Therapeutic Targets and Translational Endpoints in Fragile X Syndrome

Celine Ellen François de Esch

ISBN: 978-94-6182-448-6

Cover design and layout: Celine de Esch

Thesis layout and printing: Off Page, Amsterdam

Publication of this thesis was financially supported by:

Erasmus University Rotterdam, Department of Clinical Genetics, Erasmus MC

J.E. Jurriaanse Stichting

Benedictus, the Netherlands

Novartis Pharma B.V.

Therapeutic Targets and Translational Endpoints in Fragile X Syndrome

Therapeutische aangrijpingspunten en translationele eindpunten voor het fragiele X syndroom

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

woensdag 25 juni 2014 om 13.30 uur

door

Celine Ellen François de Esch

geboren te Heerlen

Promotiecommissie

Promotor:	Prof.dr. R. Willemsen
Overige leden:	Prof.dr. F. Kooy Prof.dr. S.A. Kushner Prof.dr. R.J. Pasterkamp

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABC	Aberrant Behavior Checklist
ADHD	Attention Deficit Hyperactivity Disorder
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
ANOVA	Analysis of variance
Arc	Activity-regulated cytoskeleton-associated protein
APP	Amyloid precursor protein
ASD	Autism Spectrum Disorders
CA1	Cornu Ammonis 1 region
CaMKII	Calcium/calmodulin-dependent protein kinase II
CGI (-S/-I)	Clinical global impression (-severity/-initiation)
ChIP	Chromatin immunoprecipitation
cKO	Conditional knockout
cON	Conditional ON
Cre	Cre recombinase
CTEP	2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4 yl)ethynyl)pyridine
DHPG	(S)-3,5-Dihydroxyphenylglycine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EEG	Electro-encephalo gram
EGTA	Ethylene glycol tetraacetic acid
Emx	mouse homologue of the *Drosophila* homeobox gene empty spiracles
ERK	Extracellular regulated kinase
FM	Full mutation (>200 CGG repeats)
FMR1	Fragile X mental retardation gene 1
FMRP	Fragile X mental retardation protein
FRAXA	Fragile X Association
FXPOI	Fragile X-associated premature ovarian insufficiency
FXS	Fragile X syndrome
FXTAS	Fragile X-associated tremor/ataxia syndrome
g	Grams
GABA	γ (gamma)-aminobutyric acid
Gad	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gp	Group
GSK-3	Glycogen synthase kinase 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ID	Intellectual disability
iPSC(s)	Induced pluripotent stem cell(s)
KO	Knockout
LC-MS	Liquid chromatography and mass spectrometry

LTP/LTD	Long-term potentiation/ Long-term depression
mACh	Muscarinic Acetylcholine receptor
mGluR	Metabotropic glutamate receptor
MMP	Matrix metalloproteinase
MPEP	2-Methyl-6-(phenylethynyl)pyridine
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NES	Nuclear export signal
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate
NOS	Nitric Oxide Synthase
OCD	Obsessive-compulsive disorder
PAK	P21-activated protein kinase
PBS	Phosphate buffered saline
PCR	Polymarase chain reaction
PET	Positron emission tomography
PI3K	Phosphoinositide 3-kinase
PPI	Prepulse inhibition
PSD	Postsynaptic density
PV	Parvalbumin
SAPAP	Synapse-associated protein 90/postsynaptic density-95-associated protein
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
S	Seconds
SEM	Standard error of the mean
SN(s)	Synaptoneurosome(s)
SPSN	Sociability and preference for social novelty
SST	Somatostatin
STEP	Striatal-enriched tyrosine phosphatase
STR	Stranger
uFM	Unmethylated full mutation
UTR	Untranslated region
VABS	Vineland adaptive behavior scale
VAS	Visual Analog Scale
WAIS	Wechsler Adult Intelligence Scale
WT	Wild type

CHAPTER 1

TRANSLATIONAL ENDPOINTS IN FRAGILE X SYNDROME

Celine EF de Esch, Shimriet Zeidler and Rob Willemsen

Neuroscience & Biobehavioral Reviews, 2014 (adapted version).

DISCOVERY OF FRAGILE X SYNDROME

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 fragile X 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 fragile X syndrome is focused on therapeutic interventions that target the underlying molecular mechanisms of these synaptic defects.

CLINICAL, BEHAVIORAL AND NEUROANATOMICAL PHENOTYPE OF FRAGILE X SYNDROME

Diagnosis of fragile X syndrome is usually made around the age of three years, when children present with developmental or behavioral problems [11, 12]. 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 visuo-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 macroorchidsm. Medical issues may be recurrent otitis media in childhood and gastro-oesophageal reflux in infants [12-15]. The physical symptoms in females are usually milder than in males [14].

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, 16], 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, 17]. 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 eyeblink conditioning [18, 19]. They also show deficits in prepulse inhibition of startle response (PPI) [20, 21]. Neurophysiological and neuroanatomical features include EEG anomalies and epileptic seizures with an incidence of 13–18% in male children with FXS. Seizures often resolve during childhood [12].

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 toward a defect in development and maturation of the dendritic spines [7, 8, 22, 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].

ROLE OF FMRP IN 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*) [33-37], zebrafish (*Danio rerio*) [38-42], mouse (*Mus musculus*) [43, 44] 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.

Normally, FMRP is highly expressed in brain and testes and has four major isoforms between 70 and 80 kDa. The protein can bind to RNA trough one of its three RNA-binding domains [45, 46]. The protein also contains a nuclear localization signal (NLS) and a nuclear export signal (NES) which facilitate shuttling of the protein between nucleus and cytoplasm

[47]. However, in neurons the majority of FMRP is present in the in the cell body as well as in dendrites and their specialized protrusions named spines [48]. FMRP has been shown to associate with specific dendritic mRNAs as well as with polyribosomes responsible for protein translation [49, 50]. FMRP blocks the initiation of ribosome assembly and stalls the elongation of actively transcribing ribosomes; additionally it targets mRNAs for degradation via the RISC complex. This evidence points at a negative regulator role for FMRP in mRNA translation [49, 51-53]. Consequently, the *Fmr1* KO mouse shows increased protein synthesis in total brain homogenates and isolated synaptoneurosomes, which contain presynaptic and postsynaptic components [54-56]. 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 were synapses are formed and retracted in response to external stimuli, a process believed to underlie learning and memory [57]. 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 degradation [58]. 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 [59, 60]. It was shown that *Fmr1* KO mice display an exaggerated hippocampal mGluR-LTD, and an increased level of AMPAR internalization [61, 62]. Based on these observations, the mGluR theory was proposed to explain many features of fragile X syndrome. According to this theory, Gp 1 mGluR stimulation results in local mRNA translation at the spine. FMRP acts as a major brake of this pathway, thus lack of FMRP results in a higher protein synthesis rate, and consequently AMPA receptors are internalized at a higher rate. This results in a more immature dendritic spine phenotype as observed in fragile X syndrome [63]. These findings started the search for Gp 1 MGluR modulating drugs as potential therapies for fragile X syndrome.

However, additional downstream signaling pathways connect the mGluR signaling to the FMRP-regulated mRNA translation. Most studied in FXS are the extracellular signal-regulated kinase (ERK) pathway and the mammalian target of rapamycin (mTOR) pathways, both of which have been shown to be deregulated in FXS [56, 64-70]. ERK is abundantly expressed in the central nervous system and is activated by phosphorylation via multiple extracellular ligands including neurotransmitters and growth factors. ERK is proven to be involved in both long-term potentiation and long-term depression [71]. mTOR affects cell growth, proliferation, and autophagy in all tissues and is a necessary signaling component of protein-synthesis-dependent synaptic plasticity and memory [57, 72]. By now, additional therapies have been developed based on altered ERK or mTOR signaling.

SYNAPTIC TARGETS AND THERAPEUTIC TREATMENT STRATEGIES IN FXS

Initially, antagonist of the Gp 1 mGluR signaling, like MPEP, Fenobam, AFQ056/Mavoglurant and CTEP were tested first. Later, new therapeutic strategies were based on target proteins of FMRP, including phosphoinositide 3-kinase (PI3K) and matrix metalloproteinase 9 (MMP-9) and key signaling proteins such as endocannabinoid 2-arachidonoyl-sn-glycerol, mTOR or ERK [69, 70, 73-76]. In addition to the NMDA and AMPA receptors, the neuronal Kv4.2 potassium channels and the M1 muscarinic acetylcholineric receptors are also known to be involved in FXS. Last but not least, the inhibitory γ-aminobutyric acid (GABA) system with its two classes of receptors ($GABA_A$ and $GABA_B$) has shown to be deregulated in fragile X syndrome as well. A schematic overview of therapeutic interventions currently in FXS research is presented in Figure 1.

The compounds that act on the glutamate receptors (NMDA, AMPA, mGluR5), GABA and muscarinic receptors (M1-5) are shown. Some compounds listed act downstream of the glutamate receptors, namely on one of the cascade proteins that influence the FMRP activity.

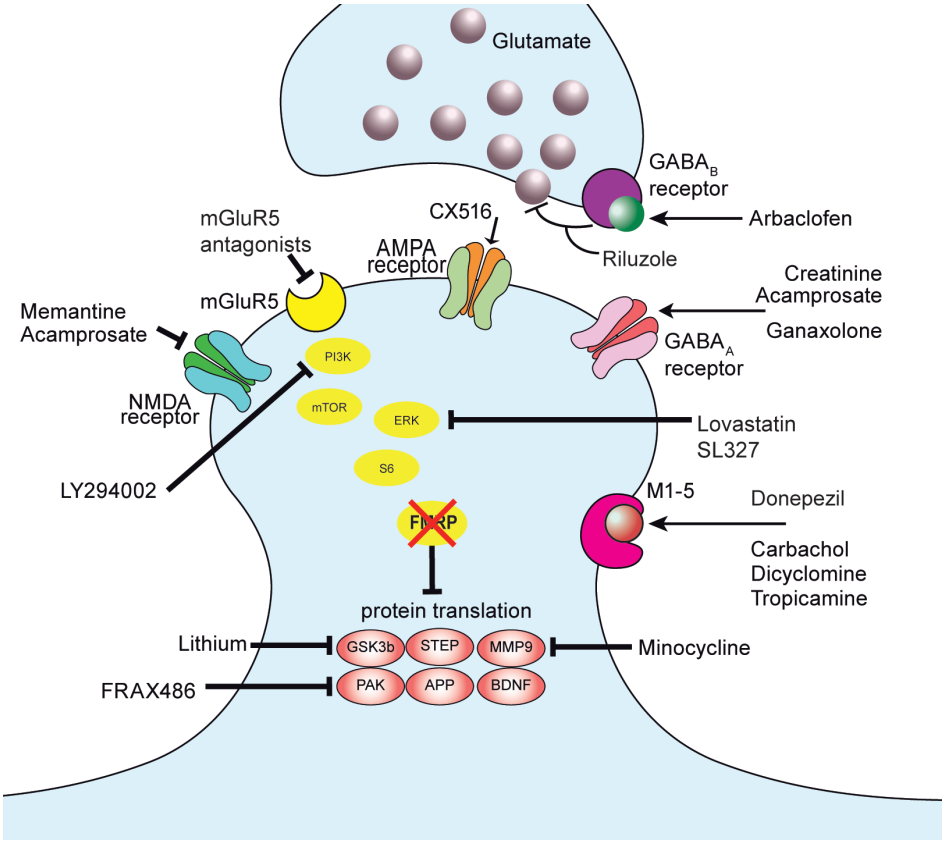


Fig. 1. Synaptic targets of the therapeutic interventions in fragile X syndrome.

