p=0.203 -0.7 (0.45)	p=0.760 -0.4 (0.55)	p=0.24 -0.3 (0.46)	p=0.081 0.0 (0.47)
Hyperactivity	-1.7 (0.64)	-1.0 (0.67)	p=0.946 -1.6 (0.68)	p=0.226 -1.9 (0.79)	p=0.590 -0.6 (0.66)	p=0.598 0.3 (0.84)	p=0.232 -1.5 (0.67)	p=0.218 -0.6 (0.71)
Inappropriate speech	-0.9 (0.38)	-0.8 (0.33)	p=0.864 -0.2 (0.41)	p=0.415 -0.9 (0.38)	p=0.224 0.2 (0.40)	p=0.231 0.0 (0.4)	p=0.797 0.2 (0.40)	p=0.663 0.1 (0.34)
Social avoidance	-1.2 (0.36)	-1.1 (0.31)	p=0.185 -0.2 (0.39)	p = 0.952 -0.6 (0.35)	p=0.051 -0.8 (0.37)	p=0.091 -0.9 (0.38)	p=0.042* -0.3 (0.38)	p=0.055 -0.6 (0.32)
			p=0.052	p = 0.231	p=0.474	p=0.612	p=0.074	p=0.220

Data are presented as least square mean (SE) unless specified otherwise.

* Indicates statistical significance at 5% level. Bid: twice daily.

Table 3. CGI-I score at week 12 per treatment, for all patients and divided by methylation status.

Statistic	Placebo		Mavoglurant 25 mg bid		Mavoglurant 50 mg bid		Mavoglurant 100 mg bid	
	Adult	Adolescent	Adult	Adolescent	Adult	Adolescent	Adult	Adolescent
CM stratum n	N=20 20	N=22 22	N=22 15	N=8 8	N=20 17	N=6 6	N=20 15	N=17 15
Mean (SE)	3.5 (0.20)	3.1 (0.18)	3.2 (0.22)	3.3 (0.30)	3.5 (0.21)	3.8 (0.34)	3.5 (0.22)	3.5 (0.22)
Mavoglurant –PBO (95% CI)			-0.3 (-0.9, 0.3)	0.2 (-0.5, 0.9)	-0.1 (-0.6, 0.5)	0.7 (-0.1, 1.5)	0.0 (-0.6, 0.6)	0.4 (-0.2, 1.0)
p-value			0.272	0.586	0.814	0.083	0.951	0.163
PM stratum n	N=24 22	N=19 18	N=22 19	N=23 23	N=22 21	N=21 21	N=25 20	N=22 20
Mean (SE)	3.6 (0.21)	3.4 (0.21)	3.4 (0.22)	3.5 (0.19)	3.0	3.2 (0.20)	3.6	3.5 (0.20)
Mavoglurant –PBO (95% CI)			-0.2 (-0.8, 0.4)	0.1 (-0.5, 0.6)	-0.5 (-1.1, 0.1)	-0.2 (-0.8, 0.4)	0.0 (-0.5, 0.6)	0.1 (-0.4, 0.7)
p-value			0.498	0.798	0.080	0.480	0.884	0.628
All patients n	N=44 42	N=41 40	N=44 34	N=31 31	N=42 38	N=27 27	N=45 35	N=39 35
Mean (SE)	3.5 (0.14)	3.3 (0.14)	3.3 (0.15)	3.5 (0.16)	3.2	3.3 (0.17)	3.6	3.6 (0.15)
Mavoglurant –PBO (95% CI)			-0.3 (-0.7, 0.2)	0.2 (-0.2, 0.6)	-0.3 (-0.7, -0.3)	0.1 (-0.4, 0.5)	0.0 (-0.4, 0.4)	0.3 (-0.1, 0.7)
p-value			0.229	0.367	0.118	0.737	0.966	0.144

CGI-I: Clinical Global Impression-improvement scale; PBO: placebo; CM: complete methylation; PM: partial methylation; bid: twice daily.

Table 4. Repetitive Behaviors Scale – Revised (RBS-R) total score at baseline, Week 12 and change from baseline to Week 12 for all patients.**A**

Adult population	Placebo	Mavoglurant 25 mg bid	Mavoglurant 50 mg bid	Mavoglurant 100 mg bid
RBS-R Total Score	N=44	N=44	N=42	N=45
Baseline				
Mean (SD)	26.3 (18.84)	24.4 (18.78)	27.7 (19.91)	24.2 (16.14)
Week 12				
n	42	34	38	35
Mean (SD)	21.5 (14.74)	18.8 (17.30)	24.8 (18.71)	20.3 (17.12)
Change from baseline to Week 12				
LS Mean (SE)	-5.1 (1.53)	-4.4 (1.69)	-2.8 (1.60)	-3.4 (1.65)
Mavoglurant -PBO Diff (95% CI) p-value		0.7 (-3.8, 5.2) 0.747	2.4 (-2.0, 6.7) 0.289	1.7 (-2.7, 6.2) 0.443

B

Adolescent population	Placebo	Mavoglurant 25 mg bid	Mavoglurant 50 mg bid	Mavoglurant 100 mg bid
RBS-R Total Score	N=41	N=31	N=27	N=39
Baseline				
Mean (SD)	29.2 (18.99)	30.2 (17.36)	27.2 (19.17)	29.9 (19.35)
Week 12				
n	40	31	27	36
Mean (SD)	22.3 (16.94)	25.9 (15.59)	21.4 (17.43)	27.4 (20.27)
Change from baseline to Week 12				
LS Mean (SE)	-6.0 (1.66)	-3.4 (1.93)	-5.7 (2.08)	-0.6 (1.74)
Mavoglurant -PBO Diff (95% CI) p-value		2.5 (-2.6, 7.6) 0.329	0.3 (-5.0, 5.6) 0.915	5.3 (0.6, 10.1) 0.028*

(A) adult study and **(B)** adolescent study. Data presented as least squares means (SE) and difference in least squares mean between each treatment group and placebo with two-sided 95% confidence interval. * Indicates statistical significance at 0.05 level. Bid: twice daily; CI: confidence interval; Diff: difference; LS: least squares; PBO: placebo; RBS-R: Repetitive Behaviors Scale – Revised; SD: standard deviation; SE: standard error.

Table 5. Social Responsiveness Scale (SRS) total score at baseline, Week 12 and change from baseline to Week 12**A**

Adult population	Placebo	Mavoglurant 25 mg bid	Mavoglurant 50 mg bid	Mavoglurant 100 mg bid
SRS-A	N=44	N=44	N=42	N=45
Baseline				
n	41	41	33	40
Mean (SD)	92.8 (31.08)	90.8 (22.75)	93.9 (28.39)	93.7 (26.44)
Week 12				
n	39	31	32	31
Mean (SD)	86.7 (27.62)	82.1 (23.47)	84.5 (32.76)	88.2 (26.85)
Change from baseline to Week 12				
LS Mean (SE)	-6.6 (2.66)	-5.7 (2.98)	-9.0 (2.94)	-6.6 (2.94)
Mavoglurant		0.9	-2.4	0.0
-PBO Diff		(-7.0, 8.7)	(-10.2, 5.4)	(-7.8, 7.8)
(95% CI)				
p-value		0.825	0.542	0.994

B

Adolescent population	Placebo	Mavoglurant 25 mg bid	Mavoglurant 50 mg bid	Mavoglurant 100 mg bid
SRS	N=41	N=31	N=27	N=39
Baseline				
n	38	31	27	35
Mean (SD)	100.4 (28.38)	98.6 (23.27)	93.4 (22.03)	98.8 (22.81)
Week 12				
n	37	31	27	32
Mean (SD)	93.1 (29.79)	93.1 (22.07)	86.6 (24.28)	94.2 (23.37)
Change from baseline to Week 12				
LS Mean (SE)	-6.0 (2.58)	-5.5 (2.88)	-7.8 (3.10)	-4.2 (2.78)
Mavoglurant		0.6	-1.8	1.9
-PBO Diff		(-7.1, 8.2)	(-9.8, 6.2)	(-5.6, 9.4)
(95% CI)				
p-value		0.882	0.657	0.623

All patients (CM and PM) (A) adult study and (B) adolescent study. Data presented as least squares means (SE) and difference in least squares mean between each treatment group and placebo with two-sided 95% confidence interval. * Indicates statistical significance at 0.05 level. Bid: twice daily; CI: confidence interval; Diff: difference; LS: least squares; PBO: placebo; SD: standard deviation; SE: standard error; SRS: social responsiveness

Table 6. Test of Everyday Attention in Childhood (TEA-Ch)

CNS VS Modules	Placebo N=44	Mavoglurant 25 mg bid N=44	Mavoglurant 50 mg bid N=42	Mavoglurant 100 mg bid N=45
BASELINE non-missing assessments, n	13	8	12	11
Visual memory (VIM), n (%)	12 (92.3)	7 (87.5)	12 (100.0)	11 (100.0)
Symbol digit coding (SDC), n (%)	9 (69.2)	8 (100.0)	8 (66.7)	9 (81.8)
Perception of emotion (POET), n (%)	6 (46.2)	6 (75.0)	9 (75.0)	6 (54.5)
Part 2 of the 3-part CPT, n (%)	9 (69.2)	8 (100.0)	11 (91.7)	9 (81.8)
Part 3 of the 3-part CPT, n (%)	9 (69.2)	7 (87.5)	10 (83.3)	8 (72.7)
WEEK 12 non-missing assessments, n	13	8	13	10
Visual memory (VIM), n (%)	11 (84.6)	8 (100.0)	13 (100.0)	10 (100.0)
Symbol digit coding (SDC), n (%)	11 (84.6)	7 (87.5)	10 (76.9)	9 (90.0)
Perception of emotion (POET), n (%)	6 (46.2)	6 (75.0)	9 (69.2)	7 (70.0)
Part 2 of the 3-part CPT, n (%)	9 (69.2)	6 (75.0)	12 (92.3)	9 (90.0)
Part 3 of the 3-part CPT, n (%)	7 (53.8)	6 (75.0)	11 (84.6)	10 (100.0)

*Test of Everyday Attention in Childhood (TEA-Ch) change from baseline in raw scores for the 3 subsets during the double-blind treatment period for all patients. Summary statistics are presented for the change from baseline after 12 weeks of treatment in each subtest raw score by treatment group. n = number of patients with non-missing baseline and post-baseline values. * Lower scores denote improvement. Bid: twice daily.*

Table 7. CNS Vital Signs

All Patients	Placebo N=41	Mavoglurant 25 mg bid N=31	Mavoglurant 50 mg bid N=27	Mavoglurant 100 mg bid N=39
Map Mission: # of targets correctly identified				
n	32	28	20	31
Mean (SD)	1.4 (7.69)	0.9 (6.76)	2.9 (5.55)	1.0 (7.13)
Walk/Don't walk: total correct				
n	25	20	16	22
Mean (SD)	-0.3 (4.59)	-0.8 (2.79)	-1.3 (3.09)	0.2 (3.35)
Opposite worlds*				
Same world total time				
n	26	22	16	20
Mean (SD)	-13.9 (25.26)	-7.3 (22.11)	2.6 (17.54)	-2.2 (10.95)
Opposite world total time				
n	24	20	14	20
Mean (SD)	-11.5 (33.59)	-4.2 (48.42)	-27.0 (59.83)	-6.1 (11.97)

Number and percentage of patients scoring better than results achieved by chance (probability) on the modules of the CNS Vital Signs (CNS VS), analyzed for all patients. Assessment performed at three selected sites only. CPT: Continuous Performance Test; bid: twice daily.

Table 8. Adverse events (AE) from baseline to Week 12, by treatment (safety population)

		Placebo	Mavoglurant 25 mg bid	Mavoglurant 50 mg bid	Mavoglurant 100 mg bid
		n (%)	n (%)	n (%)	n (%)
Adult		N=44	N=44	N=42	N=45
Adolescent		N=42	N=31	N=27	N=39
Event					
Patients with any AE (≥10% in any group)	Adult	27 (61.4)	25 (56.8)	29 (69.0)	37 (82.2)
	Adolescent	26 (61.9)	25 (80.6)	14 (51.9)	34 (87.2)
Headache	Adult	2 (4.5)	1 (2.3)	4 (9.5)	4 (8.9)
	Adolescent	4 (9.5)	4 (12.9)	0	10 (25.6)
Dizziness	Adult	1 (2.3)	1 (2.3)	0	8 (17.8)
	Adolescent	0	0	1 (3.7)	1 (2.6)
Insomnia	Adult	0	1 (2.3)	4 (9.5)	8 (17.8)
	Adolescent	0	0	0	6 (15.4)
Vomiting	Adult	2 (4.5)	3 (6.8)	5 (11.9)	4 (8.9)
	Adolescent	0	2 (6.5)	1 (3.7)	6 (15.4)
Nasopharyngitis	Adult	2 (4.5)	3 (6.8)	3 (7.1)	6 (13.3)
	Adolescent	6 (14.3)	7 (22.6)	6 (22.2)	5 (12.8)
Upper respiratory tract infection	Adult	2 (4.5)	3 (6.8)	2 (4.8)	4 (8.9)
	Adolescent	4 (9.5)	2 (6.5)	1 (3.7)	5 (12.8)
Decreased appetite	Adult	0	0	1 (2.4)	4 (8.9)
	Adolescent	1 (2.4)	0	1 (3.7)	4 (10.3)

Bid: twice daily.

3.4. Safety

During the treatment period, most adverse events (AEs) took place in the 100 mg bid group (82.2% for the adult and 87.2% for the adolescent population). AEs were more frequent in the placebo group than in some of the treatment groups. AEs are shown and discussed more extensively in Berry-Kravis *et al*, supplementary data ¹⁸. Briefly, most frequent AEs were mild and included dizziness, insomnia, headache, and vomiting (Table 8). There were four serious AEs (SAEs) reported in the adult and two in the adolescent population. In the placebo group one patient had lobular pneumonia and one patient showed convulsions after clonidine, administered for insomnia. In the mavoglurant groups, three patients showed agitation, of which one had also upper respiratory infection and one reported also visual hallucination and insomnia. One patient had appendicitis leading to hospitalization. No deaths occurred during the studies.

Discontinuations are summarized in Figure 2. In adults (Figure 2A), most discontinuation due to AEs were in the 100 mg bid (13.3%), followed by 50 mg bid (4.8%), 25 mg bid (4.5%), and placebo (2.3%), mostly because of insomnia or agitation in the mavoglurant groups. In the adolescent population (Figure 2B), two patients discontinued. One patient in the placebo group had medical history of facial nerve paralysis and showed a relapse which was treated with prednisone. Another patient in the 100 mg bid group developed hyperhidrosis, insomnia, psychomotor hyperactivity, tachycardia, and inappropriate affect. Mavoglurant seemed to be better tolerated in adolescents than in adults.

No clinically significant findings in medical examination, ECGs, or laboratory tests were reported. Laboratory measures showed no clinical relevant activation of the hypothalamic-pituitary-adrenocortical axis and no relevant differences in prolactin were measured. NPI-Q scores did not indicate increased psychiatric symptoms.

4. Discussion

In this paper we present the results of two large, double-blind, placebo-controlled clinical trials, conducted in adolescent and adult patients with FXS, in order to assess safety and potential improvement of specific FXS symptoms after mavoglurant treatment. Our data does not show any improvement in behavior or cognitive scales after 12 weeks of mavoglurant treatment, compared with placebo.

The primary outcome measure (improvement of the ABC-CFX total score in patients with FXS with completely methylated *FMR1* gene promotor) was not met, as well as the key secondary outcome measure (improvement of ABC-CFX total score in FXS patients with partially methylated *FMR1* gene promotor). Also, all other secondary behavioral outcome measures (ABC-CFX subscale scores, CGI-I, RBS-R, and SRS) did not improve after mavoglurant treatment, compared with placebo. For some groups, some of the (sub)scales even scored statistically significant worse than placebo. However, these differences were small, inconsistent and possibly due to the large number of

measurements per patient population. Unfortunately, the cognitive measures were not reliable due to the small number of patients who were able to perform these tests adequately. Based on initial power calculations, the number of patients per treatment arm in both studies was enough to be able to show an effect-size of at least 1.15 for the primary outcome measure, corresponding to a treatment difference of 17 points in the ABC-CFX between mavoglurant and placebo¹⁸.

As was shown in previous studies, mavoglurant seems to be well tolerated and safe^{11,22,23}. AEs occurred more often in patients receiving the higher doses of mavoglurant, but observed AEs were similar to those reported in previous studies and mostly not severe enough to discontinue. The highest dose was slightly better tolerated in the adolescent group. Mavoglurant is metabolized through the CYP3A4 liver enzyme. It has been reported that during development, the baseline CYP3A4 activity changes, and is higher in children compared to adults²⁴, which might account for a better tolerability. However, the differences between individuals are large and the CYP3A4 activity might be influenced by chronic mavoglurant administration. Randomization was effective for most measures, besides some groups who scored slightly higher on ABC-C and ABC-CFX.

Both studies aimed to test efficacy of mavoglurant, a new, selective, and non-competitive mGluR5 antagonist^{10,25}. Pre-clinical research in the past decade has revealed that the mGluR5 pathway is involved in the pathogenesis of FXS. In the absence of FMRP, the mGluR5 pathway is disturbed, leading to enhanced long-term depression^{9,26}. Interestingly, genetic^{8,27} or pharmacological reduction of mGluR5 activity in FXS animal models (e.g. mice, zebra fish, flies), restores several FXS features, including molecular, electrophysiological, morphological, behavioral, and cognitive features (reviewed in^{7,28-31}). These successful pre-clinical results in animal models paved the way for a phase 2 randomized, placebo-controlled and crossover clinical trial in 30 patients with FXS. This trial showed significant improvement in ABC-C, CGI-I and RBS-R compared to placebo in a subset of patients with a completely methylated *FMR1* gene promotor¹¹. These promising results have led to the initiation of the current two large randomized, placebo-controlled clinical trials.

Based on this previous trial, in our trials stratification for the methylation status was implemented. Patients were stratified into a completely methylated (no *FMR1* expression) or partially methylated group (reduced *FMR1* expression). Our current results, however, show no difference in efficacy between these two groups, based on methylation status in blood samples. Previous studies have investigated the relation between *FMR1* expression mosaicism and clinical phenotype. *FMR1* expression studies in blood and hair roots using an FMRP test have demonstrated that patients with FXS are often size mosaics (cells with methylated full mutation and cells with unmethylated premutation) or methylation mosaics³²⁻³⁴. This phenomenon was illustrated by the presence of cells expressing normal levels of FMRP in (severely) affected patients with FXS, some even reaching 30% of positively labelled FMRP cells. Thus, low number of cells with normal FMRP expression is not sufficient to reduce FXS severity. Moreover, a recent study showed individuals with FXS and mosaic genotypes do not have significantly

lower number of seizures, hyperactivity or autism spectrum disorder severity³⁵. Based on these studies, dividing FXS patients into completely methylated *FMR1* gene promotor and partially methylated *FMR1* gene promotor in blood is probably not a relevant form of stratification.

After many successful results of mGluR5 antagonists in pre-clinical FXS animal model studies and the previous phase 2 clinical trial, this lack of efficacy of mavoglurant is unexpected and highly disappointing for patients, care-givers, and all others involved. Also other larger clinical trials in FXS using mGluR5 antagonists showed negative results (NCT01015430, NCT01517698, NCT01750957, results briefly presented in <http://www.fraxa.org/roche-reports-clinical-trial-negative-results/>).

Several possible explanations could be raised for these negative results. An obvious explanation could be that the mGluR5 theory is inapplicable or only partially applicable in explaining the FXS features in patients. In pre-clinical research, animal models are often selected to model specific features, rather than modeling the complete disease³⁶. In addition, important cellular, molecular, and genetic mechanisms in humans are different from animal models. Moreover, animals are kept under very controlled circumstances and represent a very specific inbred population, very unlike our patient population. In fact, lack of translation from mice to human is a very common phenomenon in biomedical research³⁷.

Additionally, hurdles in study design aspects are another plausible explanation for the lack of efficacy in our studies. Overall, many clinical trials addressing treatment of cognitive genetic disorders are not able to translate preclinical studies into therapy, possibly due to a low quality of design. Reviews have shown that only 30% of clinical trials in these patients groups reach acceptable Jadad scores and less than 5% of clinical trials have led to effective therapy³⁸. Notably, important limits of this study are large placebo effects that were measured across all treatment groups, as is demonstrated in the placebo run-in period and which, to a lesser extent, continues during the 12 weeks of treatment. Randomized placebo-controlled clinical trials are considered as the gold standard in drug efficacy research, although placebo effect is a well-known mechanism complicating clinical drug efficacy testing, explicitly in neurodevelopmental disorders³⁹. However, with large placebo effects, possible small drug effects could be overshadowed. In this study, caregivers and clinicians assessed outcome measures, with high expectations for the drug under study, which may explain the large placebo effects. In addition, a large placebo effect probably also explains why previous open label trials did seem to be successful, without replication in the larger placebo-controlled trials. We cannot exclude a relevant effect of mavoglurant that has possibly been missed, due to the used endpoints. Outcome measures used to assess efficacy of targeted treatments in FXS clinical trials have been subject of debate in the past years^{37,40}. ABC-C, CGI-I, RBS-R and SRS are validated for assessments of symptomatic treatment for autism^{41,42} and were selected for that reason. ABC-C has been modified to cover the most prominent features in FXS patients: the ABC-CFX used in these studies¹⁶. These and other caregiver or clinician assessed outcome measures are mainstream and currently

the only available validated outcome measures, despite them being prone to large placebo effect, being non-objective and possibly not sensitive enough to detect subtle improvements with ceiling or floor effects. Bailey *et al*⁴³ investigated the implementation of CGI-I in the current adolescent trial. The authors show CGI-I, which is clinician rated, is mostly based on parental reports and lack guidelines and as such, is not an objective assessment. Also, it could not substantiate new or unexpected domains to measure effect, possibly due to small numbers per item. The cognitive outcome measures we used, could not be interpreted due to the small number of patients who were able to perform them. It is not known whether the used outcome measures represent the effects that are to be expected of targeted treatment for neurodevelopmental disorders. There is no consensus on what tests should be used⁴⁰. In addition, outcome measures from animal studies that have shown improvement, are often non-applicable in clinical trials with patients. There is clearly a need for more robust, reliable and relevant outcome measures for these clinical trials in the future. Pre-pulse inhibition^{44,45} and the eye blink test^{46,47} are examples of existing, objective endophenotypes that have been tested in FXS. However, the clinical relevance of improvements in these tests is not known, they are very time-consuming and have not been validated for clinical trial settings. Currently, many research efforts focus on development of new functional outcome measures based on endophenotypes, like eye tracking for social gaze⁴⁸ and functional near-infrared spectroscopy^{49,50}. Also, pathway-related biomarkers like ERK and AKT activation⁵¹, MMP9⁵² and S6⁵³ might be beneficial to explore efficacy of drugs (reviewed in⁵⁴).

Another aspect in trial design is the age at treatment initiation. Our trials were conducted in adults and adolescents, and the results of post hoc analysis did not implicate age to be relevant in the effect of mavoglurant. However, mavoglurant has not been tested in younger children, where targeted treatment for a neurodevelopmental disease is more likely to be effective. Some studies in *Fmr1* KO mice show that treatment with mGluR5 antagonists in young mice is effective, but not at a later age. This has been demonstrated for spine morphology, cognitive, electrophysiology, and molecular features⁵⁵⁻⁵⁸. In contrast, some studies showed treatment at an older age is still beneficial in FXS mice⁵⁸⁻⁶⁰, however, it is not known whether that is the case in humans. Extending the clinical trials to children potentially could result in a measurable treatment effect. This might become clear in the near future, since recently, a clinical trial with mavoglurant in children has been announced (<http://www.fraxa.org/clinical-trial-fragile-x-children/>). The duration of therapy might also be an important issue. Average clinical trials are not designed to capture long-term treatment effects. Some studies in *Fmr1* KO mouse indicated acute treatment is not enough and chronic treatment is needed to rescue some of the FXS phenotypes. For example, Michalon *et al*, showed that longer treatment of *Fmr1* KO mice leads to more correction in the open field test than shorter treatment⁵⁸. Possibly, mavoglurant treatment in patients will only show an effect when administered for more than a few months, or even a few years. This raises the issue that not only more reliable outcome measures are needed, but also outcome measures suitable for

measuring long-term effects.

Besides the above mentioned translational and trial design challenges, another far less investigated issue regards the molecular pathogenicity of FXS itself. In the past, both pre-clinical and clinical studies only targeted a single relevant pathway each^{28,37,61}. FMRP is an RNA-binding protein involved in the post-synaptic translational control of many target RNA's⁶², but its role in many other pathways and cell compartments, including the presynaptic compartment, becomes increasingly clear, for example from studying missense mutations^{63,64} and other cell types⁶⁵. These alternative affected pathways elucidate the tremendous complexity of FXS pathogenesis and leads us to believe that treating FXS might require targeting more than one pathway simultaneously, using combinational therapy.

5. Conclusion

In conclusion, our two large, double-blind, placebo-controlled clinical trials of 12 week-mavoglurant treatment in adolescent and adult patients with FXS did not demonstrate evidence to support efficacy of mavoglurant compared with placebo. These results are disappointing in the light of the pre-clinical evidence for beneficial effects of mGluR5 antagonists in FXS animal models. Some aspects of clinical trial design and the complex pathophysiology of FXS should be better considered in the development of future trials. To assess efficacy of drugs for neurodevelopmental disorders in a reliable manner, we need objective, specific and sensitive primary outcome measures. In addition, future trials should probably be conducted at a younger age, for a longer duration, and most likely using a combinational therapy to achieve improvement for this complex disease.