In absence of FMRP a subset of mRNAs is up-regulated at the synapse, for example GSK-3, STEP, MMP-9 and amyloid precursor protein APP, these can also serve as a therapeutic target. The NMDA and AMPA receptor equilibrium is deregulated in fragile X syndrome. Therapies aimed at lowering NMDA receptor signaling are memantine or acamprosate 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, a catalytic subunit of the key signaling molecule PI3K, can be lowered by exposure to a PI3K antagonist [74]. Deregulated ERK expression can be altered by an MEK1/2 inhibitor like SL327 or more indirectly by lovastatin [56, 77]. The increased GSK-3 activity found in different brain areas of Fmr1 KO mice can be lowered by lithium [78]. Increased p21-activated kinases (PAK) levels can be down regulated by PAK inhibitors such as FRAX486 [79]. 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 minocycline [73]. The altered inhibitory $GABA_A$ signaling in Fmr1 KO mice and can be targeted by the neuroactive steroids alphaxalone and ganaxalone. Acamprosate 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) [80, 81]. $GABA_B$ agonists used in fragile X syndrome are baclofen and its active enantiomer arbaclofen (or R-baclofen).

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 recently a mouse model for the I304N mutation, *Fmr1* I304N [82]. All are available in different backgrounds [43, 44, 83-85]. To date, several translational endpoints have been described for *Fmr1* KO mice, including an altered dendritic spine phenotype (Fig. 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 (see [86] 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 [54-56, 74]. Increased ERK phosphorylation has been observed in the cortex of *Fmr1* KO mice [76, 87]. mTOR phosphorylation and activity are up regulated in the hippocampus of *Fmr1* KO mice as well as the levels of 110β, a catalytic subunit of PIKE, and the PI3K enhancer PIKE-L [70, 74]. In addition, basal levels of striatal-enriched protein tyrosine phosphatase (STEP), a protein believed to play a role in synaptic plasticity, is elevated in *Fmr1* KO mice [88]. 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 [18, 61, 62]. However, not all *in vivo* studies on postsynaptic receptor levels show similar results [89, 90].

Some studies reported unaltered levels or an increase in the postsynaptic scaffolding proteins SAPAP3 and PSD-95 in *Fmr1* KO mice [91-93]. 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 mice [94]. Although these postsynaptic measures would provide a good

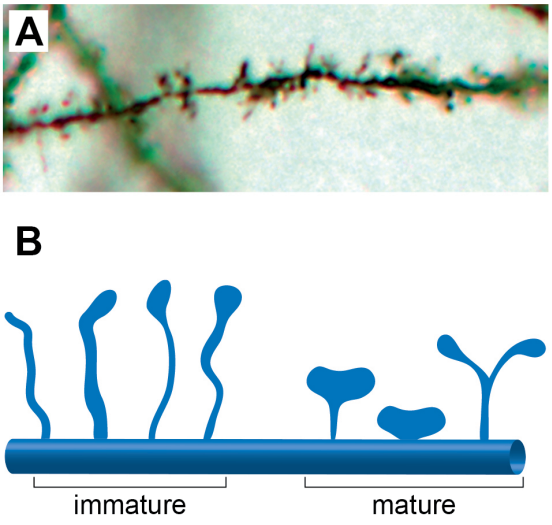


Fig. 2. Morphology of dendritic spines. Photomicrograph (A) shows a detail of the dendrite from a murine *Fmr1* KO hippocampal neuron stained with Golgi impregnation staining method. The Golgi technique selectively impregnates single neurons with silver chromate and allows visualization of dendrites and axons. Photomicrograph (B) shows graphical representation of dendritic spine morphologies defined as mature or immature.

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.

In addition to the mGluR signaling pathway, the inhibitory γ-aminobutyric acid (GABA) system has shown to be involved in the pathogenesis of fragile X syndrome 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 transport [95-98]. Electrophysiological experiments also suggested a decrease in efficiency of the GABAergic transmission in *Fmr1* KO mice [99, 100]. 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 found [101]. 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 phenotype [102]. Among the most robust behavioral phenotypes are increased locomotor activity and increased exploratory behavior consistent with observations in human fragile X patients [83, 87, 103] *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 seizures [104-106]. Autistic-like behavior such as repetitive behavior and aberrant social behavior has been shown in *Fmr1* KO mice as well [84, 85, 107, 108]. Subtle deficits have been detected in anxiety tests and learning tasks [87, 109]. However, thus far there are only few robust behavioral assays available for drug screening, *eg* susceptibility to audiogenic seizures.

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. A number of Gp1 mGluR antagonists, mostly directed against the mGluR5 receptor, are available and have been tested for their effects on spine plasticity. In *Fmr1* KO mice, 2-Methyl-6-(phenylethynyl) pyridine (MPEP) administration has shown to offset the abnormal PPI, increased anxiety, increased susceptibility to audiogenic seizures and repetitive behaviors as well as restoring the cortical spine morphology [105, 110-113]. Due to MPEPs low specificity it was later interchanged by a more specific and safer antagonist fenobam [114, 115]. Fenobam treatment successfully restored the abnormal PPI and the impaired locomotor learning [116, 117]. AFQ056/Mavoglurant was also able to offset the PPI as well as the aberrant social behavior and spine morphology in *Fmr1* KO mice [107, 118, 119]. Recently, a new mGluR5 antagonist 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine) (CTEP), became available [120]. Treatment with CTEP offsets learning and memory deficits, hyperactivity and increased locomotor activity [87]. Chronic treatment of young adult *Fmr1* KO mice with CTEP resulted in a normal dendritic spine density, but also in a normalized protein synthesis level and LTD [87]. One compound targeting the mGluR1 receptor has also been tested on the behavior of *Fmr1* KO mice, namely JNJ16259685 [(3,4-dihydro-2H-pyrano-[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone]. This compound only decreased the number of repetitive behaviors and failed to rescue any other abnormal behaviors of *Fmr1* KO mice [111]. Treatment with the GSK-3 inhibitor lithium, resulted in a normal dendritic spine phenotype and a normal LTD response in *Fmr1* KO mice [78, 121-123]. Administration of the MMP-9 inhibitor minocycline promoted spine maturation *in vivo* [73]. 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 were also reversed by lithium and minocycline which suggests broader effects than the inhibition of GSK-3 or MMP-9 alone [78, 121-124]. Treatment of *Fmr1* KO mice with the MEK1/2 inhibitor SL327 resulted in a reduced audiogenic seizure susceptibility [76]. 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 improved [125-127]. $GABA_A$ 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, respectively [128]. $GABA_B$ agonists used in fragile X syndrome are racemic baclofen and its active enantiomer arbaclofen, a novel R-baclofen prodrug. 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 [129-131]. Recently, 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 [79, 132].

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 [73, 105, 133, 134]. 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 [62, 91]. The expression of p110β is elevated in *Fmr1* KO hippocampal neurons [74]. In addition, cultured hippocampal *Fmr1* KO neurons were shown to have a reduced stimulus-induced localization of $GABA_A$ receptor δ subunit mRNA [135].

THERAPEUTIC INTERVENTION STUDIES IN PRIMARY Fmr1 KO NEURONS

Treatment with MPEP or fenobam resulted in a normalized spine phenotype of cultured hippocampal *Fmr1* KO neurons [105, 113]. The AFQ056/Mavoglurant has also been proven to have beneficial effects on spine morphology after treatment of cultured *Fmr1* KO hippocampal cells mice [107, 118, 119]. Another approach to normalize the spine phenotype found in fragile X mice is to use an NMDAR antagonist, like memantine [136]. Memantine treatment stimulated synapse formation and promoted dendritic spine maturation in cultured cerebellar granule cells of *Fmr1* KO mice [137]. After treatment with lithium, *Fmr1* KO mice showed a rescue of the spine phenotype [78, 121-123]. Administration of the MMP-9 inhibitor minocycline also promoted spine maturation *in vitro* [73]. 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 [62, 74].

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 D. melanogaster (fruitfly) and Danio rerio (zebrafish). For example, homozygous *Fmr1* null fruit flies show many phenotypes associated with fragile X syndrome [138, 139]. 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 phenotypes [140]. 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 age [38]. However, adult *Fmr1* KO fish seem to show behavioral and synaptic abnormalities [39]. 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.

HUMAN FXS STEM CELLS

The *Fmr1* KO mouse model provides the first step in testing therapeutic interventions, however a human cell model would facilitate the translation of the animal phenotypes and the rescue thereof, to human patients. One option for a human cell model is the use of embryonic stem cells derived from pre-implantation genetic diagnostics. Studies on human fragile X embryonic stem cells showed that FMRP is expressed during early embryonic development, but that epigenetic silencing of *FMR1* occurs upon differentiation [5, 141, 142]. Only very recently it was discovered that the silencing of the *FMR1* gene in human embryonic stem cells is caused by the *FMR1* mRNA which forms an RNA-DNA duplex during early development, however the mechanisms which maintain the silencing in time are still unclear [143]. Nonetheless, these human cells are only very limited available not in the last part because of ethical reasons which makes them not suitable as a cell model. In 2007, it became possible to model fragile X syndrome, as well as other genetic neurodevelopmental disorders such as Rett syndrome, *in vitro* by creating patient-specific induced pluripotent stem cells (iPSCs) [144]. Surprisingly, 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 states [145-147]. Later, it was shown that it is possible to reactivate the *FMR1* gene by the use of a different reprogramming cocktail [148]. These findings lead to the conclusion that standard reprogramming using the four transcription factors is not sufficient to create naive pluripotent FXS stem cells. Nevertheless, by differentiating FXS iPS cells into functioning mature neurons a relevant human cell model became available [149, 150]. 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.

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 the clinic. In the past years, extensive work has been done to develop targeted treatment strategies for fragile X syndrome, 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.

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 (*eg* 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 [151]. It assesses behavior on five 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 [152]. This modified ABC, named ABC-FX, has now been applied in clinical trials [153]. 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) [154]. 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 Analog Scale (VAS) consists of a continuous line between two points that 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 [155, 156]. 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 was used to measure different FXS symptoms (see Table 1).