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Supplementary data

Supplementary Table 1. Participating principal investigators and their location

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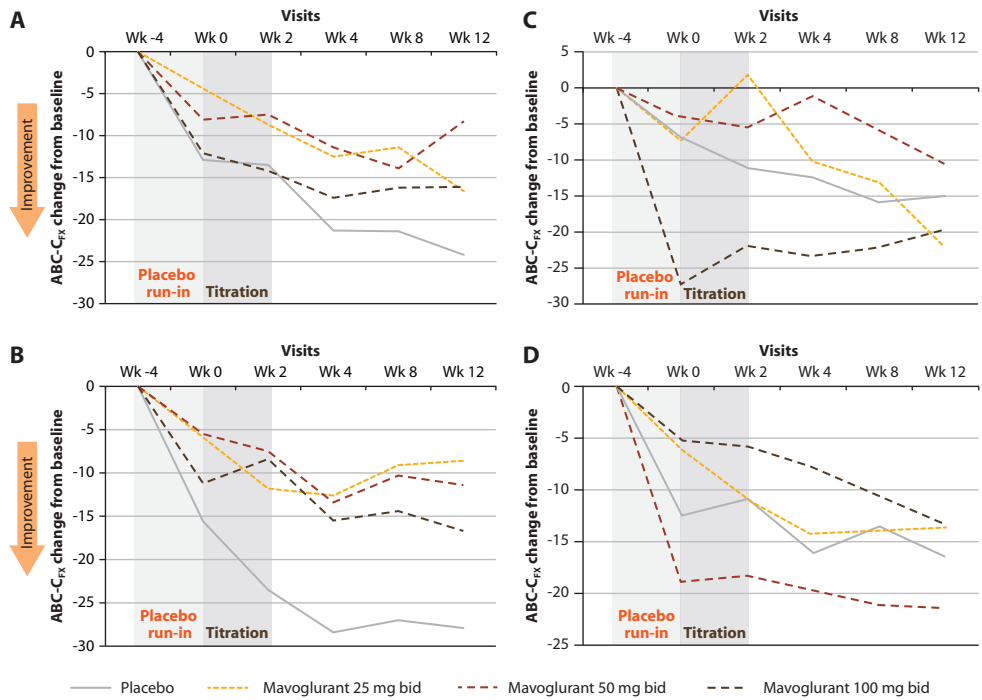
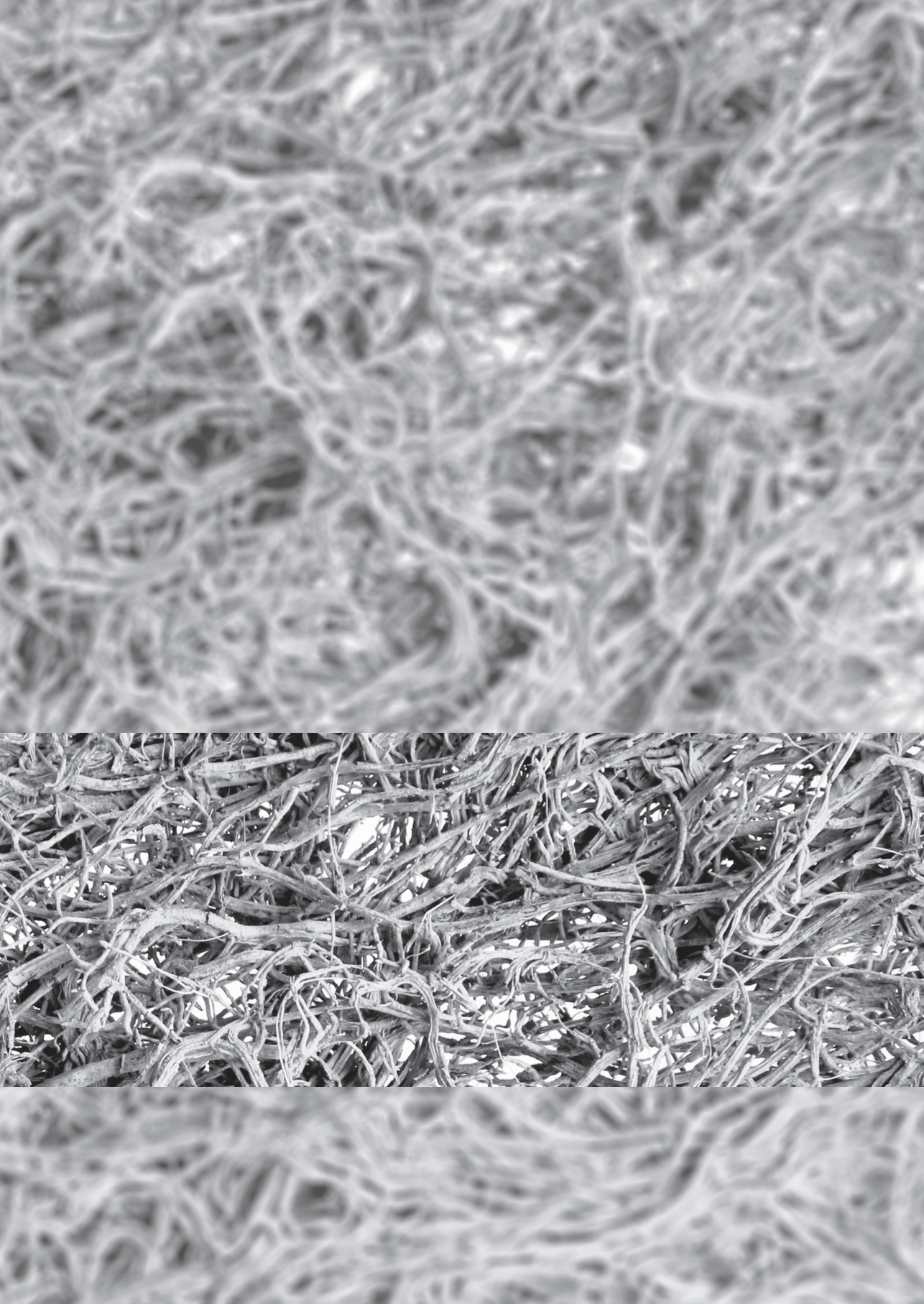


Figure S1. ABC-CFX mean change from baseline including placebo run-in. (A) CM stratum in adult population, (B) PM stratum in adult population, (C) CM stratum in adolescent population and (D) PM stratum in adolescent population. Change in ABC-CFX total scores from run-in (Week 4) represented as means. ABC-CFX, Aberrant Behavior Checklist-Community Edition using the FXS specific algorithm; CM, complete methylation; PM, partial methylation; Wk, week



CHAPTER 6

The quests for targeted therapy in Fragile X syndrome

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Abstract

Fragile X syndrome (FXS) is the commonest monogenetic cause of intellectual disability and autism-spectrum disorders. Although there is no effective therapy, greater understanding of disturbed neuronal pathways has introduced options for targeted therapy. But while many FXS phenotypes were improved in pre-clinical studies with drugs targeting these pathways in the FXS mouse model, attempts to translate these animal-model success stories into treatment of patients in clinical trials have been extremely disappointing. Complicating factors, particularly in animal studies, include mouse inbred strains, variability in functional studies between laboratories, publication bias and lack of reliable and objective primary outcome measures in both mice and patients. Possibly most important, however, is one factor that has been little explored: the complexity of the molecular imbalance in FXS and the need to simultaneously target several different disturbed pathways and different cellular compartments. New well-conceived animal studies should generate new, more productive approaches to the quest for targeted therapy for FXS.

1. Introduction

Hopes for the cure of many severe genetic diseases were raised by the identification of their genetic causes, the elucidation of pathophysiological mechanisms, and the subsequent identification of specific targets for therapy in animal models. One striking example is the story of fragile X syndrome (FXS), the most prevalent inherited cause of X-linked intellectual disability and autism. Identification of the *FMR1* gene in 1991¹ was followed by the development of the *Fmr1* knockout (KO) mouse model², which lacks a functional *Fmr1* gene and has phenotypes that resemble the human disorder in several domains, including behavior, biochemistry, electrophysiology, and spine morphology. Greater understanding of the molecular mechanisms and pathways involved in FXS led to the development of targeted therapeutic strategies intended to reverse the intellectual and behavioral problems of patients with FXS. These pre-clinical studies in *Fmr1* KO mice led to successful therapeutic intervention studies that paved the way for clinical trials. Since then, however, hope has been tempered by the discovery that it is less straightforward than first assumed to translate encouraging results in the *Fmr1* KO mouse model into therapy in the clinical setting. If we can identify the factors that complicated the research and caused the clinical trials to fail, we may find new strategies in the search for a cure.

FXS occurs in about 1:4000 males. Due to its X-linked nature, it is less prevalent in females (1:6000). It is characterized by developmental delay and intellectual disability, with specific impairments in cognitive, executive, and language performance. Behavioral problems include autistic and repetitive behavior, hyperactivity and impulsivity, anxiety, mood disorders, and sleeping problems. Epilepsy, laxity, hypotonia, ear infections, and other medical problems are prevalent. The severity of cognitive, behavior and medical problems varies greatly between patients³. Current treatment is symptomatic, including non-pharmacological behavioral and educational therapy, but also extending to pharmacological treatment for anxiety, aggression, and ADHD.

FXS is caused by the abnormal expansion of a naturally occurring CGG repeat in the 5' UTR of the *FMR1* gene to over 200 units. This triggers methylation of the *FMR1* gene, resulting in silencing of the gene and absence of fragile X mental retardation protein (FMRP), the encoded protein. Although FMRP is expressed relatively highly in the brain, where it functions as an RNA-binding protein with many postsynaptic targets, presynaptic targets have also been identified⁴. One important role of FMRP is local translational modulation at the postsynaptic terminal.

2. Molecular pathways and potential targets for therapy

Lack of FMRP leads to severe disruption of synaptic function and plasticity. Two decades of research have shown defects in major excitatory glutamatergic and inhibitory GABAergic pathways, leading to imbalanced excitatory/inhibitory function and intracellular signaling (e.g., ERK, GSK3 and mTOR) in *Fmr1* KO mice⁴. Emerging evidence suggests that

several other neurotransmitter systems are also involved, such as the endocannabinoid and muscarinic acetylcholinergic systems, APP signaling, and cytoskeleton remodeling (reviewed in ⁵). Strikingly, however, the vast majority of research studies have focused on the postsynaptic function of FMRP, ignoring its translation-independent presynaptic function ⁶ (see Figure 1 for major pathways and potential targets for therapy). Reports of murine findings have described correction of one or more (endo)phenotypes by targeting, for example the glutamatergic (metabotropic glutamate receptor, mGluR5) or GABAergic (GABA_A or GABA_B receptors) pathways, or by targeting proteins regulated by FMRP ⁵.

3. Clinical trials

Regarding the possibility of a cure for FXS, these pre-clinical data seemed extremely promising, and provided the basis for clinical trials. In 2006, clinical trials with FXS patients started ⁷, initially pilot studies with an open label construction, sometimes placebo controlled, but with short intervention times and small sample sizes. The first results showed that, overall, many of these drugs were safe. Measurement with questionnaires for parents or clinicians, which were focused primarily on behavior and not at the level of cognitive functioning, also suggested that many of them induced improvement. Although some trials also used biochemical outcome measures or more objective endophenotypes, the clinical significance for these outcome measures is unclear. Even better results seemed to be yielded by stratification for methylation status and protein levels (as used in the trial for mGluR5 antagonist AFQ056 by Jacquemont *et al* ⁸), or for severity of symptoms. Unfortunately, however, almost none of these success stories were reproduced in the larger, placebo-controlled clinical trials that followed. For instance, prior open-label trials of minocycline were associated with improvements in behavior ⁹, however, the controlled trial only showed modest global benefits ¹⁰. Overall, many trials were terminated because they did not meet their predesignated primary endpoints. For an overview of all clinical trials see clinicaltrials.gov (last accessed on 24 July 2015).

4. Clinical trials revisited

So what went wrong? The evidence from pre-clinical research seemed strong. But little of the successful targeted therapy in animal model studies were translated to the actual treatment of FXS patients. The disappointing results may have been caused by a number of complicating factors. The first is the limitations of the *Fmr1* KO mouse, which, due to its overlapping phenotypes with FXS and comparable cellular mechanisms, is the most widely used and validated animal model in FXS research. But although it is probably the best model for FXS, its phenotypic features vary greatly between different genetic backgrounds. Mouse model studies often try to bypass variability by using only

one specific inbred genetic mouse line and without balance for gender. However, as FXS patients are clinically and genetically much more varied than inbred mouse lines, individual patients might respond differently to treatment. The second reason is the poor reproducibility between different genetic backgrounds, which may indicate lack of translation to the variable human genetic and environmental background. Third, many of the mouse phenotypes are subtle and strain specific, and several phenotypes have never been reproduced by other research groups. Thus, as each research group tends to develop its own test battery, it is difficult to compare data. This is compounded by publication bias towards the success stories, a known phenomenon in medical research from which the FXS animal model field has not escaped. The absence of a platform for the negative data might therefore conceal a situation that is closer to reality, in which the effect of current targeted treatment options is less robust. Neither should one underestimate the effects of selective reporting as a cause of clinical trial failure. Fourth, assessing a drug's safety and correction of a phenotype in an established animal model is a necessary proof-of-concept in drug discovery. However, such results do not always translate to humans. The outcome measures used in FXS mice studies – such as the behavioral tests and spine morphology measurements – do not directly correlate with measurable human phenotypes and are often unsuitable as outcome measures in clinical trials. Ameliorating one or two phenotypes in a mouse does not imply an overall correction of the disease, and might not be picked up in a clinical trial setting, where other outcome measures are used.

One of the most important limitations of current trials might be the shortage of reliable, objective and feasible outcome measures for monitoring improvement. Although they are widely used and validated, questionnaires are not objective and are sensitive to considerable placebo effects; as they may not be sensitive enough to pick up small changes, they may also reach a floor effect too quickly. This reveals a serious need for more objective outcome measures. These should be not only clinically relevant, but also easily translatable between humans and mice.

Problems of a different order involve the optimal age at onset of therapy, the time of therapy needed to measure treatment effect, the optimal dosage, and the development of extensive tolerance. Although studies in the FXS mouse model suggest that some phenotypes might be ameliorated even in adulthood, it is not known whether this is also true in humans. We should add that intensity of participation in clinical trials entails a selection bias towards the less severely affected patients. In addition, considering the broad range of clinical and behavioral features in FXS, we believe that a single drug will be unlikely to correct all these phenotypic features in FXS patients; combined treatments with multiple compounds might be inevitable.

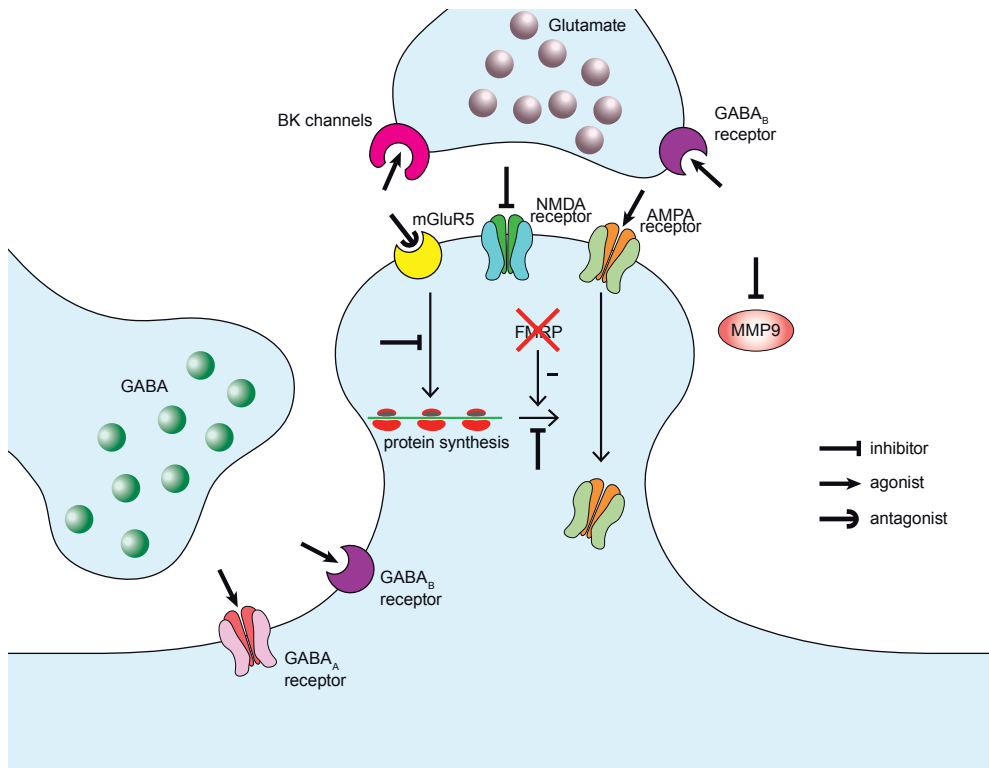


Figure 1: Potential therapeutic targets for FXS. Simplified schematic representation of a glutamatergic excitatory and GABAergic inhibitory synapse lacking FMRP. Several types of drugs/modulators/compounds (inhibitor, agonist and antagonist) can interfere with different pathways disturbed in FXS. These targeted therapeutic strategies may lead to the reversal of behavioral and cognitive impairments in patients (adapted from ¹⁴).

5. Conclusion and future prospects

While many publications have consistently proved that several pathways are involved – such as the glutamate and GABAergic pathways – review of the current literature shows that these pathways alone cannot be the entire story. As an RNA binding protein, FMRP binds hundreds of target RNAs, many of which are known to be involved in the molecular pathways underlying autism and intellectual disability ¹¹. FMRP is present not only in the postsynaptic compartment, but also in the presynaptic compartment, the cytosol, the nucleus and in other non-neuronal cell types, such as astrocytes, where its function is not completely clear ⁴. Absence of FMRP may lead to a complex disruption of the molecular balance in the neurons and surrounding cells. To restore this balance,

it is likely that targeting only one pathway is not enough, but several different pathways and proteins will need to be targeted. The *FMR1* gene itself cannot yet be targeted directly. Thus far, attempts for transcriptional therapy of FXS by modulating chromatin activation were hampered by the toxicity of the compounds caused by unspecific and genome-wide effects¹². The only way to compensate wholly or partly for the absence of FMRP is by using a combination of drugs to simultaneously target the major pathways and important proteins involved. For such combined treatment, we propose to target pathways in different compartments/neurons rather than different pathways in the same compartment. This idea has emerged from experimental data showing that pathways in the postsynaptic compartment are often interconnected. For example, a useful addition to any combined treatment may be to target BK channels (presynaptic) and mGluR5 pathway (postsynaptic) simultaneously; or to target pathways in inhibitory neurons and excitatory neurons. This might turn out to be the key to finding a cure for FXS patients.

6. Expert opinion

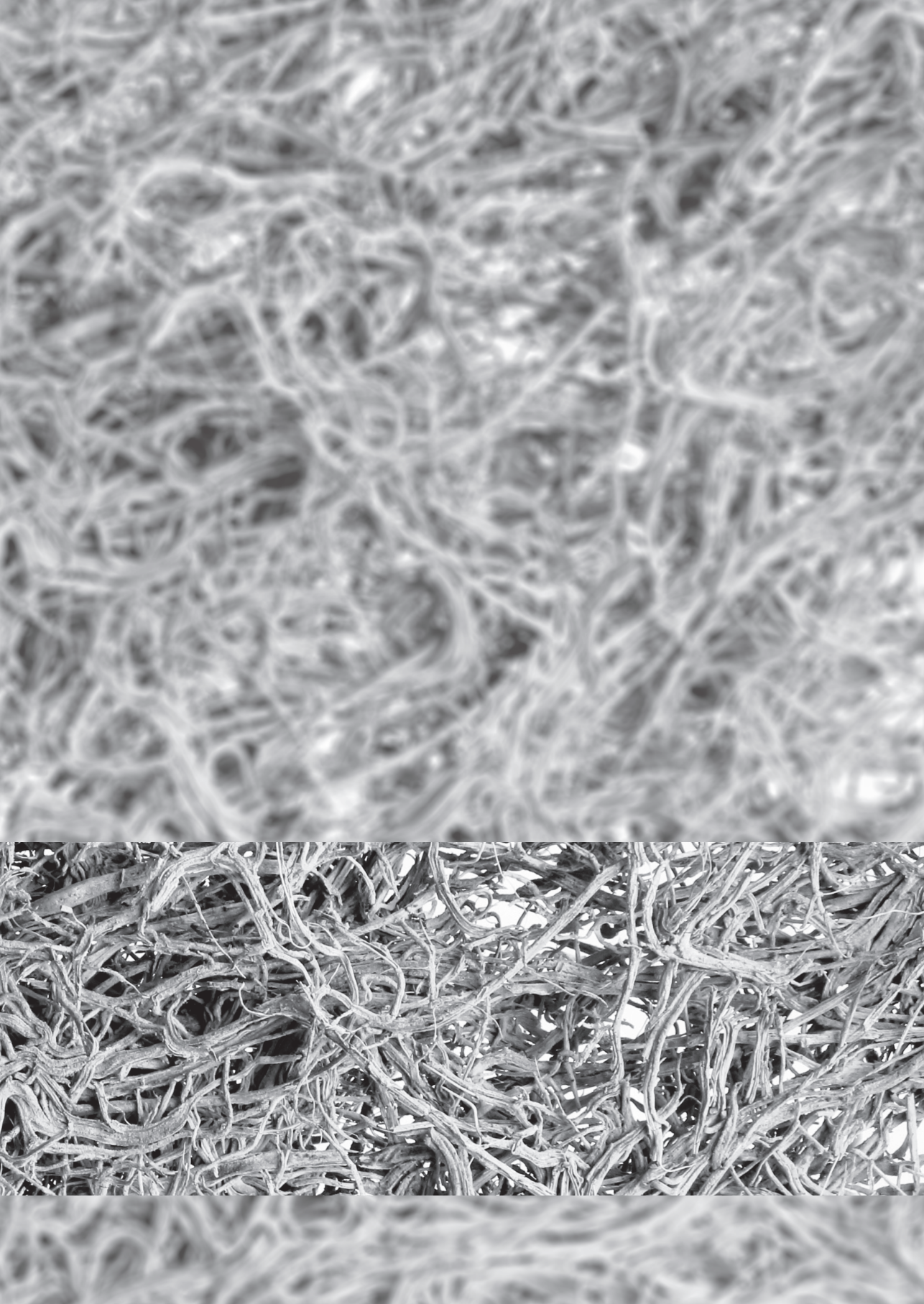
Twenty years of preclinical research has led to the identification of most of the involved pathways underlying the pathophysiology of FXS. Subsequently, their potential in serving as targets for therapy has been successfully tested in the FXS mouse model. However, the translation of these promising findings into the clinic has been rather disappointing so far. Besides overall translational disadvantages like variability between inbred strains, non-reproducibility and publication bias, two important aspects seem striking. The first is the clear need for more robust, objective and reproducible outcome measures for monitoring treatment efficiency. The second aspect regards the complex molecular imbalance due to absence of FMRP. While most studies are focused on one impaired pathway only, the key solution for finding a cure for FXS might be targeting multiple pathways and proteins simultaneously, in order to restore this imbalance. In the coming years, animal studies aimed at a multi-target approach as well as a search for reliable outcome measures, including sensitive biomarkers and relevant robust functional phenotypes, should provide us with new preclinical data in the quest for targeted therapy for FXS. In parallel, studies on clinical trial design should focus on the development of relevant objective endophenotypes to determine efficacy and reduce large placebo effects.

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CHAPTER 7

Fragile X syndrome: new therapeutic strategies

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Summary

BACKGROUND Fragile X syndrome (FXS) is the most common hereditary cause of intellectual disability and autism spectrum disorders. Targeted treatment is lacking. The past decades have revealed an enormous amount of knowledge on the involved molecular pathways, introducing potential targets for disease modifying therapy.

GOAL An overview of the development of targeted treatment for FXS.

METHOD Several important publications were collected and listed.

RESULTS Preclinical animal model studies with targeted interventions were promising. However, translation to the clinic has been disappointing.

CONCLUSION Targeted treatment for FXS is necessary and could be beneficial in other causes of autism spectrum disorders. Translational, study design and outcome measures factors have possibly led to the disappointing results. To clinically implement future therapeutic strategies and to facilitate research, clustering of patient care and expertise is required. In addition, this improves patient care, for example by the recent medical guideline for children with FXS.

Key-words: fragile X syndrome, targeted treatment, clinical trials

CHAPTER 7

Fragiele X syndroom: nieuwe therapeutische strategieën

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Samenvatting

ACHTERGROND Het fragiele X syndroom (FXS) is de meest voorkomende erfelijke oorzaak van een verstandelijke beperking en autisme spectrum stoornissen. Een gerichte therapie ontbreekt voorslugs. De afgelopen decennia brachten een explosie aan kennis over de betrokken moleculaire processen en daarmee potentiële aangrijpingspunten voor gerichte therapie.

DOEL Een overzicht van de ontwikkeling van gerichte therapie voor FXS.

METHODE Een aantal belangrijke publicaties zijn verzameld en op een rij gezet.

RESULTATEN Preklinische diermodelonderzoeken tonen veelbelovende resultaten van gerichte interventies, maar vertaling naar de kliniek is voorslugs teleurstellend.

CONCLUSIE Een gerichte therapie voor FXS is nodig en zou mogelijk kunnen worden toegepast bij andere oorzaken van autisme en verstandelijke beperking. Aspecten op gebied van translatie, studiedesign en uitkomstmaten spelen mogelijk een rol bij de teleurstellende resultaten. Voor de klinische implementatie van toekomstige therapeutische strategieën en het coördineren van onderzoek, is bundeling van zorg in een expertisecentrum noodzakelijk. Bovendien wordt zorg bevorderd door onder andere de recent ontwikkelde medische leidraad voor kinderen met FXS.

Trefwoorden: fragiele X syndroom, gerichte therapie, klinische trials

Inleiding

Het fragiele X syndroom (FXS) is de meest voorkomende erfelijke oorzaak van een verstandelijke beperkingen. De diagnose wordt bij 1% van de jongens met een ontwikkelingsachterstand gesteld ^{1,2}. Met name wanneer een ontwikkelingsachterstand gepaard gaat met psychiatrische problematiek als autisme-gerelateerd gedrag, ADHD, agressie of angststoornissen, wordt de diagnose overwogen. De gedragsproblemen vlakken veelal af na de puberteit, maar uiterlijke kenmerken worden des te opvallender. Daarnaast kan somatische problematiek voorkomen, waaronder epilepsie, recidiverende otitis media en strabisme ^{1,3}. De diagnostiek en behandeling van FXS worden uitvoerig besproken in de recent verschenen Nederlandse leidraad voor zorg van kinderen met FXS (ENCORE) en in internationale richtlijnen ^{1,4}. De verschijnselen leiden vaak tot een hoge mate van ziektebelasting. Desondanks is de huidige behandeling zeer beperkt en bestaat voornamelijk uit psychologische interventies, aangepast onderwijs en medicamenteuze behandelingen.

FXS wordt veroorzaakt door een mutatie in het *FMR1* gen, dat op het X-chromosoom ligt en codeert voor het FMR-protein (FMRP) ⁵. Sinds de ontdekking van dit gen, zijn er vele stappen gemaakt in het ontrafelen van de onderliggende moleculaire mechanismen, mede met behulp van het FXS muismodel. Deze muus vertoont vele overeenkomsten met het FXS fenotype bij mensen, waaronder afwijkingen op het gebied van cognitie, sociaal gedrag, hyperactiviteit en elektrofysiologie ⁶. Ook worden moleculaire kenmerken waargenomen en een afwijkende morfologie van de neuronale dendritische uitlopers, de zogenoemde dendritische spines ⁶. Begrip van de onderliggende moleculaire pathways, heeft potentiële aangrijpingspunten voor gerichte farmacologische therapie aan het licht gebracht ⁷. En inderdaad, medicatie die hierop aangrijpt, bleek succesvol te zijn in diermodellen, waarna de stap naar de mens snel is gemaakt. Aanvankelijk leken deze medicamenteuze interventies in de mens veelbelovend in kleine trials, maar grotere studies zijn stuk voor stuk beëindigd wegens gebrek aan effectiviteit. In dit artikel worden aangrijpingspunten besproken die zijn toegepast in de klinische trials en worden oorzaken genoemd die mogelijk hebben bijgedragen aan de discrepantie tussen resultaten van preklinische studies en klinische trials. De problemen waar men tegenaan is gelopen bij FXS, gelden veelal ook voor andere ontwikkelingsstoornissen waarvoor een therapie in ontwikkeling is, waaronder autisme ⁸. Daarnaast zouden nieuwe therapieën voor FXS mogelijk kunnen worden geëxtrapoleerd naar de gehele patiëntengroep met autisme, vanwege de fenotypische en moleculaire overeenkomsten van deze aandoeningen ⁹.

FMRP en aangrijpingspunten voor gerichte therapie

FMRP speelt een belangrijke rol in de regulatie van synaptische plasticiteit¹⁰. Synaptische plasticiteit reguleert de mate van de connectiviteit tussen zenuwcellen, wat essentieel is voor ontwikkeling, leervermogen, het vastleggen van geheugen, regulatie van gedrag en aanpassing aan interne en externe stimuli^{10,11}. De synaptische plasticiteit is maximaal tijdens de zogenoemde kritische periode in de eerste levensjaren, maar blijft van belang gedurende het hele leven. Gebrek aan FMRP, zoals bij FXS, leidt tot verstoring van de synaptische plasticiteit via verschillende moleculaire 'pathways'^{7,11}. Belangrijke pathways waarin defecten zijn aangetoond bij FXS, zijn de metabotrope glutamaat receptor 5 (mGluR5) pathway en de gamma-Aminoboterzuur (GABA) pathway, die tezamen in de zenuwcel de excitatie-inhibitie balans verzorgen. Daarnaast heeft FMRP een functie in verschillende andere neurotransmitter receptoren, intra- en extracellulaire pathways, ionkanalen en zelfs in niet-neuronale celtypes^{7,10,12}. Dieronderzoek heeft zich de afgelopen jaren gericht op verschillende van de bovengenoemde aangrijpingspunten, waaronder mGluR5, GABA_A of GABA_B, MMP9, 5-HT7 serotonine receptor, endocannabinoid pathway en componenten van de mGluR5 signaling pathway^{7,12,13}. Vele van deze studies tonen verbetering van verschillende kenmerken van het FXS muismodel, waaronder cognitieve functies, sociaal gedrag, hyperactiviteit, angst, repetitief gedrag, vatbaarheid voor epileptische aanvallen, morfologie van de spines, sensorimotor gating afwijkingen en moleculaire kenmerken^{7,12}.

Gerichte moleculaire therapie in klinische trials

De eerste klinische trial met FXS patiënten, die therapie gericht op de moleculaire onderliggende basis testte, werd in 2006 gepubliceerd. De studie maakte gebruik van CX516, een AMPA-receptor agonist, wat de mate van synaptische transmissie reguleert¹⁴. Deze dubbelblinde, placebo-gecontroleerde fase 2 klinische trial van 4 weken, testte het effect van CX516 in 48 volwassenen met FXS, maar toonde geen verbetering in de gehele groep in verschillende uitkomstmaten, waaronder een aantal geheugentesten en gedragsscorelijsten. Wel was er een significante verbetering van de 'clinical global impression' in een subgroep van patiënten die antipsychotica gebruikten. Deze trial werd gevolgd door trials met o.a. de GSK3 modulator lithium, de mGluR5 receptor antagonist fenobam, mavoglurant en basimglurant, de MMP9 antagonist minocycline, de GABA_B receptor agonist arbaclofen, de GABA_A receptor agonist ganaxolone, de acetyl-cholinesterase remmer donepezil en meer^{7,13}. Opvallend is dat een aantal kleinere trials aanvankelijk effect toonde in een specifieke subpopulatie, met kleine aantallen. Desalniettemin konden deze resultaten niet worden gerepliceerd in de grotere placebo-gecontroleerde trials met mavoglurant, basimglurant, arbaclofen en donepezil⁷. Alleen de dubbelblinde placebo-gecontroleerde trial met minocycline bij 55 kinderen met FXS gedurende 3 maanden toonde een verbetering op de Clinical Global Impression en een 'visueel analoge schaal' van de meest ernstige verschijnselen.

Echter, het effect was zeer klein, en er werden veel bijwerkingen van dit antibioticum gemeld ¹⁵. Van de overige medicamenten is het slechts bij één kleine trial gebleven. Enkele dubbelblinde, placebo-gecontroleerde trials lopen nog, waaronder trials met ganaxolone, acamprosate en bumetanide.

De meest gebruikte uitkomstmaat is de 'Aberrant Behavior Checklist' (ABC), met een FXS-specifieke gevalideerde versie, de ABC-FX ¹⁶. Dit is een scorelijst die wordt ingevuld door de ouders of verzorgers. Ook de andere veel gebruikte uitkomstmaten, zoals de Clinical Global Impression en Repetitive Behavior Scale, bestaan uit scorelijsten die worden ingevuld door de ouders of de arts ^{13,17}. Ondanks de aangetoonde effectiviteit van deze scorelijsten bij klinische trials met symptomatische therapie voor patiënten met autisme ¹⁸, blijkt er sprake te zijn van een groot placebo effect in bovenstaande klinische trials met FXS patiënten. Het placebo effect is een veel voorkomend probleem in dit soort trials, waarschijnlijk vanwege de subjectiviteit van de uitkomstmaten ¹⁹. Meer objectieve uitkomstmaten als cognitieve testen bleken vaak onvoldoende uitvoerbaar. Andere uitkomstmaten, die gericht zijn op endofenotypes en biomarkers, zijn vaak onvoldoende onderzocht op klinische relevantie.

Uitdagingen van nieuwe therapeutische strategieën

Opvallend genoeg heeft geen enkele trial geleid tot een effectieve therapie, ondanks de preklinische successen en het gerichte karakter van de medicamenten. Er zijn verschillende mogelijke redenen te bedenken voor deze discrepantie, waaronder het trial design en de gebruikte uitkomstmaten ²⁰. Zoals hierboven besproken zijn de gebruikte uitkomstmaten subjectief en niet ontworpen voor pathway-modificerende therapieën voor ontwikkelingsstoornissen. Nieuwe objectieve en gevalideerde uitkomstmaten zijn noodzakelijk, alvorens potentiële medicamenten worden geëvalueerd.

Een tweede belangrijk aspect betreft de vertaalbaarheid van diemodelstudies met medicatie naar de mens. Ondanks de grote voordelen van diere modellen en de noodzaak ervan voor het ontwikkelen van medicatie, ondervinden vele vakgebieden problemen in de translatie van diere modellen naar mensen. Dit gebrek aan translatie heeft er de laatste jaren toe geleid dat farmaceutische bedrijven, die van oudsher geïnteresseerd waren in de ontwikkeling van psychofarmaca, afzien van verder ontwikkeling van medicamenten, ondanks de grote sprongen die worden gemaakt in het ontrafelen van biologische mechanismen ²¹.

Een derde aspect, betreft de complexiteit van de functie van FMRP. Gezien de verscheidene betrokken pathways, die allen met elkaar samenhangen, is het wellicht noodzakelijk om op verschillende pathways tegelijk aan te grijpen door middel van een combinatietherapie. Ondanks de complexiteit van trial design bij combinatietherapie, zijn er nieuwe trials aangekondigd die een combinatie van twee medicamenteuze interventies gaan testen bij FXS patiënten (<https://clinicaltrials.gov/>). Echter, deze zijn niet of nauwelijks preklinisch getest.

Conclusie

De gedetailleerde ontrafeling van de moleculaire pathofysiologie van ontwikkelingsstoornissen zoals FXS heeft aangrijpingspunten voor gerichte therapie aan het licht gebracht die in diermodellen veelbelovende resultaten hebben opgeleverd. Ondanks de preklinische succesverhalen, zijn de resultaten van klinische trials vooralsnog teleurstellend. Er is een grote pathofysiologische en etiologische overlap van FXS en andere genetische oorzaken van autisme spectrum stoornissen en verstandelijke beperking. Mogelijk zal een gerichte therapie voor FXS ook toepasbaar zijn op deze veel grotere patiëntengroepen. Nieuwe modelsystemen en objectieve klinisch relevante uitkomstmaten en endofenotypes zijn essentieel bij het vervolg van de ontwikkeling van een gerichte therapie voor FXS en andere (monogenetische) ontwikkelingsstoornissen. Zolang gentherapie, waaronder CRISPR-CAS, verre toekomstmuziek zijn voor hersen-gerelateerde ontwikkelingsstoornissen, zal wellicht een combinatietherapie, dat op verschillende pathways tegelijk aangrijpt, onvermijdelijk blijken. Om onderzoek naar toekomstige therapieën te faciliteren, en resultaten te implementeren in de kliniek, is het noodzakelijk kennis en ervaring van FXS te vergroten binnen een multidisciplinair expertisecentrum. Patiënten profiteren van de beste gespecialiseerde zorg voor deze specifieke aandoening en kunnen gemakkelijker bereikt worden voor nieuwe initiatieven. Door het Nederlandse FXS expertisecentrum, ingebed in ENCORE (Erfelijke Neuro-Cognitieve Ontwikkelingsstoornissen Rotterdam Erasmus MC) is mede op basis van die opgedane ervaring een actuele leidraad ontwikkeld voor diagnostiek en zorg van kinderen met het FXS en worden nieuwe uitkomstmaten ontwikkeld voor onderzoek naar toekomstige behandelingen.

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Supplement

Leidraad voor diagnostiek en behandeling van kinderen met het fragiele X syndroom

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1. Introductie

Deze leidraad bevat een overzicht van lichamelijke, psychische en ontwikkelingsproblematiek, voorkomend bij kinderen met het fragiele X syndroom (FXS). Daarnaast worden adviezen gegeven ten aanzien van begeleiding en behandeling. De leidraad is bedoeld voor alle professionals die te maken krijgen met FXS. Hoewel FXS een relatief frequente erfelijke oorzaak is van verstandelijke beperking en autisme spectrum stoornissen (ASS) met een prevalentie geschat op 1:4000-1:7000 jongens, zal een gemiddelde arts of zorgverlener slechts sporadisch in aanraking komen met de aandoening. Daarnaast kunnen kinderen met FXS, vanwege de verstandelijke beperking, vaak maar beperkt communiceren over pijn en ongemakken. Kennis en bewustzijn van mogelijke problemen, kan de zorg voor deze groep verbeteren. Ook behoeven kinderen met FXS vaak een specifieke aanpak van hun problematiek. Voor FXS bestaat geen Nederlandse richtlijnen. Wel is er een informatieve huisartsbrochure van het VSOP en een informatiefolder uit 2009 van het NVAVG. Amerikaanse richtlijnen zijn eveneens verouderd en vaak niet geheel toepasbaar op het Nederlandse zorgsysteem. Om de zorg voor FXS te bundelen en te bevorderen is in 2013 het Fragiele X Syndroom Expertisecentrum opgericht in het Erasmus Medisch Centrum te Rotterdam. Het expertisecentrum is een samenwerkingsverband van verschillende disciplines en is een onderdeel van Expertisecentrum ENCORE (Erfelijke Neuro-Cognitieve Ontwikkelingsstoornissen Rotterdam Erasmus MC). In dit tijdperk waarin grote sprongen worden gemaakt naar een gerichte moleculaire therapie, is het van belang om nieuwe therapeutische opties op waarde te schatten, voordat we deze klinisch kunnen implementeren.

Deze leidraad is tot stand gekomen door middel van het bestuderen en samenvatten van de beschikbare literatuur. De adviezen zijn geformuleerd op basis van de best beschikbare literatuur, toegepast op het Nederlandse zorgsysteem, en waar gedegen adviezen ontbreken, aangevuld met onze eigen expertise. Omdat het expertisecentrum tot voor kort beperkt was tot kinderen, is deze leidraad gericht op kinderen met FXS. Huidige initiatieven houden zich bezig met de transitie naar de volwassen zorg en op termijn zal een leidraad voor volwassenen volgen. In de meeste gevallen zijn de adviezen in deze richtlijn ook toepasbaar op volwassenen. Deze leidraad beperkt zich tot FXS en gaat slechts in beperkte mate in op de uitgebreide problematiek van de premutatie en FXS geassocieerde aandoeningen. De premutatie wordt door een ander pathofysiologisch mechanisme veroorzaakt en geeft een ander klinisch beeld.

2. Inleiding

2.1. Fragiele X syndroom (FXS)

FXS is de meest voorkomende erfelijke vorm van verstandelijke beperking en ASS. Ongeveer 1-6% van patiënten met ASS en 1% van de patiënten met verstandelijke beperking heeft FXS^{1,2}. Daarnaast komt een scala van gedragsproblemen, psychiatrische problemen en medische problemen voor. De mate waarin patiënten zijn aangedaan is zeer variabel, deels afhankelijk van het geslacht en het vóórkomen van genetisch mozaïcisme. De geschatte prevalentie is ongeveer 1:4.000-7.000 mannen. Ondanks het X-gebonden karakter kunnen ook vrouwen aangedaan zijn. De prevalentie van de volle mutatie bij vrouwen wordt geschat op 1:8.000-11.000, waarvan ongeveer de helft de diagnose FXS zal hebben (zie voor uitleg de volgende alinea)³⁻⁵.

FXS wordt veroorzaakt door een mutatie in *FMR1* gen, dat codeert voor het eiwit FMRP. Het *FMR1* gen ligt op het X-chromosoom. In meer dan 99% van de gevallen betreft de mutatie een expansie van de natuurlijk voorkomende CGG repeat in de promotor van het gen. Het aantal CGG repeats in de populatie is polymorf, met 5-44 repeats in de normale situatie. Een expansie tot boven de 200 repeats, genaamd volle mutatie, leidt tot inactivatie van het gen, dat leidt tot FXS (Tabel 1)^{1,6-8}.

2.2. Andere *FMR1*-gerelateerde aandoeningen

Onder “andere *FMR1* gerelateerde aandoeningen” worden aandoeningen bedoeld, die zijn geassocieerd met de *FMR1* premutatie. De premutatie is een CGG repeat-expansie in het *FMR1* gen, van 55-200 repeats (Tabel 1). Hierbij is het gen niet geïnactiveerd, zoals bij FXS het geval is, maar is er sprake van verhoogde RNA-productie en mogelijk een schadelijk eiwit. De verschijnselen en pathofysiologie zijn anders dan van FXS, ondanks dat de premutatie en volle mutatie in de zelfde familie kunnen voorkomen. Daarnaast is de premutatie in de regel instabiel tijdens de meiose bij de vrouw, met een risico op verdere expansie en het optreden van FXS bij het nageslacht. Dit proces heet anticipatie⁹. Ook bestaat een zogenaamde “grijze zone” of intermediair allel dat bestaat uit 45-54 repeats, waarbij er mogelijk meiotische instabiliteit bestaat. De prevalentie van de premutatie die in de literatuur wordt genoemd, is zeer uiteenlopend, van 1:110-1:259 vrouwen en 1:250-1:800 mannen. De prevalentie van het intermediaire allel is ongeveer 1:20-70 vrouwen en 1:40-110 mannen voor⁹⁻¹¹.

In het verleden werd gedacht dat een premutatie geen klachten geeft, maar slechts een verhoogd risico op het krijgen van een kind met FXS, wanneer een vrouw de premutatie heeft. Inmiddels is bekend dat het hebben van de premutatie een scala aan mogelijke verschijnselen kan geven^{12,13}. De meest bekende is het Fragile X Tremor Ataxia Syndrome (FXTAS), dat voorkomt bij ongeveer 45% van de mannen en 8-16% van de vrouwen met premutatie, veelal na het 50^e jaar. Ook komt prematuur ovariumfalen

(FXPOF of FXPOI), ook wel vervroegde overgang genoemd, voor bij ongeveer 20% van de vrouwen¹⁴. Daarnaast wordt de premutatie geassocieerd met andere verschijnselen, waaronder chronische pijnsyndromen, hypertensie, hypothyreoïdie, migraine en psychiatrische problemen als angst, fobische klachten, depressie of ASS. Het is niet duidelijk in hoeverre er sprake is van een bias in de onderzochte populaties en wat de invloed is van het hebben van een kind met FXS of het hebben van FXTAS op deze verschijnselen. Toekomstig onderzoek zal wellicht meer informatie verschaffen. In deze leidraad wordt verder niet ingegaan op de premutatie en het intermediaire allel en de bijhorende verschijnselen.

Tabel 1: CGG repeat aantallen van het FMR1 en het bijbehorende ziektebeeld.

CGG-repeats	Naam	Klinisch beeld	Nageslacht
5-44	Normaal	Normaal	Normaal
45-54	Grijze zone / intermediair allel	Onduidelijk	Klein risico op enige expansie bij maternale transmissie
55-200	Premutatie	FXTAS, FXPOI, etc	Risico op expansie tot > 200 repeats bij maternale transmissie, afhankelijk van aantal repeats
>200	Volle mutatie	FXS	Altijd >200 bij maternale transmissie

3. Genetische Diagnostiek

De diagnose FXS wordt bij jongens gemiddeld rond het 3^e jaar gesteld, aldus buitenlandse literatuur¹⁵. Getallen voor Nederland zijn niet beschikbaar, maar ook hier geldt dat er een aanzienlijke tijd zit tussen de eerste zorgen van ouders, vaak al voor of rond het eerste jaar, en de definitieve diagnose. Het stellen van de diagnose FXS is zinvol voor het kind en zijn of haar familieleden om een aantal redenen. De soms lange en belastende zoektocht naar een oorzaak voor de klachten kan worden gestaakt en de diagnose geeft ouders en familie inzicht in de aandoening, de prognose en wat hen nog meer te wachten zou kunnen staan. De behandeling en begeleiding kunnen worden gericht op het kind en de ouders, met aandacht voor specifieke kennis over de aandoening. Daarnaast kunnen familieleden en de patiënt gecounseld worden ten aanzien van hun eigen gezondheidsrisico's gerelateerd aan de diagnose (zie onder andere het kopje "andere FMR1 gerelateerde aandoeningen"), geeft het hen inzicht in eventuele eigen medische problemen en stelt het hen in staat reproductieve keuzes te maken.

3.1. Wanneer en bij wie DNA-onderzoek van het fragiele X syndroom

Verskillende internationale publicaties houden er subtiel verschillende visies over het moment waarop genetische diagnostiek zou moeten plaatsvinden ^{1,6,7,16}. Hieronder worden criteria opgesomd, toegepast op de Nederlandse gezondheidszorg.

Patiënten met (mogelijke) verschijnselen van FXS

- Kinderen met milde tot matige verstandelijke beperking, motorische ontwikkelingsachterstand, spraak-taalachterstand of ASS, met name wanneer deze gepaard gaan met gedragsproblemen en somatische kenmerken van FXS. Dit geldt zowel voor jongens als meisjes, ongeacht de aanwezigheid van uiterlijke kenmerken. Bij ongeveer 1-6% van deze populatie wordt een *FMR1* mutatie vastgesteld. Vanwege de brede differentiaaldiagnose zouden SNP Array en metabole diagnostiek tevens moeten worden overwogen. Geïsoleerde gedragsproblemen zoals ADHD zijn over het algemeen geen indicatie.
- Volwassenen met verstandelijke beperking zonder een diagnose kunnen getest worden indien er behoefte is aan een diagnose vanuit de familie, of in het kader van zorg voor de patiënt.
- Bij baby's en jonge kinderen van een moeder met een *FMR1* (pre)mutatie, kan overwogen worden in een vroeg stadium DNA onderzoek in te zetten, indien zich opvallende kenmerken van FXS voordoen, zoals hypotonie, gastro-oesofageale reflux en prikkelbaarheid. Het verdient de voorkeur om pas te testen wanneer er duidelijke verschijnselen zijn, of wanneer dit consequenties heeft voor de begeleiding van het kind, in verband met het recht op autonomie van het kind.
- Bij patiënten bij wie in het verleden de diagnose met cytogenetische technieken is vastgesteld, kan de diagnose met huidige DNA-technieken worden bevestigd, voor een accuratere diagnose en adviezen voor familieleden.
- Wanneer het vermoeden op Sotos syndroom of Prader-Willi syndroom bestaat, zonder moleculaire bevestiging hiervan, kan DNA diagnostiek van het *FMR1* gen worden overwogen, vanwege de beschreven Sotos en Prader-Willi fenotypes bij FXS ^{17,18}.

Familieleden

- Asymptomatische personen met FXS in de familie: In het kader van cascadescreening, bij vrouwelijk familieleden met kinderwens of tijdens een zwangerschap en bij familieleden die zelf behoefte hebben aan duidelijkheid over mogelijk dragerschap, in verband met risico's voor henzelf en eventueel familieleden. Wanneer in het verleden cytogenetische technieken zijn gebruikt, kan de test herhaald worden met recente technieken, ook wanneer deze in het verleden een normale uitslag had gegeven, omdat deze niet accuraat waren, met name ten aanzien van de premutatie.
- Personen met symptomen van de premutatie: Bij vrouwen met POF of verhoogd FSH voor het 40^e jaar en patiënten met een vermoeden op FXTAS op basis van klinische criteria¹³ of bij patiënten met late-onset (intentie-) tremor, ataxie of parkinsonisme zonder andere oorzaak, kan DNA onderzoek worden overwogen.

3.2. Genetische testen

DNA-onderzoek van het FMR1 gen: Bij het overgrote deel van de gevallen wordt FXS veroorzaakt door een CGG repeat expansie in het *FMR1* gen. Wanneer DNA-onderzoek wordt aangevraagd, dan worden standaard het aantal CGG-repeat en de methyleringsstatus bepaald in de klinisch genetische laboratoria in Nederland. De sensitiviteit bedraagt >99%, vanwege het missen van ongeveer <1% microdeleties of puntmutaties^{6,19}.

Sanger sequencing en exome/genome sequencing: Vanwege counselingsaspecten en de brede differentiaaldiagnose van FXS, adviseren wij deze testen via een klinisch geneticus te laten verlopen. Wanneer CGG repeatanalyse geen afwijkingen toont en er een sterke klinische verdenking is op FXS, kan sanger sequencing van het *FMR1* gen worden overwogen. Omdat momenteel exome sequencing laagdrempeliger wordt verricht, zullen er steeds meer *FMR1* mutaties via die techniek worden opgespoord^{20,21}. Een verlengde CGG repeat kan echter in de meeste gevallen niet met exome/genome sequencing worden opgespoord en zal apart moeten worden aangevraagd.

Haarworteltest: Bij de haarworteltest wordt gekeken naar FMRP expressie in haarwortels. Dit betreft een non-invasieve functionele test die plaatsvindt in het Erasmus MC. Het percentage FMRP dat tot expressie komt in haarwortels, correleert met het verstandelijke en gedragsfenotype, meer dan bijvoorbeeld DNA-onderzoek op lymfocyten²². Haarworteltest heeft een toegevoegde diagnostische waarde, als aanvulling op DNA-onderzoek, bij mozaïcisme of als voorspellende informatie bij jonge meisjes met de volle mutatie.

Ook kan deze test informatie geven wanneer een onbekende puntmutatie wordt vastgesteld ²³.

Overige punten van aandacht:

Mozaïcisme: Bij veel patiënten met FXS is er sprake van mozaïcisme, vanwege het instabiele karakter van de volle mutatie. Wanneer er een hoog percentage mozaïcisme is (premutatie of ongemethyleerde volle mutatie), kan er een milder klinisch beeld voorkomen. Mozaïcisme kan worden opgespoord met DNA-onderzoek op lymfocyten of met de haarworteltest, wanneer deze in een voldoende hoge percentage aanwezig is ^{24,25}.

AGG interrupties: Er zijn aanwijzingen dat de aanwezigheid van AGG interrupties in de CGG repeats de kans op expansie verkleinen²⁶. De precieze bijdrage van AGG interrupties aan het risico op expansie is echter nog onvoldoende in kaart gebracht. AGG interrupties worden vooralsnog niet bepaald bij DNA-onderzoek.

3.3. Klinische diagnostische criteria

Er bestaan verschillende versies van klinische criteria voor FXS (zie de tabellen in Bijlage 1) ²⁷⁻³¹. Hiermee kan de waarschijnlijkheid van de diagnose wellicht worden verhoogd, echter de diagnostische waarde is waarschijnlijk zeer beperkt met de huidige laagdrempelig toegankelijke technieken. De kenmerken van FXS zijn vaak aspecifiek, ontstaan vaak later in het leven en er is een grote variatie van het klinisch beeld, zodat het ontbreken van kenmerken onvoldoende is om klinisch de diagnose uit te sluiten. De diagnose zal altijd met DNA-onderzoek bevestigd of uitgesloten moeten worden.

3.4. Differentiaal diagnose

Met name in de vroege kinderjaren zijn de verschijnselen van FXS aspecifiek. Alle andere (X-gebonden) genetische oorzaken van non-syndromale of mild syndromale ontwikkelingsachterstand, verstandelijke beperking en/of autisme kunnen differentiaal diagnostisch worden overwogen. Dit geldt vaker voor syndromen waarbij macrocefalie voorkomt, zoals het Sotos syndroom en andere overgroeisyndromen. Hieronder vallen ook mutaties in het *FMR2* gen die leiden tot milde verstandelijke beperking zonder de lichamelijke verschijnselen en familie-anamnese passend bij FXS.

Verworven oorzaken van verstandelijke beperking en gedragsproblemen kunnen tevens overwogen worden, waaronder het foetaal alcoholyndroom. Het onderscheid kan gemaakt worden op basis van de aanwezigheid van andere kenmerken, familieanamnese, omgevingsfactoren tijdens de zwangerschap en bevalling en het DNA-onderzoek ^{2,32}.

3.5. Preconceptie diagnostiek

In Nederland wordt preconceptie screening van FXS niet standaard aangeboden, behalve wanneer er familiale belasting bestaat of wanneer de patiënt zelf verschijnselen heeft. Zie “*Wanneer en bij wie DNA-onderzoek van het fragiele X syndroom*”. Indien een ouderpaar weet of één van hen de aanleg heeft, stelt dat hen beter in staat om reproductieve keuzes te maken ^{1,16}

3.6. Prenatale en pre-implantatie genetische diagnostiek

FXS is een indicatie voor ofwel prenatale (PND) ofwel pre-implantatie (PGD) genetische diagnostiek indien de vrouw een volle of premutatie heeft in het *FMR1* gen (>200 respectievelijk ≥ 55 repeats) ^{1,26}.

Een man met een *FMR1* premutatie zal deze onveranderd aan alle dochters doorgeven, resulterend in een kans op een late-onset aandoening. PND en PGD zijn hierbij in de meeste gevallen niet geïndiceerd. Een man met een *FMR1* volle mutatie heeft zelf FXS en zal over het algemeen geen kinderen krijgen. Indien hij toch een kind verwekt, zullen alle dochters een premutatie erven. Alle zonen zullen niet de aanleg erven, omdat zij de Y chromosoom krijgen.

PND vindt plaats door middel van regulier DNA diagnostiek (CGG repeat analyse en methyleringsstatus) op embryonaal weefsel verkregen uit chorion villus afname (vlokkentest, 11-12 weken, uitslagtermijn 2-3 weken) of amniocentese (vruchtwaterpunctie 15-16 weken, uitslagtermijn 3 weken). Beiden zijn accuraat ten aanzien van de repeat-lengte. Een vlokkentest is echter minder betrouwbaar ten aanzien van de methyleringsstatus, omdat de methylering nog incompleet is in de placenta tussen 10-12 weken ^{30,33}. Indien de ouders in hun keuze het geslacht van het kind willen meewegen, kan foetale geslachtsbepaling voorafgaand worden verricht. Het is van belang te realiseren dat de ernst van de aandoening, zeker in het geval van een meisje, niet kan worden voorspeld. Ook kan er op basis van methyleringsstudies en repeat-bepaling geen prognostische informatie worden verstrekt, omdat deze onbetrouwbaar zijn in de prenatale setting.

PGD met *in vitro* fertilisatie (IVF) is tevens in veel gevallen beschikbaar voor FXS. Of een ouderpaar in aanmerking komt hiervoor, wordt beoordeeld door een PGD centrum. Naast de beperkingen van IVF, is PGD niet altijd technisch haalbaar voor FXS. Ook kan een beginnende POF een beperkende factor zijn bij de IVF procedure. Andere opties rondom een kinderwens dienen te worden besproken, waaronder het risico accepteren, eicel- (of zaadcel-) donatie en adoptie. Counseling over de interpretatie en mogelijke uitslagen, door een klinisch geneticus, psycholoog en gynaecoloog, is van belang voor een geïnformeerde keus.

3.7. Populatiescreening

FXS wordt niet op populatieniveau bepaald in Nederland (zoals de hiehprik of preconceptioneel). Argumenten die vooralsnog tegen de hiehprik pleiten, zijn het ontbreken van een gedegen behandeling en het ontbreken van een goede high throughput methode. Ook zijn er ethische bezwaren die verband houden met het zeer variabele fenotype (vooral bij vrouwen en in het geval van mozaïcisme) en de premutatie die tevens zou worden vastgesteld bij kinderen. Omdat de premutatie met name op volwassen leeftijd verschijnselen kan geven, en omdat sommige vrouwen geen verschijnselen zullen krijgen van de volle mutatie, zou de hiehprik het kind ontnemen om hierin een eigen keuze te maken.^{1,30,34} Daarnaast is er voor het presymptomatisch testen een goede counseling noodzakelijk, wat veel teweeeg zou kunnen brengen bij ouders, die a priori een laag risico hebben op FXS. Internationaal is er de laatste jaren veel aandacht voor de hiehprik voor FXS, met name vanwege het vergroten van de reproductieve opties voor ouders en hun verwanten³⁴. Studies laten zien dat in potentie de premutatie bij relatief veel mensen voorkomt en de meeste dragers zich hiervan niet bewust zijn. De diagnose FXS wordt in ongeveer 50% van de gezinnen pas gesteld nadat een volgend kind is geboren. Gezinnen met een kind met FXS geven aan dat zij een andere beslissing zouden hebben genomen ten aanzien van kinderwens, indien zij hadden geweten dat een eerder kind de aandoening had¹⁵. Dit is een belangrijk argument dat bij beslissingen rondom de hiehprik en preconceptiescreening kan worden meegewogen.

4. Klinische kenmerken, aanbevelingen voor follow-up en behandeling

Lichamelijke, ontwikkelings- en psychiatrische problematiek worden hieronder besproken, met aanbevelingen voor diagnostiek, behandeling en begeleiding. In Bijlage 2 vindt u de kenmerken en aanbevelingen samengevat.

4.1. Lichamelijke problematiek

Uiterlijke kenmerken/lichamelijk onderzoek

FXS gaat gepaard met milde en vaak aspecifieke dysmorphe kenmerken, die vaak bij kleine kinderen afwezig zijn en met de leeftijd toe nemen (zie ook Bijlage 1). De meest opvallende zijn een grote hoofdomtrek, prominent voorhoofd, een lang smal gezicht met een prominente kin en grote afstaande oren. Veel mannen hebben een relatieve macrocefalie. Het palatum kan hoog of gotisch zijn. Een palatoschisis is zelden beschreven. Er kan sprake zijn van crowding van de tanden en malocclusie. Ook kan er sprake zijn van strabismus. Opvallende macro-orchidisme wordt gezien bij ongeveer 80% van de adolescenten en volwassen mannen, en ontstaat vanaf de puberteit. De huid kan

soms zacht aanvoelen, hyperelastisch zijn en de gewrichten zijn vaak hypermobiel, met name de vingers en polsen. Ook komen pes planus (80%), scoliose, pectus excavatum, cubitus valgus en klompvoeten voor. 50% van vrouwen met volle mutatie heeft enige fysieke kenmerken van FXS ^{7,29,35-37}.

Aanbevelingen: een dysmorfologisch onderzoek is onderdeel van het diagnostisch traject. Specifieke kenmerken die behandeling behoeven worden hieronder besproken.