Table 1. Most used outcome measures in human clinical trials

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.	[18, 19]
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	[20, 21]
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	[151]
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	[175]
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	[175]
Vineland Adaptive Behavior Scale (VABS)	Caregiver or teacher input. Categories: Communication, Daily living skills, Socialization, Motor skills, Maladaptive behaviour.	Asses daily functioning and adaptive behavior	[154]
Visual Analogue Scale (VAS)	Caregiver rated continuous visual scale on which agreement or severity is pointed.	Severity of target behavior or cognition	[176]
Social responsive scale (SRS)	Caregiver rated, 65 items score list on severity of autism spectrum symptoms.	Level of impairment in reciprocal social behavior and autistic spectrum symptoms	[177]
ADHD Rating Scale (ADHD-RS)	Clinician rated.	Score ADHD symptoms Often used in clinical trials	[178]
Peabody Picture Vocabulary Test (PPVT)	Measurement of single word receptive vocabulary.	Communication and language, receptive vocabulary and verbal ability	[179, 180]
Conners Parent Rating Scale (CPRS) Conners Teacher Rating Scale(CTRS)	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	[181]
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	[182]

Table 1. Most used outcome measures in human clinical trials (*continued*)

Test	Characteristics	Measured function	Reference
Repetitive Behavior Scale (RBS)	Caregiver rated score list, 44 items, 6 domains: Stereotypic, Self-injurious, Compulsive, Ritualistic, Sameness behavior, Restricted interests	Measures repetitive behavior, a key feature of autism	[183]
Childhood Autism Rating Scale (CARS)	Clinician, teacher or caregiver rated, based on observation information of persons autism symptoms	Recognition of autism and differentiation from developmental disorders	[184]
Autism Diagnostic Observation Scale (ADOS)	Observation on social interaction, communication and play during administration of structured modules	Diagnosing, assessing and classifying autism	[185]
Children's Yale-Brown Obsessive Compulsive Scale modified for PDD (CY-BOCS-PDD)	Clinician rated, 10 item score list	Measure repetitive an compulsive behavior	[186]
Kiddie Schedule for Affective Disorder and Schizophrenia (K-SADSL PL)	Clinician scored based on interviewing caregivers and caregiver scores by parent, child and teacher.	Child specific diagnostic criteria for psychopathology	[187]
Clinical Evaluation of Language Fundamentals (CELF)	Child specific clinical test, administered by clinician, consisting of several subtests.	Communication and language skills	[188]
Expressive Vocabulary Test (EVT)	Clinical test with 1-word synonym response to visual stimuli	Assess expressive vocabulary and development	[189]
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	[190]

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 [20] in males with FXS and later by [21]. 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) [157]. 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 control [158]. The test comprises of a weak auditory stimulus that precedes a loud startling noise, which leads to a reduced startle response by the test subject. Frankland *et al*. (2004) was the first to test this in young males with FXS (10 patients and 7 controls). 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. Hessl *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 females [21]. 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 patients [159]. A second endophenotypic test in FXS is the eyeblink test [18]. 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 pathway [19]. FXS patients showed delay in eyeblink conditioning compared with controls [18]. Also long-term measurements show an aberrant response in FXS patients. Objective measures, like the PPI and eyeblink 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 execute [13].

BIOCHEMICAL BIOMARKERS IN PATIENTS WITH FXS

Preclinical research on tissue or cells of fragile X 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 [160, 161]. A recent paper quantified MMP-9 activity in peripheral blood of patients with FXS and showed a higher activity as compared with controls [161]. One trial used brain derived neurotrophic factor (BDNF), a biomarker shown to be decreased in autistic individuals and *Fmr1* KO mice [162]. 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) [163]. A significant difference in mGluR5 protein levels has also been reported in superior frontal cortex from post-mortem material of autistic children [164]. 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 synthesis [69]. Increased protein synthesis and increased phosphoinositide 3-kinase (PI3K) activity was also demonstrated in lymphoblastoid cells from FXS patients [165]. 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 signaling [166]. In contrast, an increased phosphorylation, and hence activation, of MEK1/2 and ERK1/2 was measured in cortex of post mortem brains from 4 patients with FXS (age range 10–86 years) as compared

with 7 age matched controls [77]. 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 [160, 167]. 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) method [168]. This PET method has been shown to be a reliable and reproducible method for measuring protein synthesis in human brain [169, 170]. This reduction is presumably due to the sedation effect, as was later demonstrated by comparing with sedated and not sedated *Fmr1* KO mice [168]. 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 controls [171].

CLINICAL TRIALS IN FRAGILE X SYNDROME

In FXS, the behavioral problems and intellectual disability cause a marked decrease in the patients and his 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 and Table 3 shows ongoing trials, mentioned on www.clinicaltrials.gov. Only one study has attempted to influence the *FMR1* expression, by using valproic acid [172]. 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 [172]. 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 [173]. This drug has previously shown to reduce autistic features in non-FXS children with autism spectrum disorder [155]. 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 explored in FXS patients yet, like the endocannabinoid pathway and statins. These could also 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

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

Study	Drug, dosage, treatment regimen	Mechanism	Study Design: Phase, design	Participants: N sex average age in years (min-max)
[191]	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, PL	38 male 11 female 27.9 (18-49)
[160]	Lithium 0.8-1.2 mEq/L TI 1-4 wk TR total 2 mnth	Reduces mGluR5 – dependent protein synthesis in dendrite	OL	15 male 11 (6-23)
[159]	Fenobam 50/100/150 mg Single dose	mGluR5 negative modulator	OL	6 male 6 female 23.9 (18.7-30.7)
[192]	Memantine TI: 5 mg/d TR: 15- 20 mg/d Mean 34.7 wks (range 8-104)	Uncompetitive NMDA receptor antagonist	OL	6 male 18.3 (13-22)
[171]	Donepezil 3 wk 5 mg/d 3 wk 10 mg/d	Acetyl cholinesterase inhibitor	OL	6 male 2 female 18.8 (14-44)
(Torrioli, et al., 2010)	Valproic acid TI 10-30 mg/kg, 1 mnth TR 30 mg/kg/d total 6 mnth	histone deacetylase inhibition (possible DNA demethylation of FMR1)	OL	10 male 10.6 (7-16)
[156]	Minocycline 100/200 mg/d TI 2 wk TR total 8 wk	MMP-9 inhibitor	OL	18 male 2 female 18 (13-32)
[193]	Minocycline Various 25-200 mg/d >2 wks Mean 3.5 mnth (range 2-20 wk)	MMP-9 inhibitor	Ret	43 male 7 female 13.3 (0.3-25)

Outcome measures	Main results	Side effects
VABS ADOS SNAP-IV ABC-C VAS CGI-S/I Cognitive test battery (TVPS, WJR, RBANS, PLS-4, GARS)	No significant difference	Minimal 12.5% allergic rash, 1 discontinuation
ABC-C CGI VAS VABS Cognitive test battery (ia PPVT-III, RBANS) ERK-activation	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
PPI	Significant improvement PPI Reported: calmed behavior, better eye contact	Mild sedation
CGI-S/I ABC SRS ADHD-RS (language and social behavior)	No significant improvement, 4 of 6 improved on CGI-I	Increased irritability 2 discontinued
ABC Test battery (CNT, HVLT, CBCL/ ABCL)	Significant improvement in ABC (hyperactivity, irritability), CNT, CBCL/ ABCL (attention)	Mild
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.
ABC-C VAS CGI	Significant improvement ABC-C irritability, stereotypy, hyperactivity, inappropriate speech, VAS, CGI	Minor diarrhea, seroconversion to positive ANA
Questionnaire on Likert scale (language, academic abilities, attention, behavior, physical features, side effects)	Improvement in language, attention, social communication, anxiety.	21 pt side effects, ia gastro-intestinal, discoloring nails, 4 discontinued because behavior worsening, 2 other side effects

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

Study	Drug, dosage, treatment regimen	Mechanism	Study Design: Phase, design	Participants: N sex average age in years (min-max)
[174]	AFQ056/ Mavoglurant TI 100-300 mg/d up in 1 wk, down in 1 wk TR total 4 wk WO >1 wk	Selective mGluR5 negative allosteric modulator	IIb DB, P, R, CO	30 male 25.5 (18-36)
[167]	Riluzole TI 50 mg/d, 1 wk TR 100 mg/d Total 6 wk	Inhibits glutamate release, enhances glutamate reuptake, potentiates GABA neurotrans-mission	OL	6 male 22.5 (19-24)
[153]	STX209/ arbaclofen TI 2-20 mg/d TR <12 y: 20 mg/d >12 y: 30 mg/d Total 4 wk WO 1 wk	GABAB agonist Decrease glutamate release, decrease mGluR5 activity	II DB, P, R, CO	55 male 8 female 6-11: n=24 12-17: n=22 18-40: n=17
[194]	Donepezil TI 2.5 mg/d, 4 wk TR 5 mg/d Total 12 wk	Acetyl cholinesterase inhibitor	DB, P, R	20 male 9.9 (donepezil) 8.3 (placebo) (6-15)
[155]	Minocycline 25 mg/d for weight<25 kg 50 mg/d for weight 25-50 kg 100 mg/d for weight >50 kg 3 mnth WO	MMP-9 inhibitor	DB, P, R, CO	47 male 8 female 9.01 (minocycline) 9.40 (placebo) (3.5-16)
[162]	Acamprosate TI 7 wk 1332-1998mg/d TR total 10 wk	NMDA antagonist GABAAR agonist (possibly mGluR5 antagonist)	OL	10 male 2 female 11.9 (6-17)
[161]	Minocycline 3 mnth	MMP-9 inhibitor	DB, P, R, CO	10 male

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

Outcome measures	Main results	Side effects
ABC-C CGI-S/I, efficacy index VABS RBS-R SRS VAS PPVT KITAP	Overall significant improvement in RBS-R (stereotypy, restricted interests) *Subgroup* (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
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
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	1 discontinuation for irritability
SBIQ CPRS CARS	No significant improvement	Mild
CGI-I VAS ABC-C-FX VABS EVT-2	Significant but modest improvement on CGI-I, VAS (anxiety/ mood-related behaviors)	Mild, most not significant between treatment and placebo. Most gastro-intestinal. 5 pt tooth discoloration
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	Mild, most irritability and increased repetitive behavior
MMP-9 levels CGI-I	Reduction of MMP-9 levels to normal CGI-I improvement in 9 of 10 patients	

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

Drug	Mechanism	Design	Participants: N, sex Age (years)	Outcome measures
AFQ056/ Mavoglurant	Selective mGluR5 negative allosteric modulator	IIb R, DB, P	175, both sexes 18-45	ABC-C CGI-I
AFQ056/ Mavoglurant	Selective mGluR5 negative allosteric modulator	IIb R, DB, P	160, both sexes 12-17	ABC-C CGI-I
Ganaxolone	GABAAR positive modulator	II, R, DB, P, CO	60, both sexes 6-17	ABC CGI-I PARS VAS ADAMS SNAP-IV KiTAP PPI Social Gaze (eye tracking) ERP
RO4917523	Selective mGluR5 antagonist	II R, DB, P	45, both sexes (5-13 and 16-50)	ABC CGI-S and I ADAMS RBANS VAS
Donepezil	Acetylcholinesterase inhibitor	II R, DB, P	50, both sexes 12-29	Behavior assessments and working memory tests
NPL-2009	mGluR5 antagonist	ISD, OL	12 both sexes (18-45)	PPI Not published yet while finished in 2008
STX209 arbaclofen	GABAB agonist Decrease glutamate release, decrease mGluR5 activity	III R, DB, P	200 5-11	ABC-C (Lethargy Social Withdrawal subscale) suspended for autism and FXS

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 measures: ABC-C: Aberrant Behavior Checklist – Community edition, ABC-C-FX: Aberrant Behavior Checklist – Fragile X edition, ADAMS: Anxiety, depression, and Mood Scale, 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, 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, ERP: Event-related brain potentials, 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, HVLT: Hopkins Verbal Learning Test, MMP-9: matrix metalloproteinase 9, PARS: Pediatric Anxiety Rating Scale, 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

or conceal the effect in placebo-controlled trials. Very 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. 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 [174]. 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. 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.

ACKNOWLEDGMENTS

The authors would like to thank Tom de Vries Lentsch for excellent artwork. This work was supported by The Netherlands Organisation for Health Research and Development (ZonMw) 912-07-022 (RW), E-Rare program entitled "Cure FXS" (no. EU/FIS PS09102673) (RW) and FRAXA Research Foundation (RW).

SCOPE AND AIMS OF THIS THESIS

Compelling evidence indicates that the abnormal synaptic connectivity found in FXS is associated with a broad spectrum of clinical, behavioral and neuroanatomical abnormalities. The generation of mouse models for FXS served as a pre-clinical starting point and paved the way for drug discovery research. To date, 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. However, a major challenge in preclinical studies for FXS remains the identification of reliable outcome measures using new cellular models and *Fmr1* knockout mice.

The aim of this thesis is to contribute to the development of reliable translational outcome measures and putative biomarkers that will aid in the development and evaluation of therapeutic interventions in FXS.

- The development of targeted therapies for FXS has been hindered by a lack of human cellular models. As a first step, human fragile X fibroblasts and a human fibroblast line with an unmethylated extended CGG repeat were used to generate induced pluripotent stem cells (iPS). An initial characterization of these lines is described in chapter 2.
- There is a lack of robust and reliable behavioral tests for FXS mice that can be used in the evaluation of therapeutic treatments. Since autism related behavior is such an important feature of FXS patients, the social behavior of mice was investigated using the sociability and preference for social novelty (SPSN) test and the automated tube test (ATT) (Chapter 3, 4 and 5).
- In clinical research, the effect of therapies is mainly investigated by subjective outcome measures. In order to find an objective outcome measure we searched for biochemical markers in the FXS mouse. We used quantitative western blot, to measure the expression levels of various signaling proteins and receptors in cortical synaptoneurosome preparations of FXS mice to provide a biochemical read out for the fragile X phenotype in the mouse (Chapter 4).

REFERENCES

1. Martin, J.P. and J. Bell, *A pedigree of mental defect showing sex-linkage*. J Neurol Psych, 1943. **6**: p. 154-157.
2. Lubs, H.A., *A marker X-chromosome*. Am J Hum Genet, 1969. **21**: p. 231-244.
3. Sutherland, G.R., *Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium*. Science, 1977. **197**: p. 265-266.
4. Verkerk, A.J., et al., *Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome*. Cell, 1991. **65**(5): p. 905-914.
5. Willemsen, R., et al., *Timing of the absence of FMR1 expression in full mutation chorionic villi*. Hum Genet, 2002. **110**(6): p. 601-5.
6. Irwin, S.A., et al., *Dendritic spine and dendritic field characteristics of layer V pyramidal neurons in the visual cortex of fragile-X knockout mice*. Am J Med Genet, 2002. **111**(2): p. 140-6.
7. Irwin, S.A., et al., *Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: A quantitative examination*. Am J Med Genet, 2001. **98**(2): p. 161-167.
8. Rudelli, R.D., et al., *Adult fragile X syndrome. Clinico-neuropathologic findings*. Acta Neuropathol, 1985. **67**: p. 289-295.
9. Kaufmann, W.E. and H.W. Moser, *Dendritic anomalies in disorders associated with mental retardation*. Cereb Cortex, 2000. **10**(10): p. 981-91.
10. Purpura, R.P., *Dendritic spine dysgenesis and mental retardation*. Science, 1974. **186**: p. 1126-1128.
11. Bagni, C., et al., *Fragile X syndrome: causes, diagnosis, mechanisms, and therapeutics*. J Clin Invest, 2012. **122**(12): p. 4314-22.
12. Garber, K.B., J. Visootsak, and S.T. Warren, *Fragile X syndrome*. Eur J Hum Genet, 2008. **16**: p. 666-672.
13. Gross, C., E.M. Berry-Kravis, and G.J. Bassell, *Therapeutic strategies in fragile X syndrome: dysregulated mGluR signaling and beyond*. Neuropsychopharmacology, 2012. **37**(1): p. 178-95.
14. Gallagher, A. and B. Hallahan, *Fragile X-associated disorders: a clinical overview*. J Neurol, 2011. **259**.
15. Maes, B., et al., *Phenotypic checklist to screen for fragile X syndrome in people with mental retardation*. Ment Retard, 2000. **38**(3): p. 207-15.
16. Wang, L.W., E. Berry-Kravis, and R.J. Hagerman, *Fragile X: Leading the Way for Targeted Treatments in Autism*. Neurotherapeutics, 2010. **7**(3): p. 264-274.
17. Tranfaglia, M.R., *The Psychiatric Presentation of Fragile X: Evolution of the Diagnosis and Treatment of the Psychiatric Comorbidities of Fragile X Syndrome*. Dev Neurosci, 2011. **33**: p. 337-348.
18. Koekkoek, S.K., et al., *Deletion of FMR1 in Purkinje Cells Enhances Parallel Fiber LTD, Enlarges Spines, and Attenuates Cerebellar Eyelid Conditioning in Fragile X Syndrome*. Neuron, 2005. **47**(3): p. 339-52.
19. Smit, A.E., et al., *Savings and Extinction of Conditioned Eyeblink Responses in Fragile X Syndrome*. Genes Brain Behav, 2008. **7**: p. 770-777.
20. Frankland, P.W., et al., *Sensorimotor gating abnormalities in young males with fragile X syndrome and Fmr1-knockout mice*. Mol Psychiatry, 2004. **9**: p. 417-425.
21. Hessl, D., et al., *Prepulse inhibition in fragile X syndrome: Feasibility, reliability, and implications for treatment*. Am J Med Genet B Neuropsychiatr Genet, 2009. **150B**: p. 545-553.
22. Hinton, V.J., et al., *Analysis of neocortex in three males with the fragile X syndrome*. Am J Med Genet, 1991. **41**(3): p. 289-294.
23. Irwin, S.A., R. Galvez, and W.T. Greenough, *Dendritic spine structural anomalies in fragile-X mental retardation syndrome*. Cereb Cortex, 2000. **10**(10): p. 1038-44.
24. Gothelf, D., et al., *Neuroanatomy of fragile X syndrome is associated with aberrant behavior and the fragile X mental retardation protein (FMRP)*. Ann Neurol, 2007. **63**: p. 40-51.
25. Greco, C.M., et al., *Neuropathologic features in the hippocampus and cerebellum of three older men with fragile X syndrome*. Mol Autism, 2011. **2**(1): p. 2.
26. Hessl, D., S.M. Rivera, and A.L. Reiss, *The neuroanatomy and neuroendocrinology of fragile X syndrome*. Ment Retard Dev Disabil Res Rev, 2004. **10**(1): p. 17-24.
27. Reiss, A.L., et al., *Neurodevelopmental effects of the FMR-1 full mutation in humans*. Nature Med, 1995. **1**: p. 159-167.
28. Schapiro, M.B., et al., *Adult fragile X syndrome: Neuropsychology, brain anatomy, and metabolism*. Am J Med Genet, 1995. **60**(6): p. 480-493.
29. Kim, S.Y., et al., *Fear-specific amygdala function in children and adolescents on the fragile x spectrum: a dosage response of the FMR1 gene*. Cereb Cortex, 2014. **24**(3): p. 600-13.

30. Hoeft, F., et al., *Fronto-striatal dysfunction and potential compensatory mechanisms in male adolescents with fragile X syndrome*. Hum Brain Mapp, 2007. **28**: p. 543-554.
31. Menon, V., et al., *Frontostriatal deficits in fragile X syndrome: Relation to FMR1 gene expression*. Proc Natl Acad Sci U S A, 2004. **101**: p. 3615-3620.
32. Kwan, K.Y., et al., *Species-Dependent Posttranscriptional Regulation of NOS1 by FMRP in the Developing Cerebral Cortex*. Cell, 2012. **149**(4): p. 899-911.
33. Dhami, G.K. and S.S. Ferguson, *Regulation of metabotropic glutamate receptor signaling, desensitization and endocytosis*. Pharmacol Ther, 2006. **111**(1): p. 260-71.
34. Dockendorff, T.C., et al., *Drosophila lacking dfmr1 activity show defects in circadian output and fail to maintain courtship interest*. Neuron, 2002. **34**: p. 973-984.
35. Gatto, C.L. and K. Broadie, *Temporal requirements of the fragile X mental retardation protein in the regulation of synaptic structure*. Development, 2008. **135**: p. 2637-2648.
36. Kanellopoulos, A.K., et al., *Learning and memory deficits consequent to reduction of the fragile x mental retardation protein result from metabotropic glutamate receptor-mediated inhibition of cAMP signaling in Drosophila*. J Neurosci, 2012. **32**(38): p. 13111-24.
37. Pan, L., et al., *The Drosophila fragile x gene negatively regulates neuronal elaboration and synaptic differentiation*. Curr Biol, 2004. **14**(20): p. 1863-70.
38. den Broeder, M.J., et al., *Generation and characterization of FMR1 knockout zebrafish*. PLoS One, 2009. **4**(11): p. e7910.
39. Ng, M.C., Y.L. Yang, and K.T. Lu, *Behavioral and synaptic circuit features in a zebrafish model of fragile x syndrome*. PLoS ONE, 2013. **8**(3): p. e51456.
40. Tucker, B., R. Richards, and M. Lardelli, *Expression of three zebrafish orthologs of human FMR1-related genes and their phylogenetic relationships*. Dev Genes Evol, 2004. **214**: p. 567-574.
41. Tucker, B., R.I. Richards, and M. Lardelli, *Contribution of mGluR and Fmr1 Functional Pathways to Neurite Morphogenesis, Craniofacial Development and Fragile X Syndrome*. Hum Mol Genet, 2006. **15**: p. 3446-3458.
42. van 't Padje, S., et al., *Characterisation of Fmrp in zebrafish: evolutionary dynamics of the fmr1 gene*. Dev Genes Evol, 2005. **215**: p. 198-206.
43. Bakker, C.E., et al., *Fmr1 knockout mice: A model to study fragile X mental retardation*. Cell, 1994. **78**: p. 23-33.
44. Mientjes, E.J., et al., *The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo*. Neurobiol Dis, 2006. **21**: p. 549-555.
45. Darnell, J.C., et al., *Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein KH2 domain and brain polyribosomes*. Genes Dev, 2005. **19**(8): p. 903-18.
46. Darnell, J.C., et al., *Fragile X Mental Retardation Protein Targets G Quartet mRNAs Important for Neuronal Function*. Cell, 2001. **107**(4): p. 489-99.
47. Feng, Y., et al., *Fragile X mental retardation protein: Nucleocytoplasmic shuttling and association with somatodendritic ribosomes*. J Neurosci, 1997. **17**(5): p. 1539-1547.
48. Willemsen, R., et al., *The fragile X syndrome: From molecular genetics to neurobiology*. Ment Retard Dev Disabil Res Rev, 2004. **10**(1): p. 60-7.
49. Feng, Y., et al., *FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association*. Mol Cell, 1997. **1**(1): p. 109-118.
50. Willemsen, R., et al., *Association of FMRP with ribosomal precursor particles in the nucleolus*. Biochem Biophys Res Comm, 1996. **225**: p. 27-33.
51. Darnell, J.C., et al., *FMRP Stalls Ribosomal Translocation on mRNAs Linked to Synaptic Function and Autism*. Cell, 2011. **146**(2): p. 247-61.
52. Laggerbauer, B., et al., *Evidence that fragile X mental retardation protein is a negative regulator of translation*. Hum Mol Genet, 2001. **10**(4): p. 329-338.
53. Li, Z., et al., *The fragile X mental retardation protein inhibits translation via interacting with mRNA*. Nucleic Acids Res, 2001. **29**(11): p. 2276-2283.
54. Dolen, G., et al., *Correction of Fragile X Syndrome in Mice*. Neuron, 2007. **56**(6): p. 955-962.
55. Muddashetty, R.S., et al., *Dysregulated metabotropic glutamate receptor-dependent translation of AMPA receptor and postsynaptic density-95 mRNAs at synapses in a mouse model of fragile X syndrome*. J Neurosci, 2007. **27**(20): p. 5338-48.
56. Osterweil, E.K., et al., *Hypersensitivity to mGluR5 and ERK1/2 Leads to Excessive Protein Synthesis in the Hippocampus of a Mouse Model of Fragile X Syndrome*. J Neurosci, 2010. **30**(46): p. 15616-27.
57. Richter, J.D. and E. Klann, *Making synaptic plasticity and memory last: mechanisms of translational regulation*. Genes Dev, 2009. **23**(1): p. 1-11.