Epilepsie

Epilepsie komt frequenter voor bij kinderen met FXS dan in de algemene populatie, hoewel de gerapporteerde prevalentiecijfers uiteenlopen. In hoogrisicogroepen, gerekruteerd op neurologie afdelingen, worden prevalentie cijfers tot 44% gevonden ³⁵. In bevolkingsstudies bij kinderen met FXS ligt de prevalentie lager, tussen de 10 en 18% ³⁸⁻⁴⁰. De meest genoemde getallen zijn 10-20% van de jongens en 5% van de meisjes met FXS ^{7,35,39,41,42}. Veel kinderen met FXS hebben abnormale EEG's, zonder zichtbare insulten, vaak met een patroon dat lijkt op dat van benigne focale epilepsie ^{43,44}. De grotere studies tonen een predominantie van partiële epilepsie en absences ^{35,40} met in enkele case reports een secundaire generalisatie en status epilepticus ^{45,46}. De prognose van epilepsie bij FXS is meestal gunstig en insulten verdwijnen vaak gedurende de eerste 10 jaar ⁴⁰.

Er zijn slechts milde anatomische hersenafwijkingen beschreven bij FXS, waaronder een vergroot vierde ventrikel, vergrote nucleus caudatus, vergrote hippocampus, toegenomen hersenvolume en een kleine cerebellaire vermis ^{36,47,48}. Postmortem zijn microscopische afwijkingen beschreven van de morfologie van dendritische uitlopers ⁴⁹.

Aanbevelingen: Routinematige EEG screening bij afwezigheid van klinische symptomen wordt niet aangeraden ³⁵. Bij aanwijzingen voor aanvallen (waaronder ook bijzonder gedrag, staren, nachtelijk braken), is nader onderzoek door de (kinder-) neuroloog of kinderarts aangewezen. Epilepsie bij FXS wordt volgens gangbare principes behandeld, waarbij doorgaans monotherapie volstaat ⁵⁰. Een routine MRI is niet geïndiceerd.

Groei en voeding

Voedingsproblemen bij zuigelingen met FXS zijn frequent, onder andere door gastro-oesofageale reflux (bij 11-30%) en een gestoorde mondmotoriek ^{7,29,35,50}. Chronische diarree komt bij ongeveer 11% voor ³⁵. Geboortegewicht en -lengte zijn doorgaans normaal tot licht verhoogd, waarna lengtegroei versneld verloopt, om in de adolescentie weer af te buigen. Bij meisjes is er geen verschil in de groei ten opzichte van de normale populatie ^{7,35,50}. Bij een kleine groep is er sprake van een Sotos (overgroei) of Prader-

Willi (overgewicht ten gevolge van hyperfagie en kleine lengte) fenotype ^{17,50}.

Aanbevelingen: bij voedingsproblemen is onderzoek naar een eventueel onderliggend probleem in de mondmotoriek of gastro-oesofageale reflux via de kinderarts noodzakelijk. Follow-up en behandeling is conform groei- en voedingsproblemen zonder FXS. Consultatie van een diëtiste is te overwegen bij overgewicht.

Oogheekundige problemen

Bij jonge kinderen met FXS komen frequent oogheekundige problemen voor waaronder strabismus, refractieafwijkingen (hypermetropie), astigmatisme, ptosis en nystagmus ⁷.

Aanbevelingen: in het eerste jaar volstaat onderzoek volgens het screeningsprogramma op het consultatiebureau. Bij twijfel direct verwijzing naar oogarts. Tussen eerste en tweede levensjaar is éénmalig oogheekundig onderzoek raadzaam, daarna volstaat weer het screenend onderzoek binnen de jeugdgezondheidszorg. Twijfel over de visus bij ouders is aanleiding voor verwijzing naar de oogarts.

Keel-, neus- en oorproblemen

85% van de kinderen maakt een of meer episodes van acute otitis media door voor het 5^e levensjaar ³⁵. Chronische of recidiverende otitis media komt bij 45-65% van de patiënten voor, vaak al vanaf babyleeftijd, en kan leiden tot gehoorverlies en taal- spraakachterstand ^{35,36,50}. Recidiverende sinusitis komt bij 23% van de kinderen voor. Obstructieve slaapapneu (OSAS) komt regelmatig voor, waarschijnlijk door een combinatie van faciale kenmerken, bindweefselproblemen en hypotonie.

Aanbevelingen: Bij gedragsveranderingen, koorts, hoofdpijn en braken laagdrempelig inspectie van de oren. Verder wordt geadviseerd jaarlijks het gehoor en de oren te controleren en laagdrempelig te verwijzen naar de KNO arts voor verdere diagnostiek en behandeling. Vanwege het risico op gehoorverlies bij deze groep waar taal-spraakachterstand al frequent is, is laagdrempelig ingrijpen aan te bevelen. Overmatig snurken, onrustige slaap en vermoeidheid kunnen wijzen op OSAS en behoeft verdere diagnostiek via de kinderarts in samenwerking met de KNO-arts. De jaarlijkse griep prik is waarschijnlijk zinvol, alhoewel daar geen bewijs voor is.

Cardiovasculaire problemen

In het verleden is gerapporteerd dat mitralisklepprolaps frequent voorkomt bij FXS: bij tot 50% van de volwassen mannen en 20% van de vrouwen ^{7,50}. Getallen voor kinderen zijn

niet bekend. Een recent database-onderzoek van FXS poliklinieken in de VS, waarin met name kinderen zijn beschreven, toont echter maar bij 1% mitralisklepprolaps³⁵, wat niet verhoogd is in vergelijking met de algemene bevolking. Ook milde aortaworteldilatatie is beschreven. Hypertensie komt veel voor, met name op volwassen leeftijd.

Aanbevelingen: Ondanks de wisselende getallen, wordt jaarlijkse auscultatie van het hart (door AVG, huisarts of kinderarts) aanbevolen met laagdrempelige verwijzing naar een kindercardioloog. Routinematige echocardiografie op kinderleeftijd is niet zinvol. Na de puberteit en/of in de transitieperiode, valt éénmalige controle door een (kinder-) cardioloog te overwegen. De bloeddruk kan via de huisarts of AVG worden gemonitord en eventueel gangbaar farmacologisch behandeld.

Bewegingsapparaat- en bindweefselproblemen

Kinderen met FXS hebben vaak verschijnselen die passen bij bindweefselproblematiek, waaronder een zachte hyperelastische huid, hypermobiliteit met name van de vingers, pes planus (bij 80%), scoliose (bij 20%), congenitale heupdysplasie, klompvoeten, hoog palatum, crowding van de tanden, malocclusie en hernia inguinalis^{7,35}.

Aanbevelingen: bij pronatie van de voeten kan een orthese noodzakelijk zijn. Bij het optreden van een hernia inguinalis, met name in de jonge kinderjaren, kan het kind verwezen worden naar de kinderchirurg voor verdere evaluatie en behandeling. Scoliose kan worden gemonitord d.m.v. lichamelijk onderzoek, maar is meestal mild en behoeft geen interventie. Kinderfysiotherapie of ergotherapie kan overwogen worden bij motorische problematiek.

Urogenitale en endocriene problemen en voortplanting

Enuresis komt bij ongeveer de helft van de kinderen met FXS voor, met name 's nachts. Bij jongvolwassenen komt het nog bij ongeveer een kwart voor⁵¹. Er is mogelijk een predispositie voor dilatatie van de ureteren en vesico-ureterale reflux. Macro-orchidisme is een zeer frequent, maar verder onschuldig kenmerk bij jongens met FXS. Omdat FMRP nodig is voor spermatogenese, zal sperma niet de volle mutatie bevatten, maar bevat wel de premutatie (dit heeft te maken met mozaïcisme). Er zijn beperkte aanwijzingen voor een gestoorde spermatogenese en verminderde fertiliteit bij mannen met FXS^{7,52,53}. Meisjes met de volle mutatie hebben soms te maken met een vervroegde puberteit, de oorzaak is niet bekend.

Aanbevelingen: Bij urineweginfecties en enuresis laagdrempelig verwijzen naar kinderarts, -uroloog of –nefroloog voor beeldvorming. Behandeling van enuresis is, evenals bij andere kinderen, vooral gericht op gedragsverandering, eventueel met gebruik van een alarmsysteem bij nachtelijke enuresis. Hierbij dient rekening te worden gehouden met zowel het fysieke als mentale ontwikkelingsniveau van het kind. Het wordt aanbevolen training niet uit te stellen, in verband met een verhoogd risico op het niet voelen van de aandrang. Ook dient de te worden nagegaan of er obstipatie is ⁵⁴. Online zijn er verschillende methoden en hulpmiddelen beschikbaar (bijvoorbeeld op: www.zindelijkheidstraining.com). Indien onvoldoende effect wordt bereikt, is begeleiding door een multidisciplinair incontinentieteam zinvol. Medicamenteuze interventies (desmopressine, imipramine) zijn meestal teleurstellend, maar kunnen soms ter overbrugging van een logeerperiode nuttig zijn (desmopressine). Macro-orchidisme behoeft geen behandeling. De puberteit moet bij meisjes worden geëvalueerd. Bij vrouwen met FXS die zwanger willen worden, kan wilsbekwaamheid worden geëvalueerd. Daarnaast dienen reproductieve opties te worden besproken (accepteren risico, PND, PGD, eiceldonatie, adoptie, afzien van kinderen). Zie tevens het kopje “*Puberteit en seksualiteit*”

Tandheelkundige afwijkingen

Veel kinderen met FXS hebben crowding van de tanden en malocclusie. Daarnaast hebben kinderen met FXS vaak een sterke tactiele afweer bij aanraken van de mond, waarschijnlijk door de problemen in de sensorische informatieverwerking die bij FXS veel voorkomen.

Aanbevelingen: Reguliere tandheelkundige evaluatie kan het best plaatsvinden bij een tandarts met ervaring met kinderen met gedragsproblemen en angst. Een nauwe samenwerking met het ziekenhuis,, een Centrum voor Gespecialiseerde Tandzorg (CGT), of een VG-organisatie met een gespecialiseerde tandarts is aan te bevelen, omdat narcose vaak noodzakelijk is bij tandheelkundige ingrepen.

Een neonaat met FXS

In sommige gevallen wordt er prenataal onderzoek gedaan naar FXS en wordt een neonaat geboren met een moleculaire diagnose. Over het algemeen zullen baby's in de eerste periode weinig verschijnselen hebben. Er kan soms sprake zijn van voedingsproblemen of gastro-oesofageale reflux. Ook kan er uitgesproken hypotonie zijn en frequente otitiden. Sommige kinderen zijn al vanaf hele jonge leeftijd prikkelbaar, wat in verband wordt gebracht met sensorische informatieverwerkingsproblemen zoals tactiele hyperreactiviteit. Er kan sprake zijn van aangeboren afwijkingen, waaronder heupdysplasie en klompvoeten.

Aanbevelingen: onderzoek naar heupdysplasie en beoordelen van klompvoeten is van belang bij de neonaat. Voedingsproblemen en gastro-oesofageale reflux, hypotonie en prikkelbaarheid kunnen, indien nodig, worden behandeld. Indien de diagnose al op babyleeftijd bekend is, is vervolg door de kinderarts zinvol teneinde de ontwikkeling over een langere termijn te volgen en zinvolle interventies (door kinderfysiotherapeut, logopedist, KNO-arts, orthopedagoog of psycholoog, kinderpsychiater en revalidatiearts) te coördineren.

Aandachtspunten bij narcose

Omdat kinderen met FXS snel overprikkeld raken, vaak onrustig en niet coöperatief zijn, zal narcose in vele gevallen noodzakelijk zijn, ook voor kleine ingrepen als tandheelkundige inspectie. Wanneer een kind met FXS onder narcose moet worden gebracht, is er een aantal punten dat aandacht verdient, waaronder het frequenter voorkomen van OSAS, cardiovasculaire afwijkingen, bindweefselproblematiek en het gebruik van anti-epileptica en psychofarmaca.

Aanbevelingen: ten aanzien van middel van keuze zijn er geen aanbevelingen vanuit de literatuur. Extra aandacht om stress en angst te reduceren is aan te bevelen. Zie voor meer informatie de paragrafen verderop over angst en https://www.orpha.net/data/patho/Pro/en/Fragile_X_EN.pdf.

4.2. Ontwikkeling en psychiatrische problematiek

Verstandelijke beperking, cognitieve- en leerproblemen

Bij jongens met FXS is er meestal sprake van een matige tot ernstige verstandelijke beperking. Meisjes met de volle mutatie en FXS hebben meestal een lichte tot matige verstandelijke beperking³⁶. Bij jongens is het IQ tussen de 20 en de 60 en bij meisjes tussen de 50 en 85. Bij zowel jongens als meisjes met FXS zijn het lage IQ en andere cognitieve problemen geassocieerd met het voorkomen van mozaïcisme en de hoeveelheid FMRP^{55,56}. Het is echter niet mogelijk om deze de mate van verschijnselen te voorspellen op basis hiervan. Gedurende de ontwikkeling neemt het (non-verbale) IQ van kinderen met FXS af tot het 12^e levensjaar, waarna het stabiliseert. Waarschijnlijk is er geen sprake van een achteruitgang, maar wel van een minder snelle intellectuele groei in die periode⁵⁷.

Cognitieve problemen zijn met name te zien bij taken waarbij veel hogere orde mentale capaciteit nodig is, bijvoorbeeld bij de executieve functies. Dit zijn cognitieve vaardigheden die betrokken zijn bij doelgericht gedrag, zoals het verschuiven van aandacht, het plannen van handelingen, het inhiberen van ongewenst gedrag en het

werkgeheugen⁵⁸⁻⁶⁰. Daarnaast wordt het gedrag van kinderen met FXS vaak gekenmerkt door onoplettendheid, hyperactiviteit en impulsiviteit, waardoor veel van hen ook een ADD of ADHD diagnose hebben. Ook zijn zij vaak overgevoelig voor stimuli uit hun omgeving waardoor ze mogelijk nieuwe situaties proberen te vermijden. Deze eigenschappen hebben vaak ook een nadelig effect op het leervermogen van de kinderen⁵⁹. Vergeleken met andere kinderen met een verstandelijke beperking hebben kinderen met FXS meer moeite met het verschuiven of wisselen van de aandacht, waaronder visuele of taakgerichte aandacht. Zij missen de cognitieve flexibiliteit⁶⁰. Dit blijkt ook uit het feit dat zij vaak meer tijd nodig hebben om te reageren en het moeilijk vinden om niet noodzakelijke responsen te inhiberen. Zo zullen zij sneller geneigd zijn om de instructies van een eerdere opdracht te blijven volgen hoewel een tweede opdracht al is gestart⁵⁹. Ook op het gebied van rekenen ondervinden kinderen met FXS vaak moeite. Deze problemen worden gerelateerd aan executieve functies, ruimtelijk inzicht en leesvaardigheid⁵⁸. Een klein Amerikaans onderzoek bij kinderen tussen 5 en 9 jaar liet zien dat 13 van de 15 meisjes met FXS (87%) rekenproblemen heeft, vergeleken met 44% in de algemene populatie (cijfers voor jongens zijn onbekend)⁵⁸. Zowel jongens als meisjes hebben een verminderde werkgeheugencapaciteit⁵⁹. Jongens met FXS hebben in het bijzonder moeite met het onthouden van sequentiële informatie, zoals reeksen cijfers, wat problemen oplevert bij het tellen^{57,58}. Bij meisjes nemen de prestaties af naarmate taken meer geheugencapaciteit vereisen. Daarnaast kunnen meisjes moeite hebben met het onthouden van visueel ruimtelijke informatie⁵⁸. Kinderen met FXS hebben ook meer moeite met abstract redeneren, maar zijn relatief sterk in taken waar concreet redeneren nodig is⁶⁰. Met name meisjes zijn relatief sterk in het herkennen van incomplete of verborgen afbeeldingen, en het schrijven en lezen van cijfers. Jongens zijn sterk in het onthouden van betekenisvolle informatie in een context⁵⁸. Daarnaast zijn kinderen met FXS relatief sterk in woordenschat en zinsopbouw, perceptuele integratie en het herkennen van gezichten en emoties^{59,60}. De studies waaruit bovenstaande kenmerken worden afgeleid, moeten met voorzichtigheid worden geïnterpreteerd vanwege de vaak kleine steekproefgrootte.

Aanbevelingen: Vroege stimulering van de ontwikkeling heeft voordelen voor kinderen met FXS, met behulp van een orthopedagoog of psycholoog. Voorbeelden van een ontwikkelings-stimuleringsprogramma zijn 'Kleine stapjes' of het Portage programma. Met name als er naast FXS ook autisme speelt, zijn gedragsinterventies en vroegtijdige stimulatie van de ontwikkeling van belang⁶¹. Om te achterhalen welke cognitieve of leerproblemen een rol spelen, kan een (neuro)psychologisch en/of orthodidactisch onderzoek worden verricht. Soms zitten kinderen met FXS, voornamelijk meisjes, in het reguliere onderwijs en kan ondersteuning van selectieve leerproblemen, bijvoorbeeld in de vorm van remedial teaching, voldoende zijn. Als er meer dan dit nodig is en als kinderen daarbij sociale en/of emotionele problemen ontwikkelen omdat ze chronisch worden overvraagd, kan plaatsing in speciaal (basis) onderwijs, speciaal onderwijs binnen cluster 2 (taalontwikkelingsstoornissen), 3 (verstandelijke en/of motorische

beperkingen) of 4 (ernstige gedrags- en/of leerproblemen) worden overwogen. Om de cognitieve en sociaal-emotionele ontwikkeling van kinderen met FXS te volgen, met name op transitie momenten in het onderwijs, is er bij het FXS expertisecentrum van ENCORE een follow-up programma opgestart. In dit VOLG-(Vroegtijdige Onderkenning Leer- en Gedragsproblemen) programma worden kinderen rond 3, 6, 11 en 15 jaar onderzocht met behulp van (neuro)psychologisch en kinderpsychiatrisch onderzoek. Indien nodig, kan dit onderzoek ook op indicatie buiten deze vaste momenten worden afgenomen. Hierbij is het van belang om gebruik te maken van gestandaardiseerde instrumenten voor het observeren en testen van de ontwikkeling, maar ook semi-gestandaardiseerde observatie van sociaal gedrag om ontwikkelingsstoornissen en andere psychiatrische stoornissen tijdig op te sporen en te kunnen behandelen.

Door de cognitieve en sociaal-emotionele (ontwikkelings-)problemen is ook het adaptief functioneren verstoord. Kinderen met FXS leren dagelijkse vaardigheden meestal later en hebben hier meer geduld en begeleiding bij nodig. Ondersteuning van kinderen en ouders op dit gebied door een gedragskundige (psycholoog of orthopedagoog) is daarom geregeld geïndiceerd. Eerstelijns hulp kan worden opgestart via het wijkteam of (bij jonge kinderen) integrale vroeghulp. Indien dit onvoldoende mocht blijken, kunnen het wijkteam of de huisarts doorverwijzen naar de tweede of derde lijn, zoals integrale vroeghulp, MEE, GGZ of revalidatie. Bij ernstige verstoring van de balans tussen draagkracht en draaglast voor de ouders kan ook aanvragen van een persoonsgebonden budget (PGB) worden overwogen.

Motorische ontwikkeling

Voor het tweede levensjaar worden een vertraging in het behalen van mijlpalen gezien ⁶². Hypotonie, hyperlaxiteit en stoornissen in de motorische planning en de sensorische informatieverwerking dragen hieraan bij. Het hebben van ASS en een lage IQ, hangt met de mate van achterstand samen. ^{63,64}.

Aanbevelingen: (Kinder-) fysiotherapie of ergotherapie kunnen zinvol zijn bij hypotonie, houdingsproblemen en als er moeilijkheden zijn met het verwerven van motorische en alledaagse vaardigheden die ernstiger zijn dan mag worden verwacht op basis van de algehele ontwikkeling.

Spraak/taalontwikkeling

Als spraak en taal later op gang komen, is dat vaak een van de redenen om aan FXS en/of ASS te denken ⁶⁵. Veel kinderen zijn moeilijk verstaanbaar en laten opvallende kenmerken zien, zoals echolalie, van de hak op de tak springen, favoriete onderwerpen blijven herhalen en snel en onsamenvattend praten ⁵⁹. Een minderheid van de

kinderen met FXS gaat nooit praten. Het merendeel (90%) kan communiceren, met op zijn minst één woord of gebaar ⁶⁶. Het kan ook voorkomen dat iemand met FXS alleen in bepaalde situaties niet verbaal communiceert, terwijl dit bijvoorbeeld thuis wel gebeurt (selectief mutisme) ^{59,67}. Kinderen met een combinatie van FXS en ASS hebben een tragere expressieve en receptieve taalontwikkeling in vergelijking met kinderen die alleen FXS hebben ⁶⁸. Zo zijn zij bijvoorbeeld slechter in staat om aan te geven dat zij iets niet begrijpen in vergelijking met kinderen met alléén FXS of ASS ⁶⁹.

Aanbevelingen: Ondersteuning door een logopedist kan worden ingezet als de taalontwikkeling minder vlot verloopt dan mag worden verwacht op basis van de algehele ontwikkeling. In de kinder- en jeugdpsychiatrie en in de zorg voor kinderen met een verstandelijke beperking, worden soms behandelingen aangeboden die gericht zijn op de ontwikkeling van communicatieve vaardigheden bij kinderen met ASS, zoals Pivotal Respons Treatment (PRT) en applied behavior analysis (ABA).

Sociale ontwikkeling en autisme spectrum stoornissen (ASS)

Over het algemeen zijn kinderen met FXS heel vriendelijk en verlegen, waarbij ze een bescheiden indruk maken. Kinderen met FXS kunnen veel moeite hebben met sociale contacten, zijn vaak verlegen en angstig en nemen weinig tot geen initiatief ³⁶. Naast vermijdend kunnen zij ook hyperactief en agressief reageren in sociale situaties. Mogelijk dat de overgevoeligheid en beperkingen in het omgaan met emoties van deze kinderen ertoe bijdraagt dat zij niet goed met de spanning van sociale interacties om kunnen gaan ^{36,55}. Hierdoor kan het zijn dat kinderen met FXS sociale interacties proberen te vermijden, wat het aanleren van sociale vaardigheden weer in de weg staat ⁵⁵. Kinderen kunnen snel overprikkeld zijn (ook tactiel), laten stereotiepe bewegingspatronen zien (zoals fladderen met hun handen of bijten op de hand) en andere ASS kenmerken zoals sterke verlegenheid en vermijden van oogcontact ³⁶. Verlegenheid, sociale angst, specifieke fobieën, impulsiviteit en schizotypische kenmerken kunnen leiden tot beperkingen in de sociale omgang ³⁶. Ongeveer 30 tot 75% van kinderen met FXS voldoet aan de criteria van een ASS, met een lagere prevalentie van rond 20% bij meisjes ^{38,70-72}. Kenmerken kunnen al bij zeer jonge kinderen worden gezien. In een studie werd gevonden dat de helft van 12 maanden oude kinderen met FXS, in de categorie “at risk” vielen, vergeleken met 6% van gezonde controles en 17% van broers en zussen van kinderen met ASS ⁷³. Er is veel overlap in gedrag tussen kinderen met FXS met en zonder ASS en kinderen met idiopathische ASS, maar er zijn ook verschillen. Kinderen met FXS hebben minder ernstige symptomen dan kinderen met verstandelijke beperking en idiopathische ASS, waaronder minder problemen met wederkerige sociale interacties en minder complexe stereotypieën ⁷⁴⁻⁷⁶. Bij kinderen met FXS en ASS spelen vermoedelijk sociale angst en vermijding een grotere rol ⁷⁵.

Aanbevelingen: Gezien de hoge prevalentie van ASS bij FXS is bij diagnostisch onderzoek screening met een gespecialiseerd instrument zoals de ADOS, alsmede een gerichte en bij voorkeur gestandaardiseerde ouder- en schoolanamnese aangewezen. Om de relatie tussen FXS, verstandelijke beperking en leer- en/of gedragsproblemen voldoende duidelijk te krijgen en behandeling hierop af te stemmen, wordt aangeraden om in elke levensfase (bijvoorbeeld met 3, 6, 11 en 15 jaar) minimaal eenmaal ook diagnostiek te laten verrichten bij een in FXS gespecialiseerde polikliniek, zoals bij het Erasmus MC Sophia kindziekenhuis. Ondersteuning in de vorm van psycho-educatie, ouderbegeleiding of individuele behandeling van ouders kan worden geboden door een psycholoog, orthopedagoog of maatschappelijk werker.

Prikkelverwerkingsproblemen (sensorische informatieverwerking)

Kinderen met FXS worden vaak beschreven als ‘hyperalert’⁶². Over het algemeen, en in stressvolle situaties in het bijzonder, hebben kinderen met FXS een verhoogd cortisolniveau, een hogere hartslagfrequentie en een verhoogde parasympatische tonus in vergelijking met typisch ontwikkelende kinderen. De problemen in de prikkelverwerking lijken ervoor te zorgen dat kinderen met FXS op een aangepaste wijze, overmatig, gematigd of soms zelfs helemaal niet reageren op hun omgeving^{55,59}.

Aanbevelingen: Als de problemen in de prikkelverwerking niet kunnen worden verminderd met behulp van pedagogische adviezen, kan een therapeut worden ingeschakeld die thuis is in de aanpak van problemen met de zintuiglijke informatieverwerking. Op de site van de NSSI (Nederlandse stichting sensorische informatieverwerking; www.nssi.nl) kan een gekwalificeerde therapeut in de regio gevonden worden.

Attention-deficit/ - hyperactivity disorder (ADHD)

Kinderen met FXS kunnen impulsief zijn, waardoor zij moeite hebben om ongewenst gedrag te onderdrukken. In de literatuur worden getallen genoemd van rond de 84% van de jongens en 67% van de meisjes die gediagnosticeerd of behandeld worden voor aandachtsproblemen en 66% van de jongens en 30% van de meisjes voor hyperactiviteit en impulsiviteit³⁸. In kleinere, klinische cohorten, waarbij gediagnosticeerd wordt volgens DSM criteria, wordt eveneens een hoge prevalentie van ADHD gevonden, rond 55-95% van de jongens⁷⁷⁻⁷⁹ en 35-55% van de meisjes^{80,81}.

Aanbevelingen: Diagnostiek en behandeling van ontwikkelingsstoornissen zoals ADHD kan plaatsvinden in een hierin gespecialiseerd centrum in de jeugd geestelijke gezondheidszorg (JGGZ). Medicamenteus kunnen die middelen worden overwogen die ook eerste keus zijn bij andere kinderen met ADHD (zoals methylfenidaat of dexamfetamine), alhoewel dit niet uitvoerig is onderzocht voor FXS. Er zijn weinig tot

geen placebo gecontroleerde studies die hebben gekeken naar de effectiviteit van medicatie voor ADHD bij kinderen met FXS, behoudens een kleine, inconclusieve trial met stimulantia ⁸². Twee beschrijvende cohort studies rapporteerden 70-75 % response rate bij gebruik van stimulantia en alpha-agonisten (zoals clonidine) bij kinderen met FXS en hyperactiviteit, impulsiviteit en/of milde agressie ⁸³.

Agitatie, automutilatie en agressie en andere gedragsproblemen

Bij kinderen met een verstandelijke beperking komt agressief gedrag vaker voor dan bij kinderen met een gemiddelde intelligentie. De agressie vloeit dan meestal voort uit agitatie en overprikkeling en is reactief van aard ⁸⁴. Bij FXS wordt een incidentie gerapporteerd van 80 tot 90%. Ernstige incidenten worden gerapporteerd bij 33% van de jongens en 20% van de meisjes ⁸⁵⁻⁸⁷. Vergelijken met idiopathische verstandelijke beperking is de agressie bij FXS milder van aard ⁸⁵. Automutilatie komt bij ongeveer 70% voor, vergelijkbaar met idiopathische verstandelijke beperking. Sensorische overgevoeligheid, angst en hyperactiviteit zijn vaak voorspellend voor agressieregulatie problemen ⁸⁶.

Aanbevelingen: In afwachting van specifieke, gerichte behandeling, worden gedragsproblemen psychologisch en medicamenteus behandeld volgens de binnen de kinder- en jeugdpsychiatrie gangbare richtlijnen. Hieronder valt het gebruik van antipsychotica voor het behandelen van agressieregulatieproblemen en ernstige stereotypieën. Psychologische behandeling behelst meestal gedragstherapie en begeleiding van ouders in het omgaan met dit gedrag. Ook belangrijke anderen (broertjes en zusjes, grootouders, andere familie, schoolpersoneel, oppas, etc.) kunnen hierbij worden betrokken. Bij kinderen met een lichte verstandelijke beperking of een hoger niveau wordt ook wel cognitieve gedragstherapie toegepast.