58. Malenka, R.C. and M.F. Bear, *LTP and LTD: an embarrassment of riches*. Neuron, 2004. **44**(1): p. 5-21.
59. Huber, K.M., J.C. Roder, and M.F. Bear, *Chemical induction of mGluR5- and protein synthesis--dependent long- term depression in hippocampal area CA1.* J Neurophysiol, 2001. **86**(1): p. 321-5.
60. Weiler, I.J., et al., *Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation*. Proc Natl Acad Sci USA, 1997. **94**: p. 5395-5400.
61. Huber, K.M., et al., *Altered synaptic plasticity in a mouse model of fragile X mental retardation*. Proc Natl Acad Sci U S A, 2002. **99**: p. 7746-50.
62. Nakamoto, M., et al., *Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors*. Proc Natl Acad Sci U S A, 2007. **104**(39): p. 15537-42.
63. Bear, M.F., K.M. Huber, and S.T. Warren, *The mGluR theory of fragile X mental retardation*. Trends Neurosci, 2004. **27**(7): p. 370-7.
64. Antion, M.D., et al., *Removal of S6K1 and S6K2 leads to divergent alterations in learning, memory, and synaptic plasticity*. Learn Mem, 2008. **15**(1): p. 29-38.
65. Gallagher, S.M., et al., *Extracellular signal-regulated protein kinase activation is required for metabotropic glutamate receptor-dependent long-term depression in hippocampal area CA1*. J Neurosci, 2004. **24**(20): p. 4859-64.
66. Hou, L., et al., *Dynamic Translational and Proteasomal Regulation of Fragile X Mental Retardation Protein Controls mGluR-Dependent Long-Term Depression*. Neuron, 2006. **51**(4): p. 441-54.
67. Ronesi, J.A. and K.M. Huber, *Metabotropic glutamate receptors and fragile x mental retardation protein: partners in translational regulation at the synapse*. Sci Signal, 2008. **1**(5): p. pe6.
68. Bhattacharya, A., et al., *Genetic Removal of p70 S6 Kinase 1 Corrects Molecular, Synaptic, and Behavioral Phenotypes in Fragile X Syndrome Mice*. Neuron, 2012. **76**(2): p. 325-37.
69. Hoeffer, C.A., et al., *Altered mTOR signaling and enhanced CYFIP2 expression levels in subjects with Fragile X syndrome*. Genes Brain Behav, 2012. **11**(3): p. 332-341.
70. Sharma, A., et al., *Dysregulation of mTOR signaling in fragile X syndrome*. J Neurosci, 2010. **30**(2): p. 694-702.
71. Thomas, G.M. and R.L. Huganir, *MAPK cascade signalling and synaptic plasticity*. Nat Rev Neurosci, 2004. **5**(3): p. 173-83.
72. Costa-Mattioli, M., N. Sonenberg, and J.D. Richter, *Translational regulatory mechanisms in synaptic plasticity and memory storage*. Prog Mol Biol Transl Sci, 2009. **90**: p. 293-311.
73. Bilousova, T., et al., *Minocycline Promotes Dendritic Spine Maturation and Improves Behavioral Performance in the Fragile X Mouse Model*. J Med Genet, 2008. **46**: p. 94-102.
74. Gross, C., et al., *Excess phosphoinositide 3-kinase subunit synthesis and activity as a novel therapeutic target in fragile x syndrome*. J Neurosci, 2010. **30**(32): p. 10624-38.
75. Jung, K.M., et al., *Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome*. Nat Commun, 2012. **3**: p. 1080.
76. Wang, X., et al., *Activation of the ERK Pathway Contributes to the Behavioral Deficit of Fragile X-Syndrome*. J Neurochem, 2012. **121**: p. 672-679.
77. Wang, X., et al., *Activation of the extracellular signal-regulated kinase pathway contributes to the behavioral deficit of fragile x-syndrome*. J Neurochem, 2012. **121**(4): p. 672-9.
78. Min, W.W., et al., *Elevated glycogen synthase kinase-3 activity in Fragile X mice: Key metabolic regulator with evidence for treatment potential*. Neuropharmacology, 2009. **56**: p. 463-472.
79. Dolan, B.M., et al., *Rescue of fragile X syndrome phenotypes in Fmr1 KO mice by the small-molecule PAK inhibitor FRAX486*. Proc Natl Acad Sci U S A, 2013. **110**(14): p. 5671-6.
80. Isaacson, J.S. and B. Hille, *GABA(B)-mediated presynaptic inhibition of excitatory transmission and synaptic vesicle dynamics in cultured hippocampal neurons*. Neuron, 1997. **18**(1): p. 143-52.
81. Sohn, J.W., et al., *Receptor-specific inhibition of GABAB-activated K+ currents by muscarinic and metabotropic glutamate receptors in immature rat hippocampus*. J Physiol, 2007. **580**(Pt. 2): p. 411-22.
82. Zhang, J., et al., *Altered Hippocampal Synaptic Plasticity in the Fmr1 Gene Family Knockout Mouse Models*. J Neurophysiol, 2009. **101**: p. 2572-2580.
83. Kooy, R.F., *Of mice and the fragile X syndrome*. Trends Genet, 2003. **19**(3): p. 148-54.
84. Pietropaolo, S., et al., *Genetic-background modulation of core and variable autistic-like symptoms in FMR1 knock-out mice*. PLoS ONE, 2011. **6**(2): p. e17073.

85. Spencer, C.M., et al., *Modifying behavioral phenotypes in Fmr1KO mice: genetic background differences reveal autistic-like responses*. Autism Res, 2011. **4**(1): p. 40-56.

86. Portera-Cailliau, C., *Which Comes First in Fragile X Syndrome, Dendritic Spine Dysgenesis or Defects in Circuit Plasticity?* Neuroscientist, 2011. **18**: p. 28-44.

87. Michalon, A., et al., *Chronic Pharmacological mGlu5 Inhibition Corrects Fragile X in Adult Mice*. Neuron, 2012. **74**(1): p. 49-56.

88. Goebel-Goody, S.M., et al., *Genetic manipulation of STEP reverses behavioral abnormalities in a fragile X syndrome mouse model*. Genes Brain Behav, 2012. **11**: p. 586-600.

89. Giuffrida, R., et al., *A reduced number of metabotropic glutamate subtype 5 receptors are associated with constitutive homer proteins in a mouse model of fragile X syndrome*. J Neurosci, 2005. **25**(39): p. 8908-16.

90. Till, S.M., et al., *Altered maturation of the primary somatosensory cortex in a mouse model of fragile X syndrome*. Hum Mol Genet, 2012. **21**(10): p. 2143-2156.

91. Schutt, J., et al., *Fragile X mental retardation protein regulates the levels of scaffold proteins and glutamate receptors in postsynaptic densities*. J Biol Chem, 2009. **284**(38): p. 25479-87.

92. Wang, H., S.S. Kim, and M. Zhuo, *Roles of FMRP in dopaminergic stimulation induced synapse-associated protein synthesis and subsequent AMPA receptor internalization*. J Biol Chem, 2010. **285**: p. 21888-21901.

93. Zalfa, F., et al., *A new function for the fragile X mental retardation protein in regulation of PSD-95 mRNA stability*. Nat Neurosci, 2007. **10**: p. 578-587.

94. Krueger, D.D., et al., *Cognitive dysfunction and prefrontal synaptic abnormalities in a mouse model of fragile X syndrome*. Proc Natl Acad Sci U S A, 2011. **108**: p. 2587-2592.

95. Curia, G., et al., *Downregulation of Tonic GABAergic Inhibition in a Mouse Model of Fragile X Syndrome*. Cereb Cortex, 2009. **19**: p. 1515-1520.

96. D'Hulst, C., et al., *Decreased expression of the GABA(A) receptor in fragile X syndrome*. Brain Res, 2006. **1121**: p. 238-245.

97. D'Hulst, C., et al., *Expression of the GABAergic system in animal models for fragile X syndrome and fragile X associated tremor/ataxia syndrome (FXTAS)*. Brain Res, 2009. **1253**: p. 176-183.

98. El Idrissi, A., et al., *Decreased GABA(A) receptor expression in the seizure-prone fragile X mouse*. Neurosci Lett, 2005. **377**(3): p. 141-146.

99. Centonze, D., et al., *Abnormal Striatal GABA Transmission in the Mouse Model for the Fragile X Syndrome*. Biol Psychiatry, 2007. **63**: p. 963-973.

100. D'Antuono, M., D. Merlo, and M. Avoli, *Involvement of cholinergic and gabaergic systems in the fragile X knockout mice*. Neuroscience, 2003. **119**(1): p. 9-13.

101. Olmos-Serrano, J.L., et al., *Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile x syndrome*. J Neurosci, 2010. **30**(29): p. 9929-38.

102. Errijgers, V. and R.F. Kooy, *Genetic modifiers in mice: the example of the fragile X mouse model*. Cytogenet Genome Res, 2004. **105**(2-4): p. 448-54.

103. Bakker, C.E. and B.A. Oostra, *Understanding fragile X syndrome: insights from animal models*. Cytogenet Genome Res, 2003. **100**(1-4): p. 111-23.

104. Chen, L. and M. Toth, *Fragile X mice develop sensory hyperreactivity to auditory stimuli*. Neuroscience, 2001. **103**(4): p. 1043-1050.

105. De Vrij, F.M.S., et al., *Rescue of behavioral phenotype and neuronal protrusion morphology in FMR1 KO mice*. Neurobiol Dis, 2008. **31**: p. 127-132.

106. Musumeci, S.A., et al., *Audiogenic seizures susceptibility in transgenic mice with fragile X syndrome*. Epilepsia, 2000. **41**(1): p. 19-23.

107. Gantois, I., et al., *Chronic administration of AFQ056/Mavoglurant restores social behaviour in Fmr1 knockout mice*. Behav Brain Res, 2013. **239** p. 72-79.

108. Spencer, C.M., et al., *Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome*. Genes Brain Behav, 2005. **4**(7): p. 420-30.

109. D'Hooge, R., et al., *Mildly impaired water maze performance in male Fmr1 knockout mice*. Neuroscience, 1997. **76**(2): p. 367-376.

110. Burket, J.A., et al., *Complex effects of mGluR5 antagonism on sociability and stereotypic behaviors in mice: possible implications for the pharmacotherapy of autism spectrum disorders*. Brain Res Bull, 2011. **86**(3-4): p. 152-8.

111. Thomas, A.M., et al., *Group I metabotropic glutamate receptor antagonists alter select behaviors in a mouse model for fragile X syndrome*. Psychopharmacology (Berl), 2011. **19**: p. 47-58.

112. Yan, Q.J., et al., *Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP*. Neuropharmacology, 2005. **49**: p. 1053-1066.

113. Su, T., et al., *Early continuous inhibition of group 1 mGlu signaling partially rescues dendritic spine*

abnormalities in the Fmr1 knockout mouse model for fragile X syndrome. Psychopharmacology (Berl), 2010. **215**: p. 291-300.

114. Gasparini, F., R. Kuhn, and J.P. Pin, *Allosteric modulators of group I metabotropic glutamate receptors: novel subtype-selective ligands and therapeutic perspectives*. Curr Opin Pharmacol, 2002. **2**(1): p. 43-9.
115. Porter, R.H., et al., *Fenobam: a clinically validated nonbenzodiazepine anxiolytic is a potent, selective, and noncompetitive mGlu5 receptor antagonist with inverse agonist activity*. J Pharmacol Exp Ther, 2005. **315**(2): p. 711-21.
116. Levenga, J., et al., *Potential therapeutic interventions for fragile X syndrome*. Trends Mol Med, 2010. **16**: p. 516-527.
117. Vinueza Veloz, M.F., et al., *The effect of an mGluR5 inhibitor on procedural memory and avoidance discrimination impairments in Fmr1 KO mice*. Genes Brain Behav, 2012. **11**: p. 325-331.
118. Levenga, J., et al., *AFQ056, a new mGluR5 antagonist for treatment of fragile X syndrome*. Neurobiol Dis, 2011. **42**: p. 311-317.
119. Pop, A.S., et al., *Rescue of dendritic spine phenotype in Fmr1 KO mice with the mGluR5 antagonist AFQ056/Mavoglurant*. Psychopharmacology (Berl), 2014. **231**(6): p. 1227-35.
120. Lindemann, L., et al., *CTEP: a novel, potent, long-acting, and orally bioavailable metabotropic glutamate receptor 5 inhibitor*. J Pharmacol Exp Ther, 2011. **339**(2): p. 474-86.
121. Choi, C.H., et al., *Pharmacological reversal of synaptic plasticity deficits in the mouse model of Fragile X syndrome by group II mGluR antagonist or lithium treatment*. Brain Res, 2011. **1380**: p. 106-119.
122. Liu, Z.H., D.M. Chuang, and C.B. Smith, *Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome*. Int J Neuropsychopharmacol, 2011. **14**(5): p. 618-30.
123. Yuskaitis, C.J., et al., *Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of Fragile X Syndrome*. Biochem Pharmacol, 2010. **79**: p. 632-646.
124. Dansie, L.E., et al., *Long-lasting effects of minocycline on behavior in young but not adult Fragile X mice*. Neuroscience, 2013. **246**: p. 186-98.
125. Veeraragavan, S., et al., *Modulation of behavioral phenotypes by a muscarinic M1 antagonist in a mouse model of fragile X syndrome*. Psychopharmacology (Berl), 2011. **217**: p. 143-151.
126. Veeraragavan, S., et al., *The modulation of fragile X behaviors by the muscarinic M4 antagonist, tropicamide*. Behav Neurosci, 2011. **125**(5): p. 783-90.
127. Veeraragavan, S., et al., *Genetic reduction of muscarinic M(4) receptor modulates analgesic response and acoustic startle response in a mouse model of fragile X syndrome (FXS)*. Behav Brain Res, 2011. **223**: p. 310-321.
128. Heulens, I., et al., *Pharmacological treatment of fragile X syndrome with GABAergic drugs in a knockout mouse model*. Behav Brain Res, 2012. **229**: p. 244-249.
129. Henderson, C., et al., *Reversal of disease-related pathologies in the fragile X mouse model by selective activation of GABA(B) receptors with arbaclofen*. Sci Transl Med, 2012. **4**(152): p. 152ra128.
130. Pacey, L.K., S.P. Heximer, and D.R. Hampson, *Increased GABAB Receptor-mediated Signaling Reduces the Susceptibility of Fragile X Knockout Mice to Audiogenic Seizures*. Mol Pharmacol, 2009. **76**: p. 18-24.
131. Pacey, L.K., S. Tharmalingam, and D.R. Hampson, *Subchronic Administration and Combination Metabotropic Glutamate and GABAB Receptor Drug Therapy in Fragile X Syndrome*. J Pharmacol Exp Ther, 2011. **338**(3): p. 897-905.
132. Osterweil, E.K., et al., *Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile x syndrome*. Neuron, 2013. **77**(2): p. 243-50.
133. Antar, L.N., et al., *Local functions for FMRP in axon growth cone motility and activity-dependent regulation of filopodia and spine synapses*. Mol Cell Neurosci, 2006. **32**: p. 37-48.
134. Braun, K. and M. Segal, *FMRP involvement in formation of synapses among cultured hippocampal neurons*. Cereb Cortex, 2000. **10**(10): p. 1045-52.
135. Dictenberg, J.B., et al., *A direct role for FMRP in activity-dependent dendritic mRNA transport links filopodial-spine morphogenesis to fragile X syndrome*. Dev Cell, 2008. **14**(6): p. 926-39.
136. Reisberg, B., et al., *Memantine in moderate-to-severe Alzheimer's disease*. N Engl J Med, 2003. **348**(14): p. 1333-41.
137. Wei, H., et al., *The Therapeutic effect of Memantine through the Stimulation of Synapse Formation and Dendritic Spine Maturation in Autism and Fragile X Syndrome*. PLoS ONE, 2012. **7**(5): p. e36981.
138. Wan, L., et al., *Characterization of dFMR1, a Drosophila melanogaster Homolog of the Fragile X Mental Retardation Protein*. Molecular and Cellular Biology, 2000. **20**(22): p. 8536-8547.
139. Zhang, Y.Q., et al., *Drosophila Fragile X-Related Gene Regulates the MAP1B Homolog Futsch to*

Control Synaptic Structure and Function. Cell, 2001. **107**(5): p. 591-603.

140. Chang, S., et al., *Identification of small molecules rescuing fragile X syndrome phenotypes in Drosophila*. Nat Chem Biol, 2008. **4**: p. 256-263.
141. Eiges, R., et al., *Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived from Preimplantation Genetically Diagnosed Embryos*. Cell Stem Cell, 2007. **1**(5): p. 568-577.
142. Gerhardt, J., et al., *The DNA replication program is altered at the FMR1 locus in fragile X embryonic stem cells*. Mol Cell, 2014. **53**(1): p. 19-31.
143. Colak, D., et al., *Promoter-bound trinucleotide repeat mRNA drives epigenetic silencing in fragile X syndrome*. Science, 2014. **343**(6174): p. 1002-5.
144. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors*. Cell, 2007. **131**(5): p. 861-72.
145. Bar-Nur, O., I. Caspi, and N. Benvenisty, *Molecular analysis of FMR1 reactivation in fragile-X induced pluripotent stem cells and their neuronal derivatives*. J Mol Cell Biol, 2012. **4**: p. 180-183.
146. Urbach, A., et al., *Differential modeling of fragile X syndrome by human embryonic stem cells and induced pluripotent stem cells*. Cell Stem Cell, 2010. **6**(5): p. 407-11.
147. Sheridan, S.D., et al., *Epigenetic characterization of the FMR1 gene and aberrant neurodevelopment in human induced pluripotent stem cell models of fragile x syndrome*. PLoS ONE, 2011. **6**(10): p. e26203.
148. Gafni, O., et al., *Derivation of novel human ground state naive pluripotent stem cells*. Nature, 2013. **504**(7479): p. 282-6.
149. Karumbayaram, S., et al., *Directed differentiation of human-induced pluripotent stem cells generates active motor neurons*. Stem Cells, 2009. **27**(4): p. 806-11.
150. Zeng, H., et al., *Specification of region-specific neurons including forebrain glutamatergic neurons from human induced pluripotent stem cells*. PLoS One, 2010. **5**(7): p. e11853.
151. Aman, M.G., W.H. Burrow, and P.L. Wolford, *The Aberrant Behavior Checklist-Community: factor validity and effect of subject variables for adults in group homes*. Am J Ment Retard, 1995. **100**(3): p. 283-92.
152. Sansone, S.M., et al., *Psychometric study of the Aberrant Behavior Checklist in Fragile X Syndrome and implications for targeted treatment*. J Autism Dev Disord, 2012. **42**(7): p. 1377-92.
153. Berry-Kravis, E.M., et al., *Effects of STX209 (Arbaclofen) on Neurobehavioral Function in Children and Adults with Fragile X Syndrome: A Randomized, Controlled, Phase 2 Trial*. Sci Transl Med, 2012. **4**(152): p. 152ra127.
154. Sparrow, S.S. and D.V. Cicchetti, *Diagnostic uses of the Vineland Adaptive Behavior Scales*. J Pediatr Psychol, 1985. **10**(2): p. 215-25.
155. Leigh, M.J., et al., *A Randomized Double-Blind, Placebo-Controlled Trial of Minocycline in Children and Adolescents with Fragile X Syndrome*. J Dev Behav Pediatr, 2013. **34**(3): p. 147-155.
156. Paribello, C., et al., *Open-label add-on treatment trial of minocycline in fragile X syndrome*. BMC Neurol, 2010. **10**(1): p. 91.
157. Hazlett, E.A., et al., *Prefrontal cortex glucose metabolism and startle eyeblink modification abnormalities in unmedicated schizophrenia patients*. Psychophysiology, 1998. **35**(2): p. 186-98.
158. Hessl, D., et al., *A solution to limitations of cognitive testing in children with intellectual disabilities: the case of fragile X syndrome*. J Neurodev Disord, 2009. **1**(1): p. 33-45.
159. Berry-Kravis, E.M., et al., *A pilot open-label single-dose trial of fenobam in adults with fragile X syndrome*. J Med Genet, 2009. **46**: p. 266-271.
160. Berry-Kravis, E., et al., *Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome*. J Dev Behav Pediatr, 2008. **29**(4): p. 293-302.
161. Dziembowska, M., et al., *High MMP-9 activity levels in fragile X syndrome are lowered by minocycline*. Am J Med Genet A, 2013. **161A**(8): p. 1897-1903.
162. Erickson, C.A., et al., *Impact of acamprosate on behavior and brain-derived neurotrophic factor: an open-label study in youth with fragile X syndrome*. Psychopharmacology (Berl), 2013. **228**(1): p. 75-84.
163. Lohith, T.G., et al., *Is metabotropic glutamate receptor 5 upregulated in prefrontal cortex in fragile X syndrome?* Mol Autism, 2013. **4**(1): p. 15.
164. Fatemi, S.H. and T.D. Folsom, *Dysregulation of fragile X mental retardation protein and metabotropic glutamate receptor 5 in superior frontal cortex of subjects with autism: a postmortem brain study*. Mol Autism, 2011. **2**(1): p. 6.
165. Gross, C. and G.J. Bassell, *Excess protein synthesis in FXS patient lymphoblastoid cells can be rescued with a p110beta-selective inhibitor*. Mol Med, 2012. **18**(1): p. 336-345.
166. Weng, N., et al., *Early-phase ERK activation as a biomarker for metabolic status in fragile X*

syndrome. Am J Med Genet B Neuropsychiatr Genet, 2008. **147B**: p. 1253-1257.