Twee beschrijvende cohort studies bestudeerden medicatiegebruik in patiënten met FXS. Zo'n 80% van de beschreven jongens met FXS gebruikten een stimulant, 25% gebruikte een alpha-agonist en 15% gebruikte een antipsychoticum. 25-50% van de meisjes kregen een stimulant, en 0-10% gebruikten een alpha-agonist of een antipsychoticum. De studies rapporteerden 70-75 % response rate bij gebruik van stimulantia en alpha-agonisten bij kinderen met FXS voor symptomen waaronder agressie ⁸³. Dezelfde studies beschreven een response rate voor antipsychotica rond 80%. Bij kinderen met een idiopathische verstandelijke beperking, wordt het gebruik van antipsychotica uitvoeriger ondersteund door meta-analyses ⁸⁸. Het gebrek aan onderzoek met reguliere psychofarmaca bij FXS zou echter niet mogen leiden tot onthouden van potentieel effectieve medicamenteuze behandeling. Verwijzing naar een team met ervaring op dit gebied is geïndiceerd, bijvoorbeeld het fragiele X expertisecentrum in het Erasmus MC, of een kinder- en jeugdpsychiater met ervaring op dit gebied of een AVG met een multidisciplinair team. Individuele aanpassing en titratie van medicatie is vrijwel altijd noodzakelijk. Psycho-educatie, ondersteuning en begeleiding van ouders en soms ook

voor andere leden van het gezinssysteem zoals broers en zussen kan noodzakelijk zijn als zich gedragsproblemen voordoen bij FXS. De trials die gerichte therapie hebben onderzocht, die specifiek aangrijpt op pathways die zijn verstoord bij FXS, hebben vooralsnog geen effectiviteit getoond bij grotere klinische trials ⁸⁹.

Angststoornissen

Angstsymptomen zijn een belangrijke component van het neuropsychiatrische beeld van FXS. In de grootste studie tot op heden, werd vastgesteld dat 70-85% van de jongens en 55-75% van de meisjes gediagnosticeerd waren met een angststoornis of voor angstklachten behandeld werden ^{36,38,67}. Sociale fobie en specifieke fobieën worden elk gediagnosticeerd in ruim de helft van de kinderen, mogelijk speelt hierbij hypersensitiviteit van deze kinderen voor omgevingsprikkels een rol ⁹⁰.

Aanbevelingen: Er zijn enkele niet-medicamenteuze mogelijkheden voor behandeling, waaronder ouderbegeleiding met psycho-educatie en zo nodig cognitieve gedragstherapie. Bij kinderen met een lichte verstandelijke beperking of normaal IQ kan cognitieve gedragstherapie worden overwogen. Medicamenteuze opties bij kinderen zijn zeer beperkt onderzocht. SSRI worden wel eens gebruikt, wanneer andere opties zijn uitgeput, alhoewel effectiviteit daarvan nauwelijks in deze specifieke doelgroep is onderzocht. Een klinische trial met sertraline bij kinderen met FXS gaf geen verbetering op de primaire uitkomstmaten ten opzichte van placebo⁹¹. De effectiviteit bij kinderen en adolescenten in het algemeen is niet of nauwelijks aangetoond ⁹².

Stemmingsstoornissen

Met name meisjes met FXS hebben een verhoogde kans op depressieve klachten. Ongeveer de helft van volwassen vrouwelijke FXS patiënten krijgt te maken met een depressie of dysthymie ³⁶. Ook is er sprake van overmatige verlegenheid. Over stemmingsstoornissen bij kinderen met FXS is weinig gepubliceerd, het merendeel van de studies focust zich op moeders, premutatie dragers of volwassenen met FXS. In een oude Amerikaanse studie onder 17 meisjes tussen de 4 en 27 jaar (gemiddelde leeftijd 12 jaar) werd bij 8 meisjes een stemmingsstoornis vastgesteld, 5 van hen hadden te maken met een depressie ⁸⁰. Een Amerikaanse studie onder jongens tussen de 4 en 12 jaar oud liet zien dat slechts 3% een klinisch significante score had op de angst- en depressie subschaal van een vragenlijst ⁹³.

Aanbevelingen: Als bij angststoornissen. Het is van belang te realiseren dat aanpassingsstoornissen en posttraumatische stress-stoornis (PTSS) bij deze populatie veel voorkomt, waarin life events een grote rol spelen. Bij PTSS heeft in veel gevallen een behandeling met eye-movement and desensitization and reprocessing (EMDR) de voorkeur.

Tics en stereotypieën

De prevalentie van ticstoornissen bij FXS wordt geschat op 6-16%^{35,94}, vergelijkbaar met de prevalentie van voorbijgaande tic stoornissen in de algemene bevolking⁹⁵. Het is belangrijk tics te differentiëren van stereotype bewegingen, welke vaak voorkomen bij FXS, al dan niet in het kader van een ASS. Tics zijn vaak ongewild. Ze worden gekenmerkt door plotse, snelle en korte, steeds terugkerende, niet-ritmische bewegingen of vocalisaties. Stereotypieën duren vaak langer, zijn meer ritmisch en herhalend.

Aanbevelingen: Behandeling voor tics is niet altijd noodzakelijk, en vaak kan volstaan worden met geruststelling en psycho-educatie rondom het episodisch beloop van tics. Als kinderen veel last ondervinden van tics, kunnen deze met behulp van een op tics gerichte gedragstherapie behandeld worden⁹⁶. Psychofarmaca moeten alleen overwogen worden bij zeer ernstige therapie-resistente tic-klachten. Behandeling verloopt dan volgens algemene richtlijnen voor tic-stoornissen, middels een alpha-2 agonist (clonidine, guanfacine) of een antipsychoticum (risperidon, aripiprazol). De keuze van middel kan beïnvloed worden door aanwezigheid van comorbide ADHD (clonidine, guanfacine) of agressiereguleratieproblemen (risperidon, aripiprazol).

Slaapproblemen

Veel kinderen met FXS kampen met slaapproblemen. Dieronderzoek suggereert dat dit mogelijk een rechtstreeks gevolg is van de verminderde FMRP expressie^{97,98}. 30 tot 50% van ouders met kinderen met FXS rapporteren slaapproblemen bij hun kind. Dit is hoger dan de 10 tot 20% slaapproblemen bij kinderen en adolescenten uit de algemene bevolking^{99,100}. Het gaat hierbij vaak om samengestelde problemen, met name in- en doorslaapproblemen^{101,102}, waarbij autistische kenmerken en hyperactiviteit ook een rol spelen. Slaapgebrek heeft op het kind, maar ook op de ouders en andere kinderen in het gezin een grote, negatieve lichamelijke en mentale weerslag^{101,103,104}. Een aantal studies rapporteert een verhoogd voorkomen van OSAS bij kinderen met FXS, met gerapporteerde prevalenties van 7 tot zelfs 34%^{35,102}.

Aanbevelingen: Een gedragsmatige aanpak van slaapproblematiek kan effectief zijn bij kinderen in het algemeen, ook wanneer er sprake is van verstandelijke beperking^{105,106}. Voor zover bekend heeft nog maar één studie gekeken naar de effectiviteit van een gedragsmatige aanpak voor slaapproblemen bij kinderen met FXS. Bij alle 7 kinderen met FXS was er sprake van een verbetering¹⁰⁷. Een kleine studie naar het melatonine profiel (n=13 jongens met FXS) toonde hogere melatonine spiegels zowel 's nachts als overdag vergeleken met gezonde controles¹⁰⁸. Een vier weken durende, dubbel blind, placebo gecontroleerde studie bij 18 patiënten met FXS en/of autisme toonde een positief resultaat van melatonine op sleep-onset latency en de slaapduur¹⁰⁹.

Puberteit, seksualiteit en anticonceptie

Evenals bij veel kinderen met verstandelijke beperkingen, stelt de puberteit de meeste ouders van kinderen met FXS voor lastige vragen en problemen, zoals hoe te zorgen voor een individueel aangepaste seksuele voorlichting en hoe seksueel gedrag (bijvoorbeeld masturbatie) in sociaal acceptabele banen te geleiden. Kinderen met FXS hebben in principe een normale puberteit en seksuele ontwikkeling. Zij missen echter in meer of mindere mate de cognitieve en emotionele rijping en structuur die zorgt voor een probleemloze integratie van seksualiteit in het alledaagse gedrag. Ook kunnen zij door andere prikkelverwerking bijzondere seksuele interesses hebben. Ook andere gedragingen die in het publieke domein doorgaans niet acceptabel zijn, kunnen optreden. Daarentegen lijken sommige kinderen juist asexueel te zijn. Regelmatig hebben ouders vragen over geschikte vormen van anticonceptie. De problematiek is niet anders dan bij andere adolescenten met een verstandelijke beperking. Soms is er ook de vraag naar sterilisatie op relatief jonge leeftijd, bijvoorbeeld als de gelegenheid zich voordoet om zo'n ingreep te combineren met een andere noodzakelijke ingreep onder narcose.

Aanbevelingen: Het aanleren van vaardigheden om onderscheid te maken wat wel en wat niet in het publieke domein acceptabel is en wat specifiek in het seksuele domein als ongewenst of grensoverschrijdend wordt gezien, vereist extra aandacht en meestal deskundige begeleiding vanaf lagere school leeftijd. Stichting MEE en AVG's kunnen bemiddelen in het vinden van deskundige begeleiding (meer lezen: <http://www.chp.edu/our-services/brain/neurology/fragile-x/parents/sexuality>). Ten aanzien van sterilisatie verdient het aanbeveling om uit te leggen dat sterilisatie niet helpt tegen SOA's en evenmin tegen grensoverschrijdend of hyperseksueel gedrag. De mogelijkheden voor sterilisatie bij wilsonbekwamen zijn zeer beperkt en aan voorwaarden gebonden. Nadere informatie over anticonceptie en sterilisatie kan gevonden worden in de *Handreiking kinderwens en anticonceptie bij mensen met een verstandelijke beperking* van de NVAVG (<http://nvavg.nl/wp-content/uploads/2017/02/2016-Handreiking-kinderwens-en-anticonceptie-def.pdf>).

4.3. Kwaliteit van leven

Over het algemeen zijn mensen met FXS als ze volwassen worden niet in staat om (geheel) zelfstandig te wonen. Een studie toont dat ongeveer 80% van de jongeren, met name mannen, in het ouderlijk huis woont en van de overgebleven groep woont het merendeel in een zorginstelling⁵¹. Wel is de sociale redzaamheid relatief sterk bij kinderen en volwassenen met FXS ten opzichte van andere vormen van verstandelijke beperking. Ze zijn, zeker op volwassen leeftijd, heel zorgzaam in het omgaan met anderen¹¹⁰. Voor de omgeving en met name voor de directe familie (ouders, broers en zussen) is de impact van het krijgen van een kind met een verstandelijke beperking en ernstige

gedragsproblemen groot. Toekomstverwachtingen moeten ernstig worden bijgesteld. De scores van adolescenten met FXS die hun sociale leven moeten beoordelen, zijn vergelijkbaar met die van typisch ontwikkelende adolescenten. Dit terwijl hun ouders wel een lagere beoordeling aan het sociale leven van hun kind geven ^{110,111}. Over het algemeen is de kwaliteit van leven zowel bij kinderen als hun verzorgers lager dan gemiddeld ¹¹¹.

4.4. Ouders en omgeving van kinderen met FXS

Acceptatie is voor kinderen zelf in de meeste gevallen geen probleem. In de adolescentie en de volwassenheid kunnen mensen met een lichte verstandelijke beperking of een normale cognitie (meestal de vrouwen met FXS die lichamelijke en/of psychische problemen bij zichzelf ervaren) wel last hebben van de door hen waargenomen verschillen met hun leeftijdgenoten. Voor de ouders en omgeving geldt dat zij vaak door een zwaar proces moeten. Dit proces begint eigenlijk al vóórdat de diagnose is gesteld. Het stellen van de diagnose FXS kan soms lang duren, wat als frustrerend wordt ervaren. Daarnaast is het mogelijk dat ouders opnieuw zwanger zijn of een kind hebben voordat zij een diagnose hebben ontvangen, waardoor zij mogelijk een tweede kind met FXS krijgen ¹⁵. Wanneer de diagnose is gesteld, moeten de ouders zich realiseren dat zij, gezien de levensverwachting en het ontwikkelingspatroon van de kinderen, deze zorg langdurig op zich zullen moeten nemen. Ouders van kinderen met een ontwikkelingsachterstand ervaren vaak meer stress, hebben meer fysieke symptomen en functioneren slechter op psychologisch gebied ¹¹². Naast de stress thuis, ervaren ouders vaak ook stress op andere plaatsen, bijvoorbeeld tijdens hun werk. Ouders van kinderen met FXS werken vaak minder om werk met zorgtaken te combineren. Als zij tijdens het werk ook stress ervaren, leidt dat bij deze ouders tot meer klachten dan bij ouders van kinderen zonder een ontwikkelingsachterstand ¹¹³. De klachten die ouders ervaren, worden soms vergeleken met klachten die voorkomen tijdens een burn-out of een PTSS ¹¹³.

Naast het hebben van een kind met een aandoening, is het voor ouders vaak belastend dat zij zelf een erfelijke aanleg bij zich (kunnen) dragen. Deze aanleg kan voor hun eigen gezondheid, hun kinderwens, maar ook voor andere familieleden gevolgen hebben. Hier wordt onder het kopje “*Erfelijkheidsadvies*” meer aandacht aan geschonken. Omdat dragerschap van een premutatie of een volle mutatie op zichzelf al kan leiden tot stemmingsstoornissen, angst en andere psychiatrische problemen, zijn de moeders van een kind met FXS extra kwetsbaar.

Aanbevelingen: Ouders van kinderen met een verstandelijke beperking hebben, zeker als de complexiteit van de problematiek toeneemt, vaak behoefte aan ouderbegeleiding. Als de verhouding tussen draagkracht en draaglast is verstoord, moeten soms intensievere vormen van gezinsondersteuning worden ingezet. Het is verstandig om ouders tijdig

te verwijzen naar een ouderbegeleider zoals een psycholoog, orthopedagoog of maatschappelijk werker, bij voorkeur verbonden aan een instelling voor mensen met een verstandelijk beperking. In sommige gevallen zal opvang of opname in een tehuis worden overwogen, waarvoor begeleiding via stichting MEE kan plaatsvinden. Daarnaast is het belangrijk ouders voldoende voorlichting te geven over de erfelijke aspecten van FXS zodat zij een weloverwogen beslissing kunnen maken wanneer zij gezinsuitbreiding overwegen. Ouders kunnen lotgenotencontact en steun zoeken bij patiëntenverenigingen, waaronder de Fragiele X Vereniging Nederland, oudervereniging Balans en de Nederlandse vereniging voor autisme (NVA). Momenteel worden de aanvraag en toekenning van een persoonsgebonden budget (PGB) georganiseerd vanuit de wijkteams in de gemeenten. MEE en een organisatie als Per Saldo kunnen ouders ondersteunen in de aanvraag van een PGB. Uitbreiding van opvang binnen en buiten het eigen netwerk, maar ook een logeershuis zijn vaak mogelijkheden om het gezin deels te ontlasten en het kind voor te bereiden op de transitie. Als het gezin chronisch (over) belast is, kan op termijn ook een indicatie voor de wet langdurige zorg (WLZ) bij het Centrale Indicatieorgaan voor de Zorg (CIZ, zie hun website) worden aangevraagd. WLZ-gelden kunnen worden gebruikt voor intensieve hulp thuis of voor hulp in een instelling.

Een van de mogelijke valkuilen voor gezinnen met een kind met FXS is om te vervallen in isolatie. Juist omdat mensen met FXS moeite hebben met sociale interactie is het belangrijk dat zij regelmatig in contact komen met leeftijdsgenoten. Dit kan bevorderlijk zijn voor de ontwikkeling van hun communicatieve en sociale vaardigheden ¹¹⁴. Belangrijk hierbij is een goede begeleiding om de mogelijke stress die deze sociale interacties kunnen veroorzaken te reduceren of voorkomen.

4.5. Transitie van kind naar volwassenenzorg

Opgroeien met FXS syndroom brengt uitdagingen met zich mee. De transitieperiode van 18-25 jaar houdt vaak verandering in van zorgnetwerk, dagbesteding, en woonlocatie. Deze veranderingen kunnen veel stress opleveren en dienen zeer geleidelijk plaats te vinden, om overvraging, angsten en gedragsproblemen te voorkomen. Tijdens deze levensfase verschuift de focus van het leren naar het behouden en zo mogelijk uitbreiden van vaardigheden. De ouderlijke stress blijft verhoogd tijdens de transitie van kinder- naar volwassen leeftijd als de zorg wordt overgedragen aan anderen. Het ouder worden levert extra vragen op voor ouders, zoals ‘wie zorgt er voor mijn kind als ik er niet meer ben.’

Aanbevelingen: Tijdens de tienerjaren kan een ambulante begeleider worden betrokken als vertrouwenspersoon voor de patiënt, om de ouders te ondersteunen, en om de transitie naar de volwassen levensfase te begeleiden en op die manier continuïteit in het zorgnetwerk te geven. Een breed (neuro)psychologisch onderzoek voor of in de transitieleeftijd kan een aanknopingspunten bieden voor toekomstige zorgverleners.

Het is belangrijk om tijdig na te denken over passende woon- en werkvormen in de toekomst en niet teveel veranderingen gelijktijdig te laten plaatsvinden. Tevens dienen de zorgverleners goed op de hoogte te zijn van het gedrag en de passende begeleidingsstijl. Signaleren van zorgen en zorgbehoefte van ouders en overige gezinsleden blijft belangrijk. Een AVG is in de meeste gevallen de aangewezen professional hiervoor. Bij een normale intellectuele ontwikkeling, zoals zich bij vrouwen met FXS kan voordoen, is begeleiding in de GGZ vaak op zijn plaats.

5. Erfelijkheidsadvies

Voordat genetische diagnostiek wordt ingezet, is counseling van de patiënt of ouders/verzorgers gewenst, om een geïnformeerde keuze te kunnen maken. Dit geldt vooral wanneer familieleden worden getest die geen verschijnselen hebben. Zowel voor de test als bij de diagnose is het van belang dat de adviesvrager zich realiseert dat de diagnose voor de gehele familie consequenties kan hebben. Verwijzing naar een klinisch geneticus is gewenst, voorafgaand aan de test, maar zeker nadat de diagnose bij het kind is gesteld of wanneer informatie of diagnostiek gewenst is bij een familielid zonder verschijnselen. Genetische counseling is zinvol om de patiënt of de ouders/verzorgers te informeren over de aandoening, de testuitslag, de prognose, de behandeling en begeleiding en emotionele aspecten. Ook kunnen de gevolgen voor reproductie besproken worden. Daarnaast kunnen de consequenties voor familieleden (inclusief gevolgen van een premutatie) worden besproken. Familieleden die zelf klachten hebben, of die willen weten of zij drager zijn, kunnen terecht bij de klinisch geneticus voor uitleg en counseling ten aanzien van de aandoening, de risico's en de mogelijkheden voor moleculair onderzoek en de risico's en mogelijkheden rondom kinderwens. Het is aan te bevelen om schriftelijke informatie aan patiënten mee te geven^{6,7,30,50,52}. Ook kan er verdere diagnostiek plaatsvinden, indien nodig, bijvoorbeeld een haarworteltest wanneer mozaïcisme wordt vastgesteld. Bij zowel mannen als vrouwen kan sprake zijn van mozaïcisme, dat invloed heeft op de mate van verschijnselen^{8,22}.

Herhalingsrisico en reproductieve opties

De kans op een kind met FXS is afhankelijk van het aantal CGG repeats (anticipatie), het geslacht van de ouder met de aanleg en het geslacht van het kind.

Vrouw met een volle mutatie (>200 repeats): de kans dat de aangedane allel wordt doorgegeven is 50%. In dat geval zal een zoon FXS hebben en een dochter ongeveer 50% kans hebben op FXS, met een zeer grote klinische variatie.

Vrouw met premutatie (55-200 repeats): De kans dat de aangedane allel wordt doorgegeven is 50%. Indien deze wordt overgeërfd, zou er expansie kunnen plaatsvinden

(zie Tabel 2). Hoe meer repeats, hoe groter de kans op expansie tot boven de 200 repeats (zie Tabel 2). Boven de 100 repeats is de kans op expansie tot boven de 200 rond de 100%. Waarschijnlijk is het risico op expansie hoger bij een familiegeschiedenis van FXS. Bij een expansie tot de volle mutatie, zal een zoon FXS hebben en een dochter ongeveer 50% kans op FXS. Ook kan er een premutatie (<200 repeats) worden overgeërfd, hetgeen de kans op andere (met name late-onset) problematiek geeft.

Man met volle mutatie (>200 repeats): omdat hierbij sprake is van FXS, zal hij in de meeste gevallen geen nageslacht verwekken, vanwege sociaal-emotionele en ontwikkelingsaspecten van de aandoening en mogelijk verminderde fertiliteit ^{7,52,53}. Indien een man met FXS toch een kind krijgt, zal hij aan dochters altijd een premutatie doorgeven. Zonen krijgen de aanleg niet (zij krijgen de Y chromosoom van vader).

Man met premutatie (55-200 repeats): al zijn dochters zullen de premutatie onveranderd erven. Zijn zonen erven de aanleg niet (zij krijgen de Y chromosoom van hun vader).

Grijze zone/intermediaire allel (45-54 repeats): hierbij is het risico onduidelijk. Er is instabiliteit gerapporteerd, maar geen expansie tot boven de 200 CGG repeats ⁶⁻⁸. Het kleinste repeetaantal dat geëxpandeerd is tot boven de 200 repeats was 56 CGG repeats ¹¹⁵ en 59 CGG repeats ¹¹⁶. Het is onduidelijk of de grijze zone allelen tot een klinisch fenotype leiden ^{6,7,50}. Intermediaire allelen worden vaak bij toeval ontdekt.

Er zijn verschillende *reproductieve opties*, waaronder het accepteren van het risico, PND, PGD, eicel- (of zaadcel-) donatie, adoptie en afzien van (volgende) kinderen. Daarnaast is het van belang te beseffen dat 20% van de vrouwen met een premutatie FXPOF/FXPOI krijgen, wat fertiliteitsproblemen met zich mee kan brengen.

Tabel 2: risico op expansie van het aantal maternale CGG repeats tot boven de 200 (Uit Nolin et al, 2003 ¹¹⁶)

Maternale repeats	% nageslacht met expansie naar volle mutatie (indien aangedane allel overgeërfd)
55 – 59	4
60 – 69	5
70 – 79	31
80 – 89	58
90 – 99	80
100 – 200	ongeveer 100

Psychosociale begeleiding

Het krijgen van deze diagnose kan nogal wat emoties teweegbrengen in een familie, waaronder schuldgevoelens, schaamte, boosheid en het verliezen van een toekomstbeeld. Daarnaast kan het hebben van FXS of de premutatie ook psychiatrische

problematiek met zich meebrengen, waardoor deze groep extra kwetsbaar is. Een goede begeleiding door de arts en indien nodig, een GZ-psycholoog (eventueel verbonden aan een afdeling klinische genetica of een expertisecentrum), is aan te bevelen.

6. Toekomstperspectief in het licht van gerichte therapie

Op dit moment is behandeling van FXS symptomatisch met begeleiding en medicatie. In veel gevallen is deze behandeling, zoals beschreven in deze leidraad, onvoldoende om psychiatrische en gedragsproblemen voldoende te verminderen en heeft deze weinig effect op de cognitieve ontwikkeling. De afgelopen jaren stonden in het teken van het identificeren van mogelijke targets voor een gerichte therapie voor FXS, met als doel de aandoening te genezen. De identificatie van het gen maakte het mogelijk om diermodellen te maken, waaronder de *Fmr1* KO muis. Deze diermodellen hebben onderzoekers in staat gesteld om de biologische processen die verstoord worden bij FXS te bestuderen. De kennis hierover heeft het mogelijk gemaakt om in te grijpen met medicijnen, die specifiek aangrijpen in deze verstoorde processen: targeted treatment. Bij diermodellen heeft dit geleid tot succesvolle behandeling van verschillende aspecten van de aandoening. Dit werd gevolgd door klinische trials bij patiënten met verschillende soorten medicijnen. Echter, tot nu toe is er geen medicijn dat effectief is gebleken in het behandelen van FXS, in vergelijking met placebo. Mogelijke oorzaken kunnen zijn: een gebrek aan betrouwbare uitkomstmaten om effect te meten, het design van klinische trials dat niet gebaseerd is op ziekte-modificerende behandelingen en/of beperkingen van diermodellen^{89,117,118}. De komende jaren zullen mogelijk meer duidelijkheid geven over de richting van verder onderzoek en behandeling, met aandacht voor bovengenoemde punten. Daarnaast is er momenteel onvoldoende bewijs voor gerichte medicamenteuze therapie met onder andere minocycline, diverse mGluR5 antagonisten, bumetanide, lithium en (Ar)baclofen.

Ten slotte

Inzichten op wetenschappelijk en klinisch gebied zijn aan veranderingen onderhevig. Mocht u als lezer en gebruiker van deze Leidraad wijzigingen of aanvullingen willen voorstellen, dan kunt u contact opnemen met het FXS expertisecentrum van ENCORE.

7. Afkortingen

ADD	attention deficit disorder
ADHD	attention deficit hyperactivity disorder
ASS	autism spectrum stoornis
AVG	arts voor verstandelijk gehandicapten
FXS	fragiele X syndroom
FXTAS	fragile X associated tremor and ataxia syndrome
FXPOI	fragile X associated premature ovarial insufficiency
FXPOF	fragiele X geassocieerde prematuur ovariumfalen
GGZ	geestelijke gezondheidszorg
IVF	in-vitro fertilisatie
PGD	pre-implantatie genetische diagnostiek
PND	prenatale diagnostiek
PTSS	post-traumatische stress stoornis
OSAS	obstructief slaap-apneu syndroom

8. Contact

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www.nvavg.nl

Fragiele X Vereniging Nederland

Postbus 260, 4900 AG Oosterhout

Tel: 06-14332033

Email: info@fragilex.nl

Website: www.fragilex.nl

Fragiele X verenigingen buitenland

Verenigde staten: <https://fragilex.org/>

België: <http://www.fragiel.be/>

Duitsland: <http://www.frax.de/>

9. Bronnen voor informatie

<http://www.erasmusmc.nl/encore/Poliklinieken/fraXsyndroom/>

<http://www.fragilex.nl/>

<https://fragilex.org/>

<http://www.fraxa.org/>

<http://erfelijkheid.nl/ziektes/fragiele-x-syndroom>

<http://erfocentrum.nl/pdf/paramedici/Huisartsenbrochure%20Fragiele%20X%20Syndroom.pdf>

www.pgdnederland.nl

www.nvavg.nl

www.vkgn.org

www.mee.nl

www.ciz.nl

www.persaldo.nl

www.balansdigitaal.nl

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Bijlagen bij Leidraad voor diagnostiek en behandeling van kinderen met fragiele X syndroom

Bijlage 1 Klinische criteria voor het fragiele X syndroom

Tabel Bijlage 1A. Uit De Vries *et al* ²⁷. Te gebruiken bij jongens en mannen met een verstandelijke beperking. Een score van ≥ 5 wijst op een verhoogd risico op fragiele X syndroom.

Gebied	Kenmerk	Aantal punten
<i>verstandelijke beperking in de familie</i>	aangedane broer/zus of oom/tante/neef/nicht aan moederszijde	2
	ander aangedaan familielid (passend bij X-gebonden erfelijkheid)	1
<i>gelaat</i>	brede kaak en hoog/breed voorhoofd	2
	een of enkele van deze kenmerken	1
<i>oren</i>	groot en afstaand	2
	alleen groot	1
<i>gewrichten</i>	overstreekbare pinken en duimen	2
	alleen overstreekbare pinken	1
<i>huid</i>	zacht/glad in handpalm en ruim op handrug	2
	alleen zacht/glad in handpalm	1
<i>testikels</i>	beide testikels > 30 ml	2
	één testikel > 30 ml	1
<i>gedrag</i>	verlegen/verminderd oogcontact gevolgd door vriendelijkheid, breedsprakigheid en echolalie	2
	enkele van deze kenmerken	1
Totaal aantal punten		

Tabel Bijlage 1B. Vertaald uit Maes *et al*³¹. Deze checklist is te gebruiken bij jongens en mannen van alle leeftijden met een verstandelijke beperking. Elke item levert 1 punt op, indien aanwezig. Bij een score van ≥ 17 wordt verwijzing door de auteurs aanbevolen. Sommige items zijn niet zichtbaar bij jonge kinderen. De familieanamnese is hierbij niet in beschouwing genomen.