167. Erickson, C.A., et al., *Open-label riluzole in fragile X syndrome*. Brain Res, 2011. **1380**: p. 264-70.
168. Qin, M., et al., *Altered cerebral protein synthesis in fragile X syndrome: studies in human subjects and knockout mice*. J Cereb Blood Flow Metab, 2013. **33**(4): p. 499-507.
169. Bishu, S., et al., *Regional rates of cerebral protein synthesis measured with L-[1-11C]leucine and PET in conscious, young adult men: normal values, variability, and reproducibility*. J Cereb Blood Flow Metab, 2008. **28**(8): p. 1502-13.
170. Bishu, S., et al., *Propofol anesthesia does not alter regional rates of cerebral protein synthesis measured with L-[1-(11)C]leucine and PET in healthy male subjects*. J Cereb Blood Flow Metab, 2009. **29**(5): p. 1035-47.
171. Kesler, S.R., A.A. Lightbody, and A.L. Reiss, *Cholinergic dysfunction in fragile X syndrome and potential intervention: A preliminary (1)H MRS study*. Am J Med Genet A, 2009. **149A**: p. 403-407.
172. Torrioli, M., et al., *Treatment with valproic acid ameliorates ADHD symptoms in fragile X syndrome boys*. Am J Med Genet A, 2010. **152A**(6): p. 1420-7.
173. Lemonnier, E., et al., *Treating Fragile X syndrome with the diuretic bumetanide: a case report*. Acta Paediatr, 2013. **102**(6): p. e288-90.
174. Jacquemont, S., et al., *Epigenetic Modification of the FMR1 Gene in Fragile X Syndrome Is Associated with Differential Response to the mGluR5 Antagonist AFQ056*. Sci Transl Med, 2011. **3**(64): p. 64ra1.
175. Busner, J. and S.D. Targum, *The clinical global impressions scale: applying a research tool in clinical practice*. Psychiatry (Edgmont), 2007. **4**(7): p. 28-37.
176. McCormack, H.M., D.J. Horne, and S. Sheather, *Clinical applications of visual analogue scales: a critical review*. Psychol Med, 1988. **18**(4): p. 1007-19.
177. Constantino, J.N., et al., *Validation of a brief quantitative measure of autistic traits: comparison of the social responsiveness scale with the autism diagnostic interview-revised*. J Autism Dev Disord, 2003. **33**(4): p. 427-33.
178. Zhang, S., et al., *ADHD Rating Scale IV: psychometric properties from a multinational study as a clinician-administered instrument*. Int J Methods Psychiatr Res, 2005. **14**(4): p. 186-201.
179. Price, D.R., et al., *Study of WAIS-R, Quick Test and PPVT IQS for neuropsychiatric patients*. Percept Mot Skills, 1990. **70**(3 Pt 2): p. 1320-2.
180. Naglieri, J.A. and S.I. Pfeiffer, *Stability, concurrent and predictive validity of the PPVT-R*. J Clin Psychol, 1983. **39**(6): p. 965-7.
181. Deb, S., A.J. Dhaliwal, and M. Roy, *The usefulness of Conners' Rating Scales-Revised in screening for attention deficit hyperactivity disorder in children with intellectual disabilities and borderline intelligence*. J Intellect Disabil Res, 2008. **52**(11): p. 950-65.
182. Bussing, R., et al., *Parent and teacher SNAP-IV ratings of attention deficit hyperactivity disorder symptoms: psychometric properties and normative ratings from a school district sample*. Assessment, 2008. **15**(3): p. 317-28.
183. Bodfish, J.W., et al., *Varieties of repetitive behavior in autism: comparisons to mental retardation*. J Autism Dev Disord, 2000. **30**(3): p. 237-43.
184. Mesibov, G.B., et al., *Use of the childhood autism rating scale with autistic adolescents and adults*. J Am Acad Child Adolesc Psychiatry, 1989. **28**(4): p. 538-41.
185. Lord, C., et al., *Autism diagnostic observation schedule: a standardized observation of communicative and social behavior*. J Autism Dev Disord, 1989. **19**(2): p. 185-212.
186. Scahill, L., et al., *Children's Yale-Brown Obsessive Compulsive Scale modified for pervasive developmental disorders*. J Am Acad Child Adolesc Psychiatry, 2006. **45**(9): p. 1114-23.
187. Chambers, W.J., et al., *The assessment of affective disorders in children and adolescents by semistructured interview. Test-retest reliability of the schedule for affective disorders and schizophrenia for school-age children, present episode version*. Arch Gen Psychiatry, 1985. **42**(7): p. 696-702.
188. Muma, J.R., *Semel and Wiig's CELF: construct validity?* J Speech Hear Disord, 1984. **49**(1): p. 101-4.
189. Restrepo, M.A., et al., *Performance on the PPVT-III and the EVT: applicability of the measures with African American and European American preschool children*. Lang Speech Hear Serv Sch, 2006. **37**(1): p. 17-27.
190. Randolph, C., et al., *The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS): preliminary clinical validity*. J Clin Exp Neuropsychol, 1998. **20**(3): p. 310-9.
191. Berry-Kravis, E., et al., *Effect of CX516, an AMPA-Modulating Compound, on Cognition and Behavior in Fragile X Syndrome: AControlled Trial*. J Child Adolesc Psychopharmacol, 2006. **16**(5): p. 525-40.

192. Erickson, C.A., J.E. Mullett, and C.J. McDougle, *Open-Label Memantine in Fragile X Syndrome*. J Autism Dev Disord, 2009. **39**: p. 1629-1635.

193. Utari, A., et al., *Side effects of minocycline treatment in patients with fragile x syndrome and exploration of outcome measures*. Am J Intellect Dev Disabil, 2010. **115**(5): p. 433-43.

194. Sahu, J.K., et al., *Effectiveness and safety of donepezil in boys with fragile x syndrome: a double-blind, randomized, controlled pilot study*. J Child Neurol, 2013. **28**(5): p. 570-5.

CHAPTER 2

EPIGENETIC CHARACTERIZATION OF THE *FMR1* PROMOTER AFTER REPROGRAMMING OF A HUMAN FIBROBLAST LINE CARRYING AN UNMETHYLATED FULL MUTATION INTO PLURIPOTENT STEM CELLS

Celine EF de Esch(1), Mehrnaz Ghazvini(5), Nune Schelling-Kazaryan(6), W. Widagdo (1), Shashini T Munshi(4), Erik van der Wal(3), Hannie Douben (1), Nilhan Gunhanlar(4), Steven A Kushner(4), W.W.M. Pim Pijnappel(3), Femke MS de Vrij(4), Niels Geijsen(2,6), Joost Gribnau(5)#, Rob Willemsen (1)# *

(1) Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, the Netherlands, (2) Utrecht University School for Veterinary Medicine, Dept. Companion Animals, Utrecht, the Netherlands. (3) Molecular Stem Cell Biology, Department of Clinical Genetics; Department of Pediatrics, Division of Metabolic Diseases and Genetics; Center for Lysosomal and Metabolic Diseases Erasmus Medical Center, Rotterdam, The Netherlands, (4) Department of Psychiatry, Erasmus Medical Center, Rotterdam, the Netherlands, (5) Department of Reproduction and Development, Erasmus Medical Center, Rotterdam, the Netherlands, (6) KNAW Hubrecht Institute and UMC Utrecht, Utrecht, the Netherlands
# both authors contributed equally, *Corresponding author

In revision for Stem Cell Reports, 2014.

ABSTRACT

Silencing of the *FMR1* gene leads to fragile X syndrome (FXS), the most common cause of inherited intellectual disability. To study the epigenetic modifications of the *FMR1* gene during silencing in time, we used fibroblasts and induced pluripotent stem cells (iPSCs) of an unmethylated full mutation (uFM) individual with normal intelligence. The uFM fibroblast cell line carried an unmethylated promoter region and expressed normal to slightly increased *FMR1* mRNA levels. The increased H3 acetylation and H3K4 methylation in combination with a reduced H3K9 methylation indicated an actively transcribed gene in the uFM fibroblast line. After reprogramming, the two uFM iPSC clones still carried a full mutation, however the *FMR1* promoter region was methylated and lacked *FMR1* expression. The epigenetic histone marks also indicated a repressed *FMR1* promoter. In conclusion, these findings demonstrate that the standard reprogramming procedure is not sufficient to prevent epigenetic silencing of the fully mutated *FMR1* gene.

INTRODUCTION

The most common inherited form of intellectual disability, fragile X syndrome (FXS), is caused by the absence of the *FMR1* gene product, the fragile X mental retardation protein (FMRP). In the majority of FXS patients, the transcriptional silencing of the *FMR1* gene is initiated by an expansion of a naturally occurring CGG repeat in the 5' untranslated region (UTR) of the *FMR1* gene, to more than ~200 units [1, 2]. This so called full mutation results in hypermethylation of the cytosines in the repeat region and the *FMR1* promoter region during early human embryonic development [3, 4]. This results in a lack of *FMR1* transcription and consequently an absence of FMRP. As well as hypermethylation, the *FMR1* promoter in FXS is characterized by additional epigenetic marks specific for transcriptionally repressed chromatin including reduced histone H3 and H4 acetylation, reduced histone H3K4 methylation and increased histone H3K9 methylation [5-8]. However, the timing and molecular mechanisms involved in the CGG expansion, the concomitant DNA methylation and additional epigenetic changes that occur during embryonic development are not yet fully understood. Insights into these processes may lead to a more complete understanding of the developmental processes underlying fragile X syndrome, which in turn could lead to new therapeutic strategies.

Since murine fragile X models cannot be used to investigate epigenetic *FMR1* inactivation as methylation of the full mutations does not occur, human FXS embryonic stem cells have been studied. These initial studies showed that FMRP is expressed during early embryonic development, but that epigenetic silencing of *FMR1* occurs upon differentiation [9, 10]. A further attempt to study the epigenetic changes over time made use of induced pluripotent stem cells (iPSCs) generated from human FXS fibroblasts. In contrast to human embryonic FX stem cells these pluripotent cells were shown to already carry a fully methylated *FMR1* promoter and additional heterochromatin marks, so they cannot be used to study the epigenetic silencing mechanisms in time [11-13].