Uiterlijke kenmerken	☐	Smal lang gezicht	☐	Macro-orchidisme
	☐	Hoog voorhoofd	☐	Overstreekbare vingers
	☐	Prominente onderkaak	☐	Overstreekbare gewrichten (anders dan vingers)
	☐	Grote afstaande oren		
Gedrags-kenmerken	☐	Hyperactiviteit	☐	Stereotype handbewegingen
	☐	Sensorische hypersensitiviteit	☐	Handenflappen
	☐	Impulsiviteit	☐	Vermijding oogcontact
	☐	Chaotisch gedrag	☐	Gezicht afwenden
	☐	Verlegenheid	☐	Tactiele afweer
	☐	Over-behulpzaam	☐	Snelle spraak
	☐	Conflict benadering en vermijding	☐	Overmatige spraak
	☐	Angst	☐	Perseveratie
	☐	Vrolijkheid	☐	Echolalie
	☐	Overgevoeligheid voor verandering	☐	Imitatie van eigen taal
	☐	Handbijten		

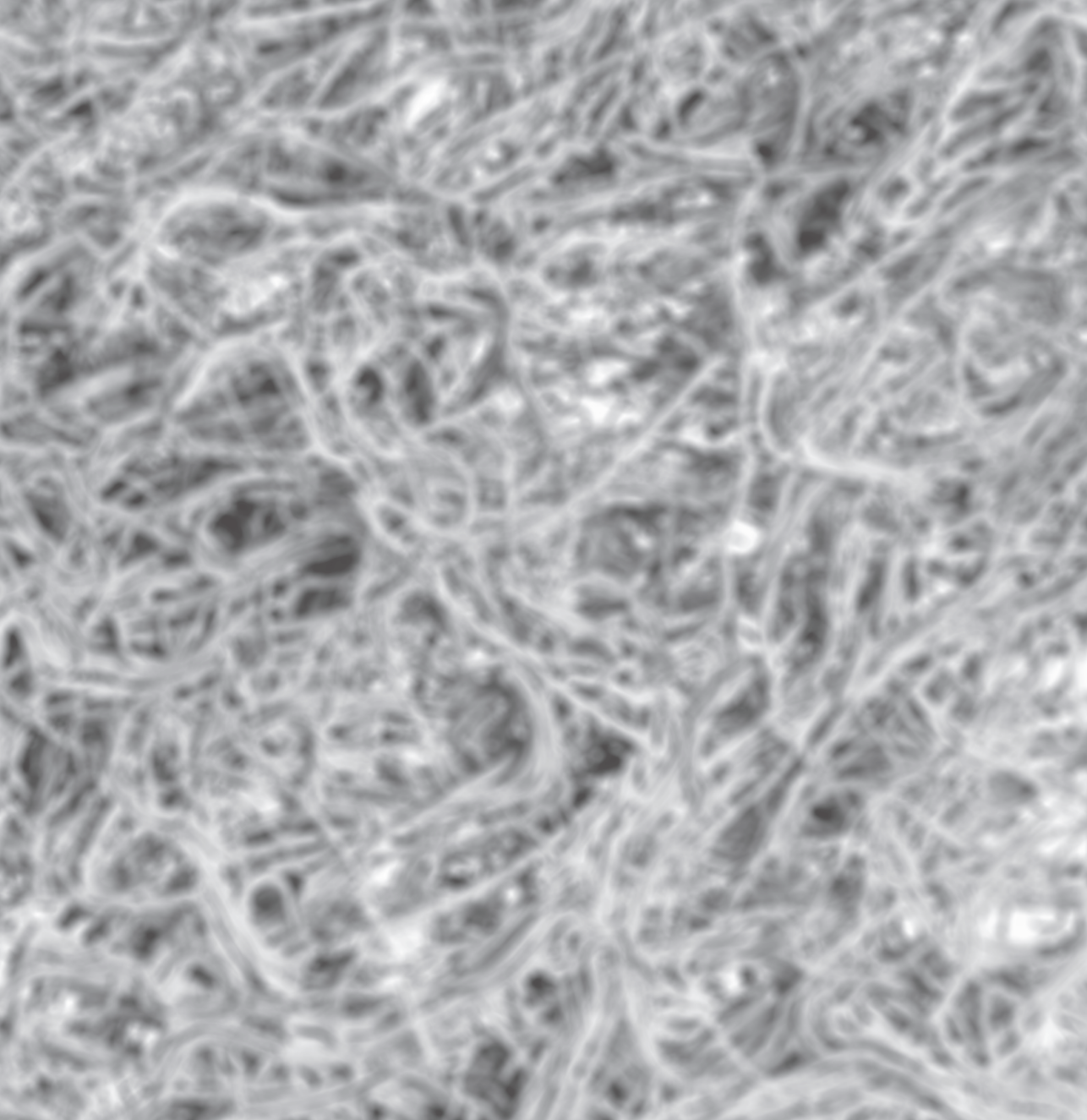
Totaal aantal punten:

Bijlage 2 Beleid en follow-up

Tabel Bijlage 2. Beleid bij diagnose en follow-up van kinderen met het fragiele X syndroom. Een kind met fragiele X syndroom dient te worden behandeld voor medische problemen, zoals ieder ander kind. Echter, specifieke kenmerken verdienen extra aandacht in de zorg voor deze kinderen. In dit tabel worden deze kenmerken genoemd en de frequentie van follow-up samengevat.

Gezondheidsproblemen		0-4 jaar en bij diagnose	5-12 jaar	≥ 13 jaar
Ontwikkeling	Motorische ontwikkeling	✓ elk half jaar	✓ jaarlijks	✓
	Cognitieve ontwikkeling	Testen bij 3 jaar	Testen bij 6 en 11 jaar	tweejaarlijks Testen bij 15 jaar
	Taal en spraak	Monitoren, vanaf 2 ^e jaar	Monitoren	Monitoren
Gedrag en psychiatrie	Passend/speciaal onderwijs	Indien van toepassing	✓	✓
	ADHD, ASS, Angststoornissen, prikkelverwerking, gedragsproblemen	Begeleiding, medicatie diagnostiek bij diagnose, bij 3 jaar en op indicatie	Begeleiding medicatie diagnostiek bij 6 en 11 jaar en op indicatie	Begeleiding medicatie diagnostiek rond 15 jaar en op indicatie
	Psychosociale begeleiding	✓	✓	✓
Groei en	Groei	✓ elk half jaar	✓ jaarlijks	✓ tweejaarlijks
Voeding	Voedingsproblemen	Op indicatie	-	-
Neurologie	Reflux	Op indicatie	-	-
	Epilepsie, cave absences en atypische aanvallen	Op indicatie	Op indicatie	Vrijwel niet
	Hypotonie	Lichamelijk onderzoek	-	-

Ogen	Strabisme, refractie- afwijkingen, myopie, nystagmus, ptosis	Op indicatie	Op indicatie	Op indicatie
KNO	Recidiverende otitiden Gehoör	✓ Frequent ✓ Jaarlijks gehoortest	✓ Frequent ✓ Jaarlijks gehoortest	Vrijwel niet
Cardio- vasculair	Slaap apneu Mitralisklep- prolaps Hypertensie	Op indicatie ✓ jaarlijks auscultatie	Op indicatie ✓ jaarlijks auscultatie	Op indicatie ✓ jaarlijks auscultatie en bloeddruk- meting
Gebit	Crowding en malocclusie	Tandheel- kundige controle, let op tactiele afweer	Tandheel- kundige controle, let op tactiele afweer	Tand- heelkundige controle, let op tactiele afweer
Bewegings- apparaat	Hyperlaxiteit en (heup)luxaties Heupdysplasie Klompvoeten Pectus excavatum Scoliose	Jaarlijks lichamelijk onderzoek Op indicatie fysiotherapie of orthopedie		
Endocrien	Menstruatie Anticonceptie/ sexualiteit	- -	Op indicatie Op indicatie bespreken	Op indicatie Bespreken
Urogenitaal	Vesico-ureterale reflux Enurese/ toilettraining Seksualiteit	Op indicatie - -	Op indicatie Op indicatie Begeleiding en anticonceptie	Op indicatie Op indicatie Begeleiding en anticonceptie
Overig Genetische counseling	Liesbreuk Kinderwens/ reproductieve opties en risico's voor familieleden	Op indicatie Bij diagnose	Op indicatie Op indicatie	- Op indicatie



CHAPTER 8

General Discussion

Fragile X syndrome (FXS) has a long history in the clinical genetics field, being one of the first diseases that has been cytogenetically mapped ¹. The identification of the causative gene, the fragile X mental retardation 1 (*FMR1*) gene ²⁻⁵, made a correct genetic diagnosis possible. Subsequently, the creation of animal models enabled the study of disease pathophysiology. Since then, many cellular functions of FMRP and the involvement of important molecular pathways have been identified, which could serve as targets for potential disease modifying therapy. In the advances that have been made, FXS has served as an example for other monogenetic diseases. The challenges we encountered in our search for a cure for FXS, do not only apply to the FXS research community in general, but also to many other neurodevelopmental disorders that are facing the development of targeted treatment. In this chapter, I will outline the most important findings in my quest for a targeted treatment for FXS, and I will place those in the context of where the field of FXS and targeted treatment development stands now. My study was aimed at targeting aberrant pathways involved in the pathogenesis of FXS, both in pre-clinical and clinical studies. In addition, my research focused on reliable and robust pre-clinical outcome measures that could be applied to assess efficacy in drug discovery.

1. The need for disease modifying therapy

The main clinical features of FXS include intellectual disabilities, executive functioning deficits, attentional problems, hyperactivity, autism spectrum disorders, anxiety, aggression, and a wide range of other psychiatric disorders. In addition, many medical problems accompany the disorder, including epilepsy, frequent otitis media, hyperlaxity, and cardiac abnormalities ⁶⁻⁸. More than the developmental delay, especially the social and behavioral deficits can lead to substantial disabilities and hamper the patients and their caregivers' normal participation in society. These problems might be aggravated, when several siblings are affected, which is often the case due to the hereditary, X-linked nature of the disorder and the frequent delay in accurate diagnosis ⁹. There is currently no targeted therapy for patients with FXS. Psychiatric and behavior problems are treated symptomatically, with supportive psychological care, educational help, and psychoactive pharmaceuticals, including stimulants, antipsychotics and antidepressants. Most of the used symptomatic drugs have not been evaluated in well-designed randomized clinical trials for FXS. The therapeutic effect of these treatments is very variable, often moderate on behavior problems with some degree of efficacy reported in 50-70% of males ¹⁰⁻¹². Some meta-analyses even advocate that there is poor evidence for pharmaceutical intervention at all ¹¹. Even less has been reported on non-pharmacological interventions, like behavioral therapies and cognitive supportive interventions for FXS, which are applied frequently in clinic. Hence, the efficacy of current treatments is difficult to estimate, but these treatments obviously do not target the molecular etiology of FXS and do not ameliorate behavioral and especially cognitive impairments. This lack of effective treatment hits a large number of patients, since FXS

is the most common monogenetic cause of intellectual disability, accounting for about 1-3% of all patients that present with developmental delay and autism in the clinic¹³. In fact, all patients with intellectual disability undergo standard *FMR1* DNA analysis in the Netherlands, when being evaluated for a genetic cause. Its frequency and chronic, life-long symptomatology, make FXS a very costly disease for society. These factors leave patients, caregivers, and society in great need of a targeted disease modifying therapy, to assist or possibly even replace current treatment.

2. From molecular pathways to therapeutic targets

In the past decades, animal models have revealed much knowledge about the function of FMRP and have identified many pathways involved in FXS. Most of those pathways are involved in synaptic plasticity, the process of changes in neuronal connectivity, which is the basis of learning, memory formation and brain development¹⁴⁻¹⁶. Short and long term changes in the strength of synapses require local protein synthesis in dendritic spines, which is partly regulated by FMRP. Upon metabotropic glutamate type 5 receptor (mGluR5) stimulation, lack of FMRP leads to disturbed regulation of translation of specific mRNAs at the synapse, leading to aberrant synaptic plasticity, also known as the “mGluR5 theory”¹⁷. Genetic reduction or pharmacological inhibition of mGluR5 in *Fmr1* KO mice, could correct several FXS characteristics, including morphological, electrophysiological, molecular, and behavioral (endo-) phenotypes¹⁸⁻²⁵. In addition, an important role has been proposed for the gamma-Aminobutyric acid (GABA)ergic system, the brains main inhibitory pathway. In *Fmr1* KO mice, studies have shown a decrease in the neurotransmitter GABA abnormalities in its metabolism and reduced expression of several GABA-receptor subunits. Moreover, the GABAergic switch from excitatory to inhibitory function, that normally occurs during birth in many animal species, seems to be delayed or absent in *Fmr1* KO mice, leading to an immature hyperexcitable state²⁶⁻³⁴. An aberrant GABAergic switch is believed to be involved in FXS, autism, and other neurodevelopmental disorders³⁵, although data about it in humans are very scarce. GABAergic agonists have shown improvement of several phenotypes in *Fmr1* KO mice, including behavior abnormalities, audiogenic seizures, aberrant spine morphology and even some phenotypes that are mostly considered mGluR5-dependent, like elevated protein synthesis and reduced AMPA receptor internalization^{33,36}.

Two chapters (chapter two and three) in my thesis focus on targeting this GABAergic system. In chapter two we report chronic treatment of *Fmr1* KO mice with bumetanide, an NKCC1 Cl⁻ channel antagonist, which is believed to force the above mentioned GABAergic switch from immature to mature function^{37,38}. We have demonstrated that bumetanide treatment results in a partial reduction of the FXS phenotype using a social behavior paradigm, the automated tube test, indicating its potential as a treatment for FXS associated social behavior symptoms. Bumetanide has been shown to ameliorate

autistic behavior in autism mouse models and has shown benefits in small clinical trials conducted with patients with autism spectrum disorders³⁸⁻⁴⁰. Currently, it is being further evaluated in larger clinical trials. However, in literature it has been reported that bumetanide poorly crosses the blood-brain-barrier and that its concentration in the brain is too low to inhibit NKCC1^{41,42}. More data about the bioavailability in the central nervous system, could enable us to improve delivery of bumetanide or even create a bumetanide prodrug that is brain-specific⁴³. Strikingly, our results clearly show an effect of oral bumetanide on social behavior in *Fmr1* KO mice. Also Tyzio *et al*³⁸ show a clear effect of oral bumetanide on electrophysiological measures and behavior in *Fmr1* KO mice and valproate exposed rats. Other publications have shown similar effects of bumetanide administration *in vivo* as well, improving features like epilepsy (reviewed in Ben-Ari *et al*⁴⁴). Although these animal studies have not measured concentrations of bumetanide in brain tissue, the results indicate that bumetanide does reach the neurons and influences their function. Our results do add up to the evidence that the developmental switch of GABAergic function, dependent on chloride levels, is important for social behavior. Since this switch in mice is believed to be around birth and has been demonstrated to be delayed in *Fmr1* KO mice⁴⁵, previous studies have focused on prenatal treatment. Treatment around birth was sufficient to correct phenotypes on the long-term, which would implicate that there is a critical window around this period and that short-term treatment around that time might be sufficient to prevent the aberrant circuit formation during this period. However, we demonstrate that later intervention, from around 4 weeks of age, does improve social behavior as well, supporting treatment efficacy later in life. We believe that whenever treatment is initiated later in life, probably a life-long treatment is warranted. Notably, in our study, we did not measure a full correction of this specific behavioral phenotype. This could either mean that targeting this GABAergic switch is insufficient, or that treatment was initiated too late, leading to irreversible aberrant inhibitory circuit formation. A previous study using the automated tube test in *Fmr1* KO mice, reported similar results after genetic reduction of mGluR5. Since this “treatment intervention” is present from conception, it precedes any critical window²⁰. Our results could be explained by lack of efficacy when targeting only one pathway, suggesting that targeting multiple pathways is necessary. Another explanation for the partial correction that we observed might be the robustness of the automated tube test. It has never been demonstrated that the FXS phenotype could be fully corrected in this test. In fact, we do not know whether that is possible at all, since the phenotype is very robust, leaving little space for nuance. Possibly even a small difference could be enlarged, due to the black and white nature of the test and the winner-loser effect. If targeting NKCC1 would prove to be effective in patients, it would probably implicate a chronic treatment, preferentially in combination with other behavioral and pharmacological interventions. Additionally, a drug specific for neurons might reduce possible side effects and enhance bioavailability.

In chapter three we report the chronic treatment of *Fmr1* KO mice with the GABA_B receptor agonist baclofen. Previous publications regarding acute treatment of *Fmr1* KO mice with baclofen, or its R-enantiomer arbaclofen, have shown rescue of several FXS

phenotypes, including molecular, morphological, behavioral and electrophysiological measures^{29,46-50}. We investigated the effect of chronic baclofen treatment on social behavior in the *Fmr1* KO mice. Two social behavior paradigms were used: the automated tube test and the three chamber sociability test. Our results show that chronic treatment of *Fmr1* KO mice with baclofen worsens the FXS phenotype in the automated tube test and does not improve the phenotype in the three chamber sociability test. Moreover, chronic baclofen treatment induces a phenotype in WT animals, resulting in behavior deficits similar to *Fmr1* KO mice.

These results are independent of the increased locomotor activity that was observed at the higher dose, but not in the lower dose. Our results were in striking contrast compared to other publications, where hyperactivity was reduced. A possible explanation for the discrepancies is the use of chronic treatment in drinking water, instead of acute treatment via intraperitoneal injections, or the different outcome measures that were used. In addition, we did not use arbaclofen or R-baclofen, but the already FDA approved racemic baclofen, containing both the potent R-baclofen and the less potent enantiomer S-baclofen. Baclofen has only been used by two other studies and only with acute treatment⁵⁰. The highest dose we used was based on pharmacokinetic studies of Henderson *et al*⁴⁶, and was comparable to the most effective acute intraperitoneal dose used there. A paradoxical effect of baclofen in patients with psychiatric diseases or spasticity and in outbred mice has been described, leading to symptoms like hyperactivity, agitation, aggression, mania, and epilepsy⁵¹⁻⁵³.

Such paradoxical effects could be due to overdose, fluctuations of blood concentrations, or the specific genetic background and specific pathway characteristics of the mice⁵⁴. Unfortunately, there is no relevant functional read out to test whether baclofen reached levels in the brain that are sufficient to assert the desired effect, but do not lead to unwanted side-effects. Moreover, the GABAergic system, like any other pathway, probably has specific functions in different brain regions and cell-types, further complicating the establishment of the ideal dosage. Possibly, this kind of paradoxical effect is responsible for the worsening of the FXS phenotype, perhaps even overshadowing a mild improvement on other behavioral facets. Patients with neurodevelopmental disorders with a known GABAergic involvement are more prone to a paradoxical effect from GABAergic drugs. This might suggest that pathway characteristics are specifically important in these patients, complicated by the difficulty to target one specific part of the pathway with a general drug^{55,56}. Moreover, baclofen is known to induce tolerance, possibly through downregulation of the GABA_B receptor, although the precise mechanism is not known and conflicting studies have been published^{49,57,58}. One possibility, especially relevant in the case of FXS, is the evidence for cross-talk between GABA_B receptors and mGluR5 receptors after chronic baclofen administration, suggesting baclofen might downregulate the mGluR5 pathway and possibly other relevant signaling pathways, influencing other canonical functions of these pathways⁴⁹. This possibility is underlined by the finding that arbaclofen treatment

also improves features that are mGluR5 dependent, including protein synthesis and AMPA-receptor internalization ⁴⁶.

Next to the mGluR5 and GABAergic pathway, many other pathways have been implicated in FXS as well, including several neurotransmitter pathways and receptors (e.g. muscarinic acetylcholine, endocannabinoid, serotonin and aminomethylphosphonic acid), intracellular signaling pathways (e.g. extracellular signal related kinase, mammalian target of rapamycin and glycogen synthase kinase-3), extracellular pathways involved in synaptic plasticity (matrix metalloproteinase 9), voltage gated ion channels (Kv3.1b and Kv4.2) and many downstream mRNA targets of FMRP (e.g. phosphatidylinositol 3-kinase, striatal-enriched protein tyrosine phosphatase, amyloid precursor protein) ⁵⁹⁻⁶⁵. Many of these pathways and proteins have been targeted using pharmacological agents, often with improvements demonstrated in animal models (reviewed in ^{62,66}). In chapter four, we report the targeting of a less known pathway that is proposed to be affected in FXS, the RhoGTPase pathway. The RhoGTPase pathway is involved in cytoskeleton remodeling, which is important for synaptic plasticity and spine maturation. Aberrant RhoGTPase function has been demonstrated to be involved in intellectual disability ⁶⁷. FMRP plays a role in the RhoGTPase pathway, possibly through interaction with CYFIP1 and CYFIP2 ⁶⁷⁻⁶⁹. We targeted the RhoGTPase pathway using CNF1, that has been previously shown to activate the RhoGTPase pathway, leading to increased synaptic plasticity in rat visual cortex ⁷⁰. We show in chapter four that CNF1 can correct the immature spine phenotype in primary hippocampal neurons *in vitro*, an anatomical phenotype present in *Fmr1* KO mice and post-mortem brain tissue from FXS patients ^{18,71-76}. In addition, our study shows that CNF1 restored cognitive features in *Fmr1* KO mice, whereas no improvement was observed in anxiety-related behavior assessment and hyperactivity. This different response might implicate that the aberrant RhoGTPase pathway in FXS is only relevant in specific brain regions and cell-types. Previous studies in WT animals have also shown that only specific cognitive tests could be modulated by CNF1 administration, while others remain unaltered ⁷⁷, confirming RhoGTPase might only be relevant in certain brain regions. Since we only studied hippocampal neurons, a brain region important for learning and memory formation, we do not know the effect of CNF1 treatment on the spine phenotype in other brain regions. For example, phenotypes such as anxiety and hyperactivity are related to the amygdala ⁷⁸, in which we did not investigate the spine morphology. Also, although spine morphology is used as an indication for spine function, in fact we do not know the exact relationship between morphology and function, and whether all relevant spine characteristics are indeed corrected ^{79,80}. Thus, the morphology of spines could probably be used as a functional read-out to assess fundamental pathological mechanism, rather than a predictor of clinical relevance.

3. Combination therapy

In chapter four we were able to rescue cognitive tasks, but not anxiety and hyperactivity, which underlines the fact that each pathway may involve specific neuronal functions and that targeting one particular pathway might ameliorate only a subset of specific FXS phenotypes. Specific functions of FMRP have been implicated previously for the R138Q missense mutation identified in a patient with intellectual disability and seizures, but without behavioral features of FXS. The point mutation revealed a specific presynaptic function of FMRP, impairing its binding to BK-channels, without impairing the canonical postsynaptic mRNA binding function of FMRP^{81,82}. This illustrates that FMRP may have many different roles in neuronal functioning, that might not all respond, when only one specific pathway is targeted. Thus, these results advocate the introduction of a combination therapy.

As is clear from the long list of pathways involved in FXS, the role of FMRP in neurons is far more complex than initially expected. The most well-known function of FMRP is its acting as a translational repressor at the postsynaptic compartment^{63,83}. Many of the target mRNAs of FMRP are coding for proteins involved in synaptic function and several have been identified to be involved in intellectual disability and autism, implicating similar pathways are interconnected in these neurodevelopmental disorders^{63,84}. Next to the postsynaptic compartment, FMRP is also present in other cellular compartments, including the neuronal presynaptic compartment, although little is known about its function there⁸². In addition, FMRP is expressed in other cell-types, for example astrocytes and oligodendrocytes, where it might have an important role during brain development⁸⁵⁻⁸⁸. Besides its canonical role in mRNA interaction, FMRP also contains domains involved in protein-protein interaction and chromatin binding sites⁸⁹. This illustrates the complexity of the many different functions of FMRP in different cell types and cellular compartments. Thus, absence of FMRP potentially disrupts multiple essential cellular pathways. Strikingly, practically all studies published thus far, targeted only one pathway each. However, a combination therapy, targeting multiple pathways in the same or different compartments simultaneously, might be inevitable for an effective treatment of FXS (chapter six)⁹⁰.

Since important excitatory and inhibitory pathways are affected, it has been hypothesized that an excitatory/inhibitory imbalance accounts for many of the FXS related symptoms^{91,92}. In chapter two we describe a combination therapy targeting the excitatory glutamatergic pathway and the inhibitory GABAergic pathway simultaneously. To our knowledge, this is the first report published using chronic combination therapy in the FXS mouse model, with a social behavior paradigm as the primary outcome measure. Unfortunately, this did not lead to an improvement, but even to worsening of the automated tube test phenotype. First, this does illustrate that targeting two pathways may create a combined effect, even though in this case it did not lead to a synergistic but rather an antagonistic effect. Second, this result underlines the delicate balance of

the involved pathways in synaptic function, which can be easily damaged. It has been proposed that each of these pathways regulates this balance in its optimal function. For example, increased as well as decreased protein synthesis in the spine, has been demonstrated to lead to equal defects in synaptic plasticity, implicating that deviations from the optimum to either side can be disrupting⁹³. Targeting both the mGluR5 and the GABA pathways, might have resulted in a deviation from this optimum via an overshoot to the one side or an antagonistic effect to the other side of the optimum, possibly due to the suggested cross-talk between these pathways⁴⁹. In both cases, it underlines the tremendous fine-tuning needed in restoring the synaptic plasticity.

Future research should be focused on alternative strategies that do justify to the complex cellular function of FMRP. These strategies may include targeting multiple pathways in different cell compartments or cell-types simultaneously. An example could be to target pre- and postsynaptic pathways. Although combination therapy does increase the complexity of clinical trial design, it has been shown to result in a synergistic effect in mice for several syndromes, including Rett syndrome⁹⁴ and tuberous sclerosis complex⁹⁵. In patients with bipolar disorder, combination therapy has been demonstrated to be more effective than single therapy⁹⁶. In fact, polypharmacy is common for many psychiatric disorders, although hardly based on rational choices of specific molecular targets. For FXS, more is known about possible targets and specific preclinical outcome measures could assist in the choices of which pathways to target. For example, studies described in this thesis may suggest that targeting RhoGTPase for cognitive improvement and GABA for social and behavioral improvement could result in a synergistic effect, especially when combined with stimulating behavioral and cognitive therapy. Also, studying the role of FMRP in astrocytes, might reveal targets affecting synaptic control and the “tripartite synapse”⁹⁷. Targeting these, in combination with postsynaptic pathways, might be more successful in restoring synaptic function. An additional reason that advocates combination therapy is the possibility for compensational mechanisms that might contribute to differences in efficacy between individuals. By targeting more than one unit of a pathway, for example the mGluR5 receptor and the downstream molecule MEK, or pre- and postsynaptic components of the mGluR5 pathway, these compensational mechanisms might be bypassed and targeting could be more specific. Combining drugs to enable synergy and reduce side effects is a trend in the field of many complex diseases, and experimental setups to test these are time- and resource-consuming. Hence, computer-based modeling integrating systems biology, pathway interactions, omics-data, and pharmacokinetic data, might introduce new options for high-throughput screening⁹⁸.

To complicate matters, the excitatory/inhibitory balance in the synapse, but also the molecular function of FMRP, might be different in different brain regions or even in individual spines^{99,100}. Thus, targets that are used for therapy are involved in various molecular pathways, which might act differently per cell type. Targeting these pathways as a whole could produce side effects. Instead, drugs that specifically target relevant

functions of the pathways without affecting unwanted functions, might introduce new promising intervention. Recently, Stoppel *et al* published a report about β -arrestin2 that couples mGluR5 specifically with downstream protein synthesis in the hippocampus, but avoids targeting alternative signaling pathways of mGluR5. Genetic reduction of β -arrestin2 resulted in rescue of audiogenic seizures and memory and cognition tests, without inducing side effects in *Fmr1* KO mice that are associated with complete mGluR5 inhibition¹⁰¹. This study is a proof of principle, showing that specific functions of pathways can be targeted, avoiding dose-limiting psychomimetic effects¹⁰¹. In addition, another recent publication demonstrates that the effect of sertraline on FXS children can be affected by polymorphisms in genes in the serotonergic pathway, like MAOA and BDNF¹⁰². Both these studies further illustrate that pharmacological interventions will probably need to be fine-tuned and personalized to the individual patient.

Although combination therapy still seems the most promising way, this idea has been slightly tilted with the publication of a new target of FMRP: diacylglycerol kinase kappa (DGKk) mRNA, important for regulating the lipids diacylglycerol (DAG) and phosphatidic acid (PA). These lipids are involved in protein synthesis and actin dynamics, regulating synaptic strength via many of the proposed pathways in FXS pathophysiology^{103,104}. The data suggests that DGKk is the main target of FMRP, and lack of DGKk leads to many of the FXS phenotypes, including molecular, morphological and behavioral. Thus, the authors suggest that targeting this protein might ameliorate FXS completely, without the need of a combination therapy. This surprising finding still needs further investigation. It is interesting to unravel why this protein has been missed previously in CLIP studies. Some technical issues have been proposed, including normalization of input, fragmentation of mRNAs before CLIP execution, and the fact that DGKk is relatively newly discovered, resulting in absence of its probes in microarrays and annotation issues¹⁰⁵. Most important, replication by other laboratories is desired and experiments should investigate whether these findings are ubiquitous, or age and region specific. These should include *in vivo* experiments with different animal models, at different stages of development. Also, for *in vitro* experiments, tissues from different brain regions should be investigated. Moreover, if DGKk is the main target of FMRP and responsible for the phenotypes of FXS, rescue experiments using DGKk overexpression should be able to demonstrate amelioration of FXS phenotypes. In addition, it is important to replicate this finding in human tissue, possibly in peripheral or iPSc-derived material, before it could be considered a new therapeutic strategy.

4. From bench to bedside

What initially seemed to be a complex and untreatable disease, unexpectedly held promise of a cure when the mGluR5 theory was proposed. In the past years, a great leap has been taken towards clinical trials for FXS patients with targeted treatments. Smaller placebo controlled trials and open label trials have tested a range of drugs

targeting different pathways, including mavoglurant, Fenobam, and basimglurant targeting mGluR5, minocycline targeting MMP9, and lithium targeting GSK3b (reviewed in chapter one and ^{62,106}). Some were even followed by larger randomized placebo controlled trials. We participated in one of the larger clinical trials, testing the mGluR5 antagonist mavoglurant (chapter five) ¹⁰⁷. Worldwide, a total of 175 adult and 139 adolescent FXS patients were randomized into mavoglurant treatment or placebo and were treated for 3 months, followed by an open label extension trial. The trial did not meet its primary and key-secondary outcome measures: improvement compared to placebo, on the parent-rated questionnaire ABC-C_{FX} in patients with fully and partially methylated *FMR1* promoter. Also other endpoints did not show improvement compared to placebo. The pharmaceutical company decided to terminate the extension study and abolish the intended pediatric trial. Other large placebo-controlled trials have tested another mGluR5 antagonist (basimglurant) ¹⁰⁸ and the GABA_B receptor agonist arbaclofen ¹⁰⁹. However, none of the larger trials were terminated prematurely and none have led to approved therapeutic interventions. The used interventions lack the desired improvements in humans, even though efficacy was previously observed in the animal studies.

4.1. Reflection on animal models and clinical trials

Now that the initial enthusiasm about the promising results in animal models has been tempered by the disappointing results of clinical trials, we face a time of reflection on what should be our next steps. We need to unravel the complicating factors, in order to learn from them and to be able to make new choices for future research. In chapter six, I discussed the - in my opinion - most important reasons for this lack of translation from mouse to human, covering 1) the suitability of animal models to model the human disease, 2) the lack of robust reliable and relevant outcome measures in mice and humans, 3) the complexity of dysfunction in the FXS brain in the absence of FMRP requiring combination therapy (which is also discussed above), and 4) the design of clinical trials. In the sections below, I will further explore these.

4.1.1. Validity of animal models and translational issues

Soon after the *FMR1* gene was identified, the first mouse model was created, the *Fmr1* KO mouse ¹¹⁰, followed by other mouse models ¹¹¹, drosophila models ^{112,113}, and zebrafish models ^{114,115} in which the gene is functionally deleted. Recently, also a rat model has been characterized ^{116,117}. As is clear from chapters two, three, and four and from many publications in the past years ^{118,119}, well-defined model systems and reliable translational endpoints are indispensable in preclinical assessment of potential targeted treatment. We used the *Fmr1* KO mouse, which is the most well studied model system for FXS. The advantages of mice as a model for neurodevelopmental disorders include

the vast knowledge on murine development and the similarities between mice and humans in development, cognition, and social behavior, compared to non-vertebrates¹¹⁸. In addition, the *FMR1* gene in humans shows strong homology with the mouse *Fmr1* gene². The *Fmr1* KO mouse seems currently the most valid available animal model for FXS. Much effort has been spent on characterizing this mouse model, first in order to understand the disease mechanisms, and later in preclinical drug testing. Although the mouse mimics the genetic cause of the disease, namely lack of FMRP, there is, unfortunately, no mouse modeling the CGG repeat expansion and subsequent methylation of the promotor region. The mutant mice that were generated with a CGG repeat expansion showed no transcriptional silencing upon CGG repeat expansion >200 units¹²⁰. An important difference between the *Fmr1* KO mouse and transcriptional silencing in the human situation, is that the *Fmr1* KO mouse lacks FMRP from conception, while in humans the *FMR1* gene is silenced in the first weeks of embryogenesis¹²¹⁻¹²³. Nevertheless, the *Fmr1* KO mouse model displays many features consistent with human FXS, including macro-orchidism, immature spine phenotype, increased seizure sensitivity, deficient prepulse inhibition, hyperactivity, repetitive behavior, social behavior deficits, and cognitive deficits, reviewed in¹¹⁸. But, despite the phenotypical similarities between the *Fmr1* KO mouse and human FXS patients, results of animal models could not yet be translated to a cure for FXS patients, reviewed in^{62,119,124}. This raises the question whether the *Fmr1* KO mouse is a suitable model of FXS and drug discovery for humans. Clearly, the *Fmr1* KO mouse should be reconsidered in its validity in drug discovery. Several disadvantages could be proposed that could have contributed to this lack of translation. Although *Fmr1* KO mice show many characteristics that are also observed in FXS patients, many of the described phenotypes are more subtle in the mouse model than in the human situation, including the cognitive and social deficits¹¹⁸. Moreover, the phenotypes vary greatly between different studies, possibly due to differences in genetic backgrounds, age, and used experimental protocols^{90,118,125}. For example, social deficits and repetitive behavior vary between FVB and C57Bl6 genetic background, showing C57Bl6 mice are probably more suitable to assess ASD features¹²⁶. In contrast, the locomotor phenotype is more pronounced in FVB mice¹²⁶. Research comparing different genetic backgrounds has shown that it can have a large impact on behavior and reaction on drug treatment^{125,127}. This underlines the limitation of generalization of the results from inbred and extremely controlled animal model setting to the outbred and uncontrolled human situation. In fact, these differences in phenotypes and efficacy of drugs between mice strains might even reflect differences between FXS patients, who can display a wide range of severity of symptoms, further complicating the drug discovery process. It is probably essential to test several (mixed) genetic backgrounds but most definitely also other animal models (e.g. rat), to check whether treatment effect is reproducible between genetic lines and species and to predict more precisely the chance of successful translation.

In addition, the differences in severity of the phenotype in mice and humans, in combination with the complete lack of efficacy of targeting the major pathways like

mGluR5 and GABA_B, might indicate differences in molecular pathophysiology between mice and humans. This is probably unlikely, since the genetic etiology is similar, and similar molecular features have been identified not only in mice, but also in other species including fruit fly and zebrafish, advocating these mechanisms are most probably similar in humans. However, there is no proof of the relevance of mGluR5 and GABA in human FXS. Possibly, other pathways are involved, or pathways are differently interconnected, demanding different approaches or targets in humans than in mice. Human specific pathophysiology's could only be unraveled with the use of model organism closer to humans, possibly even in human tissue only, reserving the *Fmr1* KO mouse and other animal models only for directive information in pathway discovery.

In the past years, many potential targets have arisen, but preclinical research has been consistently hampered by the lack of reliable, robust, and relevant outcome measures to assess efficacy of drugs. In chapter one, we reviewed the most commonly used outcome measures in *Fmr1* KO mice. Many of the outcome measures that are used in animal models, are not translatable to humans^{90,119,128-130}. Biochemical measures like increased protein synthesis and changes in synaptic receptors, electrophysiological measures on brain slices, and spine morphology measures are often used as outcome measures in mice^{66,106,118}, but these are simply unachievable in FXS patients, since the brain cannot be dissected. In our experiments, we used male *Fmr1* KO mice from C57Bl6 background¹¹¹ and measured therapeutic effects with several outcome measures, including social behavior measures, cognitive tests, and spine morphology. Behavioral abnormalities are often easier to translate, but are inconsistent across genetic backgrounds and research groups and not always translatable to humans. For example, the dominant *Fmr1* KO phenotype observed in the automated tube test that we used (chapters two and three) cannot be translated one-to-one to behavior of human FXS patients²⁰. It probably reflects ASD-like social deficits, but we can only speculate what human behaviors are modeled by this test, for example rigidity, impulsivity, disinhibition, inadequate behavior, aggression, anxiety, or a combination of those features. The same could be held true for other outcome measures that we used, like spine morphology that could not predict improvement in behavior abnormalities (chapter four). In fact we are not even certain that amelioration of these outcome measures in mice are directly correlated with clinically significant improvement in FXS patients. Moreover, the vast majority of hypotheses are based on animal model data and there is very limited knowledge about pathophysiological mechanisms involved in FXS from patient-based research. The limited patient data available is derived from either post mortem brain tissue (end stage of disease) or from peripheral tissues that is irrelevant for brain^{73,131,132}. Overall, it is unknown in what way these pathophysiological mechanisms contribute to the neurodevelopmental symptoms, and how those mechanisms should be targeted to induce improvement.

Above discussed translational factors pose a general problem of extrapolation from animal models to humans, which is encountered in all fields of medicine. For example,

in the more mature field of oncology, only 8% of therapies that were successful in animal models, and ultimately reached clinical trials, finally resulted in successful therapeutic interventions ¹³³. A recent meta-analysis showed that in research focused on targeted treatment for neurodevelopmental disorders, which is a relatively new translational field, the translational success is only 3% ¹³⁴. From 169 evaluated trials, only two interventions were FDA approved as treatment, namely diet for phenylketonuria and miglustat for Gaucher type I. The reasons identified for this low number of successes include small sample sizes and low quality of trial design. However, translational factors are probably important as well. Especially in neuropsychiatric disorders, translation from animal models has encountered many disappointments. Pharmaceutical companies were typically highly interested in psychopharmaceutical drugs, because of the high prevalence of psychiatric diseases, the lack of effective therapy, the high morbidity, and the costs for society ¹³⁵. But even though compelling evidence and possibilities are being introduced from translational research, major pharmaceutical companies are halting the development of these drugs due to limitations in translation from animal models ¹³⁵.

4.1.2. Clinical trial outcome measures and study design

Besides the above-described limitations of animal models and translation, other limitations concern the trial design. Probably, the major issues are related to the age of treatment, the treatment duration, and the lack of objective and reliable outcome measures. Randomized, placebo controlled trials are regarded as the gold standard in drug assessment, since the occurrence of placebo effect is a widely known phenomenon, especially in psychiatric drugs ^{136,137}. As described for the clinical trial with mavoglurant, a large placebo effect was measured. For example, the ABC-C_{FX} score showed during the four weeks placebo run-in period already 30% and up to 100% of the total improvement, without fading off during the trial (chapter five) ¹⁰⁷. This large placebo effect could possibly overshadow a small but relevant drug effect. Several reasons have been postulated for the occurrence of large placebo effects in psychiatry ¹³⁷. One important factor in the mavoglurant trials could have been the used outcome measures, that are based on caregiver or clinician assessed questionnaires ^{106,107,119}. Caregivers but also clinicians are not objective in their observations, especially when expectations are high. Probably more extensive training is needed to use the questionnaires correctly ¹³⁸. Chapter one gives an overview of the most used outcome measures in clinical trials with FXS patients. Many of those outcome measures are caregiver or clinician rated and thus are subjective and prone to placebo effects. In addition, none of the outcome measures have been validated for disease modifying therapy of neurodevelopmental disorders ¹³⁹. These outcome measures were mostly selected because their usability in trials with patients with autism, where they showed to be capable of assessing improvement of irritability and anxiety ¹⁴⁰. Both these symptoms are relevant for FXS, but it is not known whether improvement in these specific symptoms was to be expected from the drugs tested. Other outcome measures that are considered more objective, like cognitive tasks,

suffered from lack of power, due to unfeasibility of these tests in patients¹⁰⁷. In some trials endophenotypes were used, like pre-pulse inhibition^{106,141,142}. Those are objective, but the clinical relevance is unclear. Besides, they are hardly feasible for FXS patients to perform and very time consuming. These results have led to much debate around the used outcome measures in FXS clinical trials and in neurodevelopmental trials in general^{90,119,139}. There is a clear need for objective and relevant outcome measures that are suitable to measure effects of disease modifying therapy in neurodevelopmental disorders, to be able to fully assess new drugs. For future clinical trials, the used outcome measures should be reconsidered and future research should focus on discovering more objective and relevant outcome measures that are closer to the molecular mechanism underlying FXS. To facilitate translation, outcome measures should be developed that are similar for humans and animal models systems. These should probably include biomarkers¹²⁸, endophenotypes that are more easily performed like the eye-blink test¹⁴³, non-invasive neuroimaging techniques like functional near-infrared spectroscopy¹⁴⁴, Transcranial Magnetic Stimulation¹⁴⁵ and electroencephalogram¹⁴⁶ and quantifiable behavioral and cognitive tests like the recently published Eye Contact Avoidance Scale¹⁴⁷, instead of the now used questionnaires.

In the development of new outcome measures, it is important to recognize the differences between FXS patients and patients with other causes of autism or intellectual disability. Most outcome measures are focused on general autistic symptoms, which might disregard FXS specific differences, although some tests have been modified to better fit FXS^{148,149}. Also, the large variation of symptoms, severity and environmental background of FXS should be noted, as well as the dynamic variation of phenotypes during development. Outcome measures should be sensitive enough to overcome these individual factors and aim at measuring more precisely the underlying reasons for certain cognitive and behavioral phenotypes, that could appear similar but be a result of different disease features. Possibly, outcome measures should even be fine-tuned not only to specifically measure FXS-related symptoms, but also to be individualized per patient, to capture improvement in specific features. Other aspects of trial designs include age at start of treatment and the duration of treatment. Considering the neurodevelopmental aspects of the disease, one might assume that the earlier the intervention, i.e. before the critical age when neuronal plasticity is reduced, and the longer the treatment duration, the better the results¹⁵⁰. Data from animal models is ambiguous about the need for early intervention and the duration of treatment. Some studies have shown improvement in *Fmr1* KO mice when treated at later ages, even in adulthood^{20,21,23,24}. These findings might indicate that FXS is reversible at older age. However, other studies have shown that treatment is only effective at young age and not later in life¹⁵¹⁻¹⁵⁴, which is obviously supported by the fact that synaptic plasticity is known to be larger in younger animals and is often confined to critical windows¹⁵⁵. Clearly, it is difficult to predict the optimal age of treatment, based on the available data, but starting at a young age probably has a higher chance for success. Regarding the treatment duration, some studies show that biochemical as

well as behavioral phenotypes can be rescued within hours, while other studies showed that chronic treatment for several weeks is necessary, which might be equivalent to many months or even years in patients ²³. However, clinical trials are not designed for long term treatments and typically drug testing at a young age is not encouraged due to safety issues. Future clinical trials should aim at starting intervention at an earlier age to reach a maximum effect of treatment. This opinion is shared by many clinicians, caregivers and scientists, which is clear from the recent announcement of the investigator-initiated clinical trial with mavoglurant in children with FXS (NCT02920892). This trial will be initiated soon, even though the pharmaceutical company involved has previously terminated all mavoglurant trials in FXS, stating that extension of these trials to children is unethical in the light of the disappointing results in adults and adolescents (<https://www.fraxa.org/clinical-trial-fragile-x-children/>). Disregard of the clinical trial duration, it is assumed that any of the thus far proposed treatments would comprise of life-long medication, since these drugs do not affect the primary cause of FXS, namely the lack of expression of FMRP. In addition, solely giving a drug is probably insufficient to establish clinical effect on cognition and behavior, since these are dependent on relevant environmental stimulation ¹⁵⁵⁻¹⁵⁷. Environmental enrichment alone has shown benefit in FXS mouse models ^{158,159}. Probably drug treatment will need to be combined with other non-pharmaceutical interventions that are known to induce improvement in FXS, like training, education, and behavioral interventions. These should be integrated in the clinical trials, to investigate whether specific stimulation is needed to induce drug efficacy. New trials are planned and currently being performed, combining cognitive interventions with lovastatin (NCT02642653) and mavoglurant (NCT02920892). Another issue known from drug discovery in general is the lack of attention for drug efficacy in girls and women. This neglect could be understandable in the case of FXS, because the X-linked inheritance of the disease, that naturally leads to a less severe and more variable phenotype in women ⁷. However, the population of girls and women with FXS is still significant and they are no less in need of medical treatment than males with FXS. In clinical trials, very few females have been included and from animal models, hardly any data is available on female animals, phenotypically as well as on therapeutic interventions. Just recently a paper characterizing female heterozygous *Fmr1* KO mice has been published, showing age and gender-specific autistic like phenotypes, vocalization, and electrophysiological anomalies ¹⁶⁰⁻¹⁶². This gap in attention for females with FXS needs to be filled by studying disease phenotypes, molecular mechanisms and therapy in females, pre-clinically as well as clinically.

Although some larger clinical trials have been sufficiently powered and well-designed, most of the studies performed in FXS have been underpowered and based on open label design, which is not suitable to assess efficacy. This poor quality design of clinical trials has unfortunately been the case for many other neurodevelopmental disorders as well, with only 30% of trials reaching acceptable design quality ¹³⁴, mostly due to recruitment and financial limitations as well as undefined outcome measures. In many genetic neurodevelopmental disorders, discovery of the involved genes

enabled the generation of animal models and identification of underlying molecular pathophysiology and involved pathways, revealing possible targets for treatment ^{155,163-165}. Many of these pathways are involved in synaptic maintenance and adaptation, revealing how tight the balance of these pathways needs to be for efficient synaptic plasticity ¹⁵. Moreover, research in animal models has also revealed that synaptic plasticity can be strengthened, even in adult brain ^{70,155,166}. This implicates that restoring aberrant pathways in neurodevelopmental disorders could reduce symptoms or cure the disorders, even at an age beyond the assumed critical period. This was good news for the field, leading to an explosion of preclinical studies and clinical trials on targeted treatments, which are reviewed in ¹⁶⁵. In particular, the pathophysiological overlap between FXS and autism has appeared to be large, not only clinically, but also pathophysiologically, expanding the FXS drug discovery model to the much larger autism spectrum disorder field ^{63,93,167}. Other genetic disorders like Rett syndrome, tuberous sclerosis complex, and neurofibromatosis have been under investigation for targeted therapy as well, using transgenic animal models. These models have shown that similar neuronal pathways are involved in their pathogenesis and that targeting these pathways may reverse many symptoms ¹⁶⁵. This has led to the initiation of clinical trials for these syndromes as well. For example, for tuberous sclerosis, rapamycin has been tested in clinical trials, reducing tumor burden and epilepsy. However, this has not been replicated in larger placebo controlled trials and data on cognitive improvement remained inconclusive, due to small sample sizes and inconsistent outcome measures ¹⁶⁸. For neurofibromatosis, several randomized, placebo-controlled clinical trials have been performed, amongst others with simvastatin. Unfortunately, this has not led to cognitive or behavioral improvement compared to placebo ^{169,170}. It has been demonstrated that the used outcome measures in neurofibromatosis have weak test-retest reliability ¹⁷¹. In Angelman syndrome, treatment with minocycline and different dietary compounds affecting hypermethylation have also not been successful ¹⁷². Topoisomerase inhibitors and antisense oligonucleotides, targeting epigenetic modification of the UBE3A gene, are being evaluated now. Currently, many preclinical and clinical trials for several monogenetic disorders are ongoing, often facing similar challenges as the FXS field.

5. Future perspectives: what should we do next?

Worldwide, the past decades have been dedicated to elucidating aberrant molecular pathways and targets in FXS. These targets were important for the development of potential drugs that could modify disease characteristics and phenotypes in animal models. Despite the promising preclinical results, this has not led to successful treatment for FXS yet. Some important questions remain: is it actually possible to rescue specific phenotypes in patients with FXS, as seems the case in animal models? And how do we tackle the above-described challenges? I believe that improvement and prevention of symptoms in patients with FXS could be achieved if treatment is initiated at an early stage and combined with behavioral and psychological interventions. Even when

initiated directly after the diagnosis has been made, typically around the age of 2-3 years, I expect patients could still benefit, especially in terms of behavioral and psychiatric aspects. However, it is simply unknown what effect could be realized on cognition, and what timeframe should be addressed. Since we continue to be overwhelmed by preclinical success stories of potential treatments for FXS, and since FXS patients are still in need of a suitable therapy, choices need to be made on how to continue our quest for therapy for FXS. Moreover, the clinical and molecular overlap between autism and FXS is large ^{62,63,84,124}, implicating that treatment for FXS might reveal similar strategies for the treatment of autism as well.

First of all, the limitations of the *Fmr1* KO mouse, provoke the question whether it is a suitable model in drug discovery for FXS. Despite the large benefits of the mouse model in uncovering and modeling pathophysiological mechanisms of disease, the results of interventional preclinical trials have clearly been over-predictive for the potential treatments. Data on molecular mechanisms in FXS have shown some consistency with other animal models, including the zebrafish and fruit fly. Introducing other model systems could assist in identifying species-independent features that might account for humans as well. Further research should focus on identifying new FXS disease model systems that are more valid to use for drug screening. New possibilities might come from the *Fmr1* KO rat model, that show many similarities but also important differences from the *Fmr1* KO mouse ¹¹⁷. Also other animal models, like primates and pigs, might be more feasible with new gene editing techniques ^{173,174}. Probably a combination of several animal models, but also the use of different (mixed) genetic backgrounds of one animal, would inform us on reproducibility of therapeutic intervention studies. The use of negative controls, i.e. drugs that did not demonstrate efficacy in humans, might be helpful in evaluating new model systems.

There is currently no animal model that contains the CGG repeat expansion and recapitulates the transcriptional silencing that occurs during embryogenesis in humans. Despite many similarities between the used animals and the advantage of their use *in vivo*, they do not exactly resemble the human situation. Thus, *in vitro* cell models with patient derived materials could be used to evaluate molecular mechanisms and test drugs on human tissue. Peripheral tissue like fibroblasts and lymphoblastoid cell lines have been used, but they are irrelevant in the study of the brain phenotype. Human embryonic stem cells can model the early embryogenesis, but are very limited in their availability and it takes a long time before the *FMR1* gene is methylated ¹⁷⁵. The emergences of techniques that create induced pluripotent stem cells (iPSCs) from patient derived tissue and differentiate them into neurons, enabled researchers to study neuronal function of FXS *in vitro*. FXS iPSCs have been shown to display characteristics of FXS, including a silenced *FMR1* gene and morphological spine phenotypes ¹⁷⁶⁻¹⁷⁹. However, relevant outcome measures will need to be identified first, before iPSCs can be used in drug screening. The use of iPSCs differentiated into neuronal cells has been implicated in general for neurodevelopmental disorders, although it's *in vitro* setting is a

major drawback¹⁸⁰. This drawback might in part be overcome by the generation of brain organoids, 3D *in vitro* cell cultures mimicking organ systems, from patient derived iPSCs. Brain organoids have been demonstrated to resemble the development and function of human brain in some aspects and have been proposed as new and accessible model system for neurodevelopmental disorders including autism¹⁸¹⁻¹⁸³. This might be further developed, using technologies that mimic *in vivo* environment on a chip in a dish, the so called organ-on-a-chip^{184,185}.

The success stories of targeted treatments in animal models could have also been the result of publication bias toward the positive results. Typically, in biomedical research, it is by far easier to publish successful results, than negative data. In addition, scientists are more eager to publish their success stories, giving them access to higher impact journals, directly positively correlated with new funding^{186,187}. This creates a large gap in the available data that is necessary to appraise current knowledge. Besides, it cultivates the waste of resources, since studies might be repeated that have already failed several times, and hence were not published. Trial registration has improved the visibility of ongoing and performed clinical trials, although still not their publication, leaving many results hidden from the public¹⁸⁷. And this kind of registration does not exist for preclinical research, making it even more difficult to estimate the amount of negative data. It is essential in biomedical research, especially concerning drug development, to change the publication culture for scientists as well as journals, towards publication of negative or ambiguous data. Platforms should be created to publish negative data, while researchers should be more valued based on their experimental setups, instead of their publication rates. In addition, raw data and details on protocols, materials and environmental conditions should be publicly accessible, to enable appraisal and replication of the experiments.

Several approaches could be used when developing targeted treatment for genetic diseases. So far, most research in FXS has focused on compensating for the lack of FMRP by targeting pathways that are deregulated by its absence. This has seemed the most promising, because gene therapy still seems science fiction. In addition, accessible targets have been identified and drugs modifying these targets were readily available, sometimes even already FDA approved for other diseases. But, since FMRP targets many mRNAs, and many pathways seem to be involved, targeting only one pathway is probably not sufficient, which largely complicates drug discovery. Theoretically, the most integral strategy to cure FXS, would be to reactivate the *FMR1* gene. The presence of carriers of an *FMR1* full mutation that has escaped methylation and who have no clinical phenotype¹⁸⁸, does demonstrate that demethylation of the full mutation in the *FMR1* gene might result in a successful therapy. However, it is unknown when during development this should be established to achieve full or partial amelioration of the disease. Probably, the intervention will need to be very early, since absence of FMRP already has a major effect on differentiating neurons *in vitro*¹⁷⁹. In the past, efforts have been taken to test demethylating agents (5-azacytidine) and other histone modifying agents (valproic acid, methotrexate) *in vitro* using fibroblasts, lymphoblastoid cells,

or iPS cells from FXS patients. These agents have shown that partial and transient demethylation of the *FMR1* gene is possible, inducing transcriptional reactivation¹⁸⁹⁻¹⁹². However, the results are not consistent and these drugs are hardly usable in clinic due to toxicity and non-specificity. Only valproic acid, a known drug used to treat patients with epilepsy, has been tested in a preliminary open label clinical trial with 10 FXS patients¹⁹³. The newly available CRISPR/Cas9 technique might open a new way to edit the full mutation in the *FMR1* gene. Deletion of a part of the CGG repeat could induce transcriptional reactivation of the *FMR1* gene and led to expression of FMRP in embryonic stem cells, iPS cells and derived neurons from FXS patients^{194,195}. However, many drawbacks still exist for this technique and important questions still remain to be answered, including how to target all neurons in the brain, which efficacy could be reached at the expense of what side effects, and what off-target effects could be expected^{196,197}. Also, it is unknown how many cells in the brain will need to be reached in order to effectuate improvement. Probably, this will lead to a mosaic phenotype, that would need to be at least as high as 50-60% of the neurons containing a reactivated *FMR1* gene, to yield clinical improvement¹⁹⁸. Even if the intervention would be sufficiently efficient in targeting human neurons, still it would need to be very early during gestation, to fully prevent a phenotype¹⁷⁹. It is possible that sufficient effects might only be reached when applied at conception, preventing mosaicism and reaching all body cells. The CRISPR/Cas9 gene editing technique has been implicated in therapy for other repeat-associated disorders like Huntington's disease¹⁹⁶. In addition, it has even been tested *in vivo* in brain of mouse models, illustrating neurons can be targeted, when introduced intrathecally^{199,200}. Important drawbacks seem to be overcome, since the first clinical trials using CRISPR/Cas9 has been initiated in patients with end stage of cancer²⁰¹ (<http://www.nature.com/news/first-crispr-clinical-trial-gets-green-light-from-us-panel-1.20137>). But the research fields that are the most advanced do not involve the brain, which is probably the most difficult organ to target. In addition, the ethical dilemmas surrounding gene editing as therapeutic interventions are far more complex in neurodevelopmental disorders than in cancer, where mostly somatic cells are targeted and therapy is intended to prevent or delay imminent death. Clearly, the benefit in end stage cancer outweigh the risk more easily than in non-life threatening diseases like FXS. In addition, when germline gene editing is involved, many important ethical questions are raised about designer babies, the rights of an individual to be healthy, and the rights of people with disabilities²⁰²⁻²⁰⁴.

Before we continue to test new drugs, clinical trial design factors, should be reconsidered. For obvious safety and financial limitations in clinical trials, many choices may not have been optimal, including the used outcome measures, treatment duration, the age of participants, and the dose. Relevant and reliable outcome measures should be developed, and, in addition, be evaluated for females with FXS. Treatment should be aimed at children and should include both sexes. Because the rapid development of potential targeted treatment, there is a great need for expertise on FXS characteristics to develop outcome measures. Also, patient care and fundamental and translational

research should be combined and clustered in national expertise centers and international collaborating consortia. We have recently set up a national center of expertise in the Netherlands, in the Erasmus University Medical Center in Rotterdam, to cluster and improve patient care and research. The center is set up for children, but efforts are made to extend to adults as well. In addition, the expertise center has started collaboration with the patient organization to initiate a national expertise network. We are currently developing a clinical guideline for treatment of children with FXS (chapter seven), the first guideline for treatment of FXS in the Netherlands. This guideline is adapted to the Dutch healthcare system and is based on international guidelines, literature research and, when evidence from literature is lacking, expert opinions. The guideline is mainly aimed at healthcare workers, but can also be used by other caretakers who work with children with FXS, like teachers, and even parents. Parents of children with FXS often encounter misunderstandings about their child and need to explain their child's needs over and over again. Comparisons between FXS and other forms of autism or ID are often most achievable to explain their problems, but these disregard features that are FXS-specific and need to be addressed differently. A guideline could facilitate the care of children with FXS, which is considered a rare disease that many healthcare workers would only encounter incidentally. Moreover, a guideline could be used to implement more standard diagnostics and care in FXS. Standardized diagnostics in our expertise center includes a neuropsychiatric test battery that is measured in every stage of development of the child. In addition, these tests could be used to follow-up patients. Currently, there has been hardly any research that focused on long-term follow-up of this patient group, which is important in order to increase the knowledge of the phenotype as well as to develop clinical outcome measures. Recent research activities in our expertise center include the development of new and objective outcome measures, e.g. functional near-infrared spectroscopy¹⁴⁴ and Transcranial Magnetic Stimulation¹⁴⁵, which could potentially be used for future clinical trials. New outcome measures and well-defined phenotypical characteristics might both increase the yield of drug development.

Since 1991, a huge amount of research has been conducted, leading to impressive steps towards understanding the molecular mechanisms underlying FXS. This knowledge paved the way for translation to potential targeted therapy for FXS. However, the clinical trials so far, have not been successful and we still do not have new therapeutic options. While researchers around the world are committed to address the above-mentioned issues and revise their research strategies, our quest for a targeted treatment for FXS continues.

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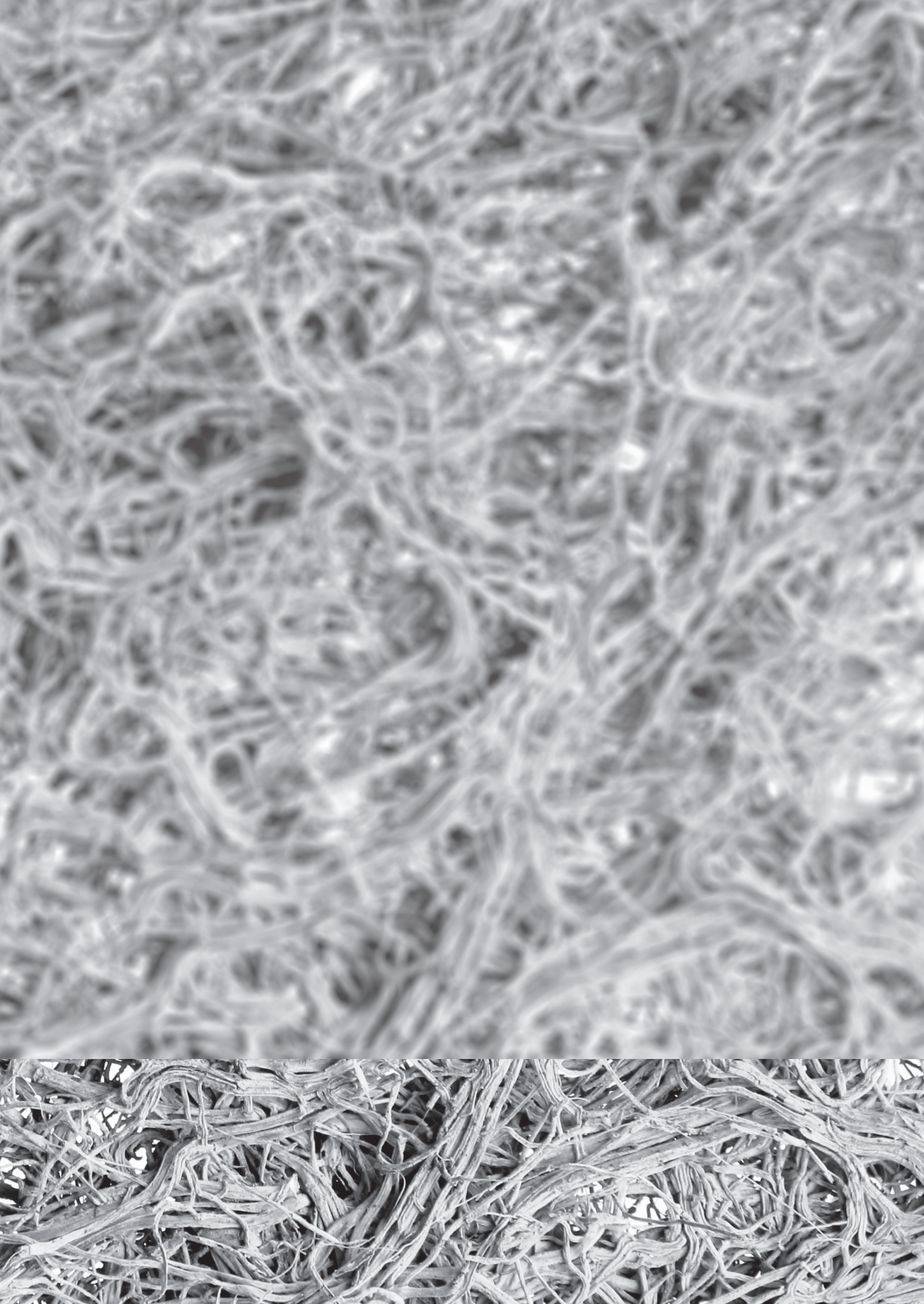
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Appendix

Summary

Intellectual disability and autism spectrum disorders are both frequent neurodevelopmental disorders in the general population, which often co-occur. In many cases the etiology is unknown. The past decades have revealed many causative genes, often with mutations in genes that are involved in synaptic plasticity. In most cases, these neurodevelopmental disorders do not have a disease modifying therapy, and are treated symptomatically. This treatment only partially reduces psychiatric symptoms and obviously does not affect cognition. Knowledge of involved genes enabled the generation and characterization of animal models that have revealed many targets for potential therapy, opening a possibility for targeted therapy. A striking example of the development of targeted treatment is fragile X syndrome (FXS). FXS is a relatively frequent cause of intellectual disability and autism spectrum disorders, due to a mutation in the X-linked *FMR1* gene. Expansion of the naturally occurring polymorphic CGG repeat in the 5' UTR of the gene, leads to transcriptional silencing and lack of the protein product, FMRP. The past years have gradually elucidated many cellular functions of FMRP, using the FXS mouse model, *Fmr1* KO mouse. An important function of FMRP is the regulation of local protein synthesis in the dendritic spines.

In **chapter one**, we summarized the molecular functions of FMRP and important cellular pathways that are disturbed in FXS, as a consequence of FMRP loss. We outlined the pathways that have been tested as targets for potential therapy in animal models. We also summarized the main used outcome measures in mice, that are used to study the efficacy of potential interventions. The results of these preclinical studies have been very promising, showing improvement of many characteristics in the mouse models. A leap to clinical trials was quickly made, resulting in a large number of small, open label clinical trials in patients with FXS. In addition, we outlined the conducted and ongoing clinical trials, as well as the most used outcome measures in clinical trials. These first trials seemed initially effective, but the larger, placebo controlled trials that followed, were all terminated prematurely due to lack of efficacy.

In **chapters two, three, and four**, we described the results from preclinical studies that we have performed in *Fmr1* KO mice. We targeted three different pathways that have been proposed to be involved in FXS: the excitatory metabotropic glutamate receptor 5 (mGluR5) pathway, the inhibitory gamma-aminobutyric acid (GABA) pathway, and the RhoGTPase pathway. The goal of these studies was to investigate whether targeting these pathways could ameliorate certain phenotypes in *Fmr1* KO mice, as a model for the disease in humans. In **chapter two and three** we focused on social behavior outcomes, since aberrant social behavior can lead to major disabilities in patients and these have been relatively little explored in FXS. In **chapter four**, other outcome measures were addressed as well, including cognitive measures, other behavior measures, and the morphology of dendritic spines.

In **chapter two**, we demonstrated that targeting the GABA pathway with bumetanide, thus forcing the GABAergic function from immature to mature, reduced social behavior deficits in the *Fmr1* KO mouse. Social behavior was measured using the social dominance paradigm, the automated tube test, in which *Fmr1* KO mice show a strong and robust dominant phenotype. The improvement of social behavior was consistent, however, only partial correction was measured. This partial correction is similar to the effect of mGluR5 reduction that was demonstrated previously. We hypothesized that combining both bumetanide treatment and mGluR5 reduction, might lead to an increased efficacy. However, instead, this led to a reduced effect of both interventions. These results suggest that these pathways are interconnected in a tightly regulated excitatory-inhibitory balance of the synaptic function, which requires more sophisticated fine-tuning. We believe that to do justice to the complex functions of FMRP, multiple pathways will need to be targeted, in different cellular compartments or even different cell-types, in order to compensate for the lack of FMRP. With this study, we were the first to attempt chronic combination therapy in FXS.

In **chapter three** we described the effect of another drug targeting the GABA_B pathway, baclofen, on two social behavior paradigms in the *Fmr1* KO mice. We measured the effect of chronic baclofen treatment on the automated tube test and on the three chamber sociability test. In both tests, no improvement was seen in *Fmr1* KO mice treated with baclofen, and even a worsening of the phenotype was seen in the automated tube test. Moreover, baclofen treatment in wildtype littermates induced a phenotype similar to the *Fmr1* KO mice, in both social behavior tests. This effect was independent of hyperactivity, which was induced by baclofen in a high dose, but not in a lower dose. Since baclofen is expected to result in sedation as a side effect, these results are highly unexpected, and might fit a paradoxical effect that has been occasionally described for baclofen. Whether our results are caused by the dose, the genetic background of our mice, the used outcome measure, or any other reason, is unknown. But regardless, they raise doubt whether baclofen, or the in clinical trials used R-enantiomer arbaclofen, is useful in clinic to treat FXS.

In **chapter four** we targeted a relatively unknown pathway, the RhoGTPase, which is important in the maintenance of dendritic spines and determining the synaptic strength. We demonstrated that targeting the RhoGTPase with CNF1, rescues an immature hippocampal dendritic spine phenotype and hippocampal and cortical dependent cognitive functions. In contrast, CNF1 did not affect anxiety and hyperactivity. These results demonstrate that targeting the RhoGTPase affects only specific domains of FXS, further illustrating the complexity of the disruption caused by lack of FMRP.

After discussing preclinical data, in **chapter five** we discussed the results of a clinical trial in which we participated: an international phase 3, randomized, placebo controlled clinical trial with adult and adolescent FXS patients. In the clinical trial the mGluR5 pathway was targeted using the mGluR5 antagonist mavoglurant. The outcome

measures were parent and clinician rated questionnaires and cognitive measures. Unfortunately, the primary and secondary outcome measures were not met and the open label extension study was terminated by the pharmaceutical company. This was a great disappointment for patients, caregivers, and clinicians, who all had high expectations. Since all larger clinical trials in FXS patients, using other drugs, have also failed to demonstrate an effect, it became clear that new directions need to be taken in FXS drug research.

This has urged us to contemplate on the possible reasons for these negative results, which we discuss in the expert opinion in **chapter six** “The quest for targeted treatment in fragile X syndrome”, as well as in the **last chapter**, the general discussion. Apparently, translation from the successful preclinical studies to patients was not as straightforward as initially expected. Major limitations can be pointed out regarding the suitability of animal models for drug research, the used outcome measures in preclinical as well as clinical trials, and the clinical trial design, that is possibly unfit to measure disease modifying therapy in neurodevelopmental disorders.

In order to guide patients and caregivers in the overwhelming amount of data from preclinical and clinical studies and in order to secure the best possible medical care, we recently set up an outpatient expertise center clinic for children with FXS. Soon, the clinic will be extended to adult FXS patients. In **chapter seven**, we presented the guideline for care for children with FXS that we are currently developing, based on up to date literature and adapted to the Dutch healthcare system.

In **chapter eight**, the general discussion, I discussed the findings from the above mentioned papers, and placed them in the context of the current research and literature. I elaborated further on possible reasons for the lack of translation from mice to human that were briefly discussed in chapter six, and suggested possible new directions of research. Importantly, the challenges encountered in our research do not only apply to the FXS research field, but also to all other fields investigating targeted therapy for neurodevelopmental disorders.

Samenvatting

Verstandelijke beperking en autisme spectrum stoornissen zijn beide ontwikkelingsstoornissen die veel voorkomen in de algemene populatie. Vaak worden ze tegelijk gezien voor bij een persoon. In de meeste gevallen is de oorzaak onbekend, maar in de afgelopen decennia zijn vele genen ontdekt die een deel van deze aandoeningen kunnen verklaren. Vele van deze genen blijken betrokken te zijn bij synaptische plasticiteit. Voor de meeste ontwikkelingsstoornissen is geen gerichte therapie voorhanden. Patienten worden symptomatisch behandeld, met een matig effect op de psychiatrische symptomen en vrijwel geen effect op de cognitie. Kennis van de betrokken genen heeft de generatie van diermodellen mogelijk gemaakt, wat aangrijpingspunten voor potentiële gerichte therapie aan het licht heeft gebracht. Een opvallend voorbeeld hiervan is de ontwikkeling van gerichte therapie voor het fragiele X syndroom (FXS). FXS is een relatief frequente oorzaak voor verstandelijke beperking en autisme spectrum stoornissen, als gevolg van een mutatie in het X-gebonden *FMR1* gen. Expansie van de van nature voorkomende CGG repeat in de 5' UTR van het gen, leidt tot inactivatie van het gen en een tekort aan het eiwitproduct, FMRP. De afgelopen jaren hebben geleidelijk vele cellulaire functies van FMRP aan het licht gebracht, waaronder de regulatie van eiwitsynthese lokaal bij de dendritische spine.

In **hoofdstuk één** gaven we een overzicht van de moleculaire functies van FMRP en belangrijke betrokken pathways die zijn verstoord bij FXS, als gevolg van het verlies van FMRP. We beschreven de pathways die zijn getest als aangrijpingspunten voor potentiële therapie in diermodellen. Ook hebben we een overzicht gegeven van de meest gebruikte uitkomstmaten in muizenstudies. De resultaten van deze preklinische studies waren veelbelovend en toonden een verbetering van verschillende kenmerken van de muismodellen. De sprong naar klinische trials werd al snel gemaakt, met als gevolg een groot aantal kleine, open label klinische trials met FXS patiënten. Wij hebben tevens een overzicht gegeven van de uitgevoerde en lopende klinische trials, tezamen met de veelgebruikte uitkomstmaten in klinische trials. De kleinere klinische trials leken aanvankelijk effectief, maar grotere, placebo-gecontroleerde trials die daarop volgden, zijn alle voortijdig beëindigd in verband met gebrek aan effectiviteit.

In **hoofdstuk twee, drie en vier**, hebben we de resultaten besproken van preklinische studies die wij hebben uitgevoerd met *Fmr1* KO muizen. We hebben drie verschillende pathways, die zijn geïmpliceerd bij FXS, als aangrijpingspunt gebruikt: de excitatoire metabotrope glutamaat receptor 5 (mGluR5) pathway, de inhibitoire gamma-aminoboterzuur (GABA) pathway en de RhoGTPase pathway. Het doel van deze studies was om te onderzoeken of aangrijpen op deze pathways fenotypische kenmerken van de *Fmr1* KO muis kunnen verbeteren, als model voor deze aandoening in mensen. In **hoofdstuk twee en drie** hebben we gefocust op sociaal gedrag als uitkomstmaat, aangezien dit kenmerk leidt tot grote beperkingen bij patiënten, terwijl dit weinig is onderzocht. In **hoofdstuk vier** hebben we ook andere uitkomstmaten gebruikt,

waaronder cognitieve maten, andere gedragstesten en morfologie van dendritische spines.

In **hoofdstuk twee**, hebben we laten zien dat aangrijpen op de GABA pathway met bumetanide, tot een verbetering leidt van het sociale gedrag in *Fmr1* KO muizen. Bumetanide forceert de GABAerge switch van een immature functie naar een mature functie. Sociaal gedrag werd gemeten met het sociale dominantie paradigma, de automatische tube test, waarin *Fmr1* KO muizen een sterk en robuust fenotype vertonen. De verbetering van sociaal gedrag was consistent, alhoewel slechts een gedeeltelijke verbetering werd gemeten. Deze verbetering is vergelijkbaar met het effect dat eerder werd gemeten met mGluR5 reductie. Wij veronderstelden dat het combineren van bumetanidebehandeling met mGluR5-reductie mogelijk kon leiden tot een versterking van het effect. Echter, in tegenstelling tot onze verwachting, leidde dit tot een vermindering van het effect van de aparte interventies. Dit suggereert dat de pathways met elkaar verbonden zijn en tezamen een balans vormen in de synaptische excitatie-inhibitiefunctie, dat mogelijk een meer verfijnde afstemming vereist. Wij geloven dat het nodig is om op multiële pathways tegelijk aan te grijpen, in verschillende cellulaire compartimenten of zelfs verschillende celtypes, om te compenseren voor het gebrek aan FMRP. Met deze studie waren we de eerste die chronische combinatietherapie hebben onderzocht.

In **hoofdstuk drie**, beschreven we het effect van een ander middel dat op de GABA pathway aangrijpt, baclofen, op twee sociale gedragstesten in de *Fmr1* KO muis. De twee gebruikte gedragstesten zijn de automatische tube test en de drie-kamer sociability test. In beide testen konden we geen verbetering zien in *Fmr1* KO muizen na baclofen-behandeling. Bovendien leidde baclofen zelfs tot verslechtering van het gedragsfenotype in *Fmr1* KO muizen met de automatische tube test en induceerde baclofen een fenotype in wildtype muizen. Dit effect was onafhankelijk van hyperactiviteit, dat in de hogere dosis werd veroorzaakt door baclofen, maar niet in de lagere dosis. Aangezien baclofen over het algemeen juist sedatie veroorzaakt, waren onze resultaten onverwacht, mogelijk passend in een paradoxale effect dat in het verleden is beschreven voor baclofen. Of onze resultaten het gevolg zijn van de gebruikte dosis, de genetische achtergrond van onze muizen, de uitkomstmaten of een andere reden, is onbekend, maar ongeacht de oorzaak, rijst dit een twijfel over de toepasbaarheid van baclofen, of zijn R-enantiomer arbaclofen, in de kliniek voor FXS.

In **hoofdstuk vier** hebben we een wat minder bekend pathway getarget, de RhoGTPase, dat betrokken is bij het onderhoud van dendritische spines en de synaptische sterkte. We hebben gedemonstreerd dat aangrijpen op RhoGTPase met CNF1 leidt tot het verbeteren van de morfologie van hippocampale dendritische spines en tot een rescue van hippocampale en corticale cognitieve functies.

Daarentegen had CNF1 geen effect op angst en hyperactiviteit. Deze resultaten tonen dat aangrijpen op RhoGTPase slechts specifieke domeinen van FXS beïnvloedt, wat de

complexiteit van de functie van FMRP onderstreept.

Nadat we preklinische data hebben bediscussieerd, werden in **hoofdstuk vijf** de resultaten besproken van een klinische trial waar we aan mee hebben gedaan: een internationale fase 3, gerandomiseerde, placebo-gecontroleerde klinische trial met volwassenen en jongeren met FXS. In de klinische trial werd op de mGluR5 pathway aangegrepen met de mGluR5 antagonist mavoglurant. De uitkomstmaten waren vragenlijsten die door de ouder of clinicus werden ingevuld en cognitieve testen. Helaas werd er geen verbetering gemeten in de primaire en secundaire uitkomstmaten en werd de open-label extensiestudie vroegtijdig beëindigd door de farmaceut. Dit was een grote teleurstelling voor patiënten, ouders/verzorgers en clinici, die allen hoge verwachtingen hadden van deze klinische trial. Aangezien alle grotere klinische trials met FXS patiënten, met andere middelen, geen verbetering hebben getoond, wordt het duidelijk dat er nieuwe stappen noodzakelijk zijn in het FXS medicatie-onderzoek.

Deze resultaten hebben ons ertoe aangezet om te reflecteren over de mogelijke oorzaken voor deze negatieve resultaten. Dit bespreken we in de expert opinion in **hoofdstuk zes** “The quest for targeted treatment in fragile X syndrome”, en tevens in het **laatste hoofdstuk**, de algemene discussie. Blijkbaar is de vertaling van succesvolle preklinische studies minder eenvoudig dan aanvankelijk werd verwacht. Grote beperkingen kunnen worden benoemd, betreffende de geschiktheid van diersystemen voor medicatiestudies, de gebruikte uitkomstmaten in preklinische en klinische studies en de studiedesign die mogelijk ongeschikt is voor gerichte therapie in ontwikkelingsstoornissen.

Om patiënten en verzorgers te begeleiden in de overweldigende hoeveelheid aan preklinische en klinische data en om de beste medische zorg te garanderen, hebben we recent een poliklinisch expertisecentrum voor kinderen met FXS opgericht. Op korte termijn wordt deze uitgebreid naar volwassenen met FXS. In **hoofdstuk zeven**, hebben we de leidraad voor kinderen met FXS gepresenteerd, dat we momenteel aan het ontwikkelen zijn op basis van de recente literatuur en toegepast op het Nederlandse zorgstelsel.

In **hoofdstuk acht**, de algemene discussie, heb ik onze bevindingen uit bovenstaande hoofdstukken besproken in de context van de huidige literatuur. Ik heb uitgeweid over mogelijke oorzaken voor de translationele beperkingen van muis naar mens, en heb nieuwe onderzoeksmogelijkheden gesuggereerd. Het is van belang te beseffen dat de uitdagingen die wij ondervinden in het FXS onderzoeksveld, ook toepasbaar zijn op andere onderzoeksvelden die gerichte therapieën bestuderen voor ontwikkelingsstoornissen.

PhD Portfolio Summary of PhD training and teaching

Name PhD student: S. Zeidler	PhD period: October 2011 - 2017
Erasmus MC Department: Clinical Genetics	Promotor: R. Willemsen
Research School: MGC	Supervisor: R. Willemsen and R.K. Hukema

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- Biomedical English Writing and Communication	2016	3 ECTS
- Laboratory animal science	2012	3 ECTS
- Statistics	2012	5.7 ECTS
- BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2012	1 ECTS
- Laboratory safety	2011	0.28 ECTS
- CPO minicourse	2012	0.28 ECTS
- Confocal course	2012	0.28 ECTS
- Research integrity	2012	2 ECTS
- Literature course	2013	2 ECTS
- Photoshop and Illustrator (molmed)	2013	0.3 ECTS
- BROK herregistratie	2016	0.28 ECTS
Specific courses (e.g. Research school, Medical Training)		
- PhD-course Biophysics and Biochemistry	2011	3 ECTS
- PhD-course Developmental cell biology	2012	3 ECTS
- PhD-course Genetics	2012	3 ECTS
- MGC Epigenetic Regulation in Health and Disease	2016	0.8 ECTS
- Research management for PhD students	2015	1 ECTS
- AMIE OIC symposium 2015	2015	0.28 ECTS
- PhD day	2015	0.28 ECTS
Work discussions of department		
- Clinical genetics work discussion	2011-2016	
- Fragile X group work discussion	2011-2016	
Seminars and workshops		
- MGC PhD-workshop 2012	2012	1 ECTS
- MGC PhD-workshop 2013 (poster)	2013	1 ECTS
- MGC PhD-workshop 2014	2014	1 ECTS
- MGC PhD-workshop 2015 (talk)	2015	1 ECTS
Presentations		
- Patient organization meeting	2013	0.5 ECTS
- Children psychiatry colloquium	2014	0.5 ECTS
- Patient organization meeting	2014	0.5 ECTS
- 17th International Fragile X and Other Early-Onset Cognitive Disorders Workshop, Strasbourg oral presentation	2015	0.5 ECTS
	2011-2017	4 ECTS
- Group work discussions	2011-2017	2 ECTS

- Department of clinical genetics research meetings	2014-2017	1 ECTS
- Journal club clinical genetics	2015	1 ECTS
- Sophia research days 2015 (oral presentation)	2015	0.5 ECTS
- FXTAS day 2015 (presentation)	2016	0.5 ECTS
- Sophia research days 2016 (slam presentation)	2017	0.5 ECTS
Sophia research days 2017 (slam presentation)	2012	1 ECTS
(Inter)national conferences		
- Fragile X and autism related disorders, GRC-meeting	2012	1 ECTS
June 2012, Boston (poster presentation)	2012	
- MGC-symposium 2012 Leiden	2013	
- NVHG symposium 2012 Arnhem	2013	1 ECTS
- FRAXA meeting 2013 (poster presentation)	2013	
- Van Gelderen symposium 2013	2014	
- MGC-symposium 2013	2014	1 ECTS
- NVHG symposium 2014 Arnhem (poster presentation)	2014	
- Opening symposium fragile X expert center	2015	1 ECTS
- MGC symposium 2014		1 ECTS
- 17th International Fragile X and Other Early-Onset Cognitive Disorders Workshop, Strasbourg (oral presentation)	2015	
	2015	
- NVHG symposium 2015 (poster presentation)	2017	
- MGC-symposium 2015		
- European society of human genetics 2017 (poster presentation)		
Other		
- Set up of Fragile X expertise centre, collaboration child psychiatry	2014	
	2014	
- Organizing MGC PhD workshop 2014	2013 – 2014	
- Activity committee 2013-2014, lecture series biomedical sciences	2017	
- Leidraad voor zorg van kinderen met het fragile X syndroom		
2. Teaching		
	Year	Workload (Hours/ECTS)
Lecturing		
- AVG onderwijs lunchreferaat presentation Fragile X syndrome	2012	
	2012	
- Minor clinical genetics	2013	
- AVG onderwijs lunchreferaat presentation X-linked MR	2013	
- Minor clinical genetics	2014	
- Minor clinical genetics	2015	
- Minor clinical genetics		
Supervising practicals and excursions, Tutoring		
- Medical students work-groups 3 days	2011	
- Medical students work-groups 3 days	2012	1 ECTS
- Junior Meds 1 afternoon	2013	
- Medical students work-groups 3 days	2013	
- Medical students work-groups 3 days	2015	
Supervising Master's theses		
- International master student 6 months internship	2013	1 ECTS
- Master student molecular medicine 12 months internship	2015-2016	2 ECTS

About the author

Shimriet Zeidler was born on March 7th, 1983 in Eilat, Israel. At the age of 9 years she moved to the Netherlands. She graduated cum laude from high school (Merletcollege in Cuijk) in 2001. She studied medicine in UMC Utrecht from 2002-2008. During that time she did an ambulance assistant course in Israel and volunteered there. Also, she did an internship in the Hawke's Bay hospital in Hastings, New-Zealand. Her research internship was in the department of microbiology, UMC Utrecht, where she investigated microbiological transmission by genotyping micro-organisms. After obtaining her MD degree, she started as junior doctor (ANIOS) in the neurology department in UMC Utrecht. From there she moved into the genetics field. First she started as a junior doctor (ANIOS) in the counseling section of the clinical genetics department in the Erasmus Medical Center in Rotterdam, in 2010. In 2011, she started her PhD in the lab of prof. dr. Rob Willemsen, at the same department. In 2013, she also started her residency in clinical genetics. In 2017, she will defend her thesis titled "Fragile X syndrome; The quest for targeted therapy", under supervision of prof. dr. Rob Willemsen and dr. Renate Hukema. The goals of her PhD research were to identify and test targets for treatment for fragile X syndrome and test these in mice and humans. Together with the pediatric psychiatry department, she initiated a multidisciplinary expert center for children with fragile X syndrome. The expertise built in this center was used to create a guideline for diagnostics and care of children with fragile X syndrome. Besides, she presented her work in several national and international conferences. Shimriet is married to Kjell Weewer and they have a daughter Sarah (born 2016).

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Zeidler S, Pop AS, Jaafar IA, de Boer H, Buijsen RAM, de Esch CEF, Nieuwenhuizen-Bakker I, Hukema RK, Willemsen R. Paradoxical effect of baclofen on social behavior in the FXS mouse model. *Under review*

Santos M, **Zeidler S**, Masini S, Bergado-Acosta J, Abekhouk S, de Esch CEF, Diana G, Bardoni B, Willemsen R, Dierssen M, Stork O. Targeting the RhoGTPase pathway rescues synapse maturation and memory in a mouse model of fragile X syndrome. *Under review*

Verkerk AJMH, **Zeidler S**, Breedveld G, Overbeek L, Huigh D, Koster L, van der Linde H, de Esch CEF, Severijnen LA, de Vries BBA, Swagemakers SMA, Willemsen R, Hoozeboom AJM, van der Spek PJ, Oostra BA. CXorf56, a dendritic neuronal protein, identified as a new gene for X-linked intellectual disability. *Under review*

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