In 1991, a familial case was reported in which two brothers with normal intelligence were shown to have a full *FMR1* mutation without the concomitant hypermethylation of the CGG repeat and the promoter region [14]. In order to unravel the molecular mechanisms behind the epigenetic silencing in fragile X syndrome, we derived iPS cells from these human fibroblasts, to analyze the epigenetic characteristics of the *FMR1* promoter after reprogramming and during differentiation. Here, we report the characterization of these iPS cells and show, unexpectedly, that the *FMR1* promoter of the unmethylated full mutation cell line becomes methylated during reprogramming and stays methylated after differentiation into neural progenitor cells.

RESULTS

Fibroblast characterization

Fibroblasts from a normal male carrying an unmethylated full mutation first described by Smeets *et al.* (1995) (uFM), fibroblasts from a clinically diagnosed male fragile X syndrome patient (14 years old, FXS), and an unrelated unaffected male control line (3 years old, Control) were analyzed for *FMR1* 5'UTR CGG repeat length, methylation status, *FMR1* expression and the histone marks associated with the *FMR1* promoter. As expected, the Control line showed a CGG repeat length within the normal range (< 55) while the uFM and the FXS line showed CGG

repeat lengths in the full mutation range (> 200) (data not shown). Also as expected, the part of the *FMR1* promoter analyzed after bisulfite conversion was not methylated in the Control and the uFM cell lines, while in the FXS cell line the *FMR1* promoter was methylated (Figure 1A). As the methylation status is predictive of *FMR1* expression, indeed the Control line showed normal expression levels, the uFM line showed normal to slightly increased *FMR1* expression while the FXS cell line did not express *FMR1* transcripts (Figure 1B).

Chromatin immunoprecipitation (ChIP) experiments showed that the *FMR1* promoter of the Control line carried active histone marks, namely H3 acetylation and H3K4 di-methylation with values similar to the positive control for active genes *APRT* and values much higher than the negative control *Crystalline*, which only serves as a positive control for repressed genes, while the inactive mark H3K9 tri-methylation was not enriched (Figure 2 A, B, C). The uFM line carried histone marks representative of an actively transcribed gene H3 acetylation and H3K4

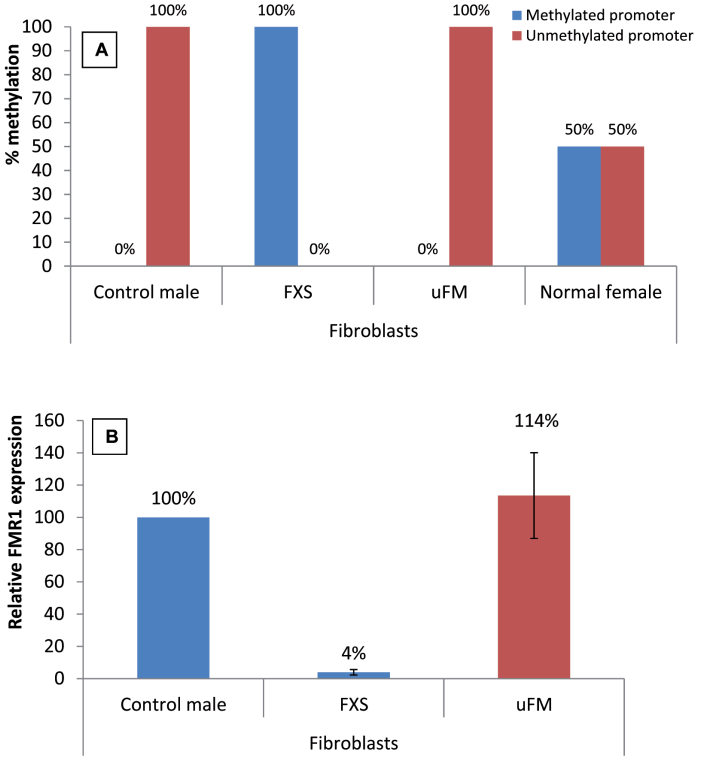


Figure 1. Methylation status and *FMR1* expression levels in the fibroblast cell lines.
A) Methylation status of a region of the *FMR1* promoter in fibroblasts of the male control line, fragile X line (FXS) and the unmethylated full mutation line (uFM). Values are ratios relative to a female fibroblast control line, for which the ratio was set to 50% for both primer sets in the methylation assay (n=2-3 separate measurements). **B**) RT-qPCR data showing *FMR1* transcript levels in fibroblasts of the male control line, fragile X line (FXS) and the unmethylated full mutation line (uFM) normalized to *CLK2* expression. Values are means ± SEM relative to appropriate male control line (n=2-3 separate measurements).

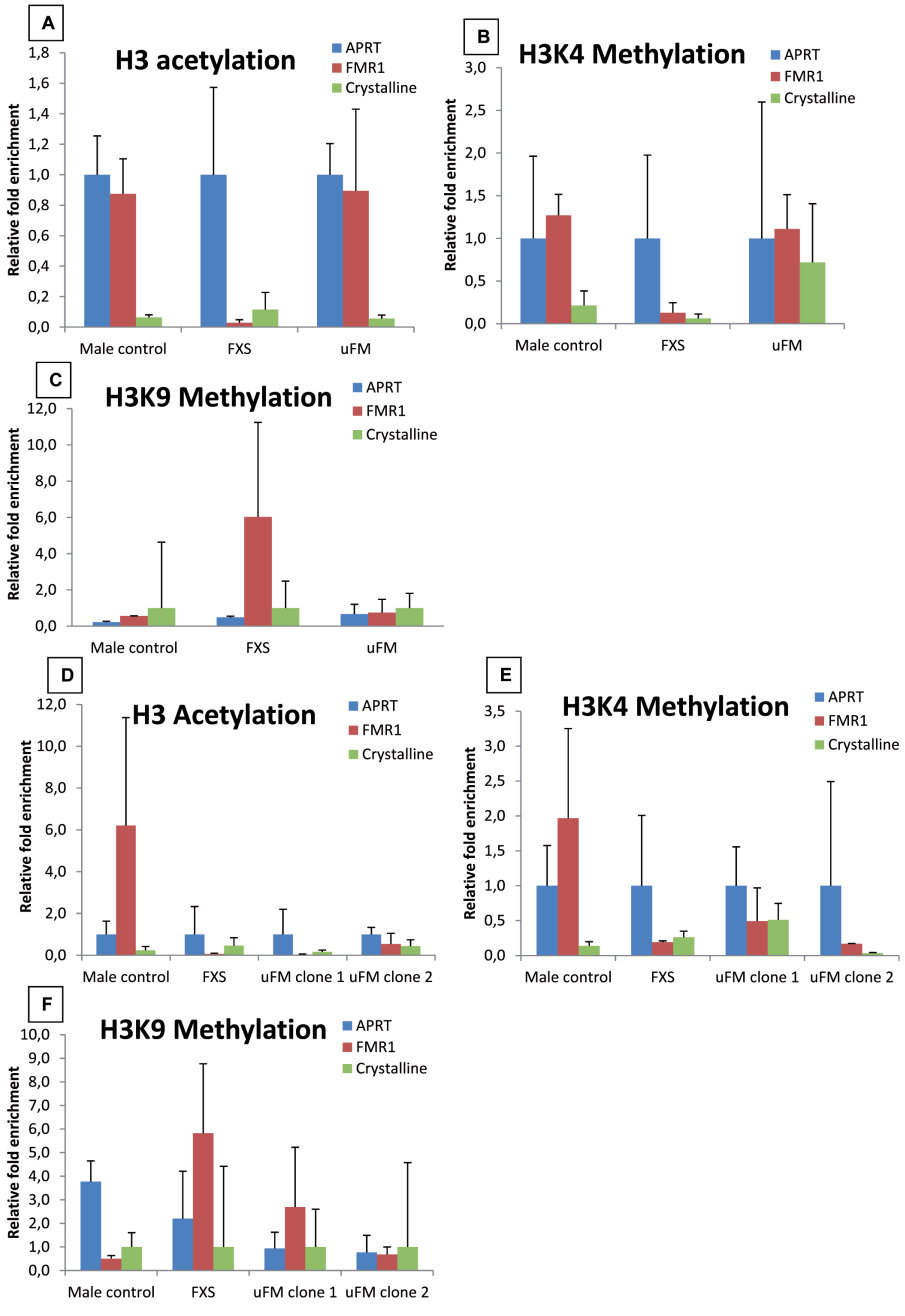


Figure 2. Chromatin immunoprecipitation analysis of H3 acetylation, H3K4 methylation and H3K9 methylation in the *FMR1* promoter of fibroblasts and iPS cells.
Chromatin immunoprecipitation analysis of H3 acetylation, H3K4 methylation and H3K9 methylation in the *FMR1* promoter of fibroblasts (**A**, **B** and **C**) and iPS cells (**D**, **E** and **F**) respectively. Results were normalized to the appropriate positive control (*APRT* or *Crystalline*) and averaged from at least two different experiments.

methylation, at similar levels as the Control line, while the inactive mark H3K9 methylation could not be detected (Figure 2 A, B, C). The *FMR1* promoter of the FXS cell line only showed the repressive mark H3K9 methylation with values higher than the positive control for the repressed mark *Crystalline* and higher than the negative control (*APRT*) (Figure 2 A, B, C).

FIBROBLAST REPROGRAMMING AND iPSC CHARACTERIZATION

The fibroblasts were reprogrammed to iPSC lines according to established protocols [15]. We analyzed one clone per line, except for the uFM line for which we analyzed two clones. All iPSC clones showed typical characteristics of pluripotent stem cells: morphology similar to that of embryonic stem cells, expression of alkaline phosphatase (Figure S1), silencing of retroviral transgenes (data not shown), reactivation of genes indicative of pluripotency (data not shown), immunoreactivity for OCT4, NANOG, Tra-1-60, Tra-1-81 and SSEA4 (Figure S2), the ability to propagate for a long time in culture (up to passage 30) and maintenance of a normal diploid karyotype (Figure S3). All cell lines generated embryoid bodies which, after differentiation *in vitro,* expressed markers of endoderm, mesoderm and ectoderm (Figure S4).

REPROGRAMMING EFFECTS ON CGG REPEAT LENGTH, FMR1 EXPRESSION AND EPIGENETIC MARKS

Analysis of the CGG repeat in the 5'UTR of the *FMR1* promoter indicated that the repeat length did not contract during reprogramming, and that the iPS clones were still within the normal or full mutation range analogous to the fibroblasts they originated from (Figure S5). As expected, the iPSC clone of the Control cell line showed *FMR1* expression, in contrast to the FXS iPSC clone which did not show *FMR1* expression. Unexpectedly, the uFM iPSC clones did not express *FMR1* either (Figure 3A). Further analysis showed that the bisulfite converted *FMR1* promoter region, was methylated in the FXS iPSC clone as well as in both uFM iPSC clones while the Control iPSC cell line did not show any methylation (Figure 3B). Thus, the originally unmethylated extended CGG repeat found in the uFM fibroblasts became methylated at some point during the reprogramming process. In addition, ChIP analysis of the *FMR1* promoter showed enrichment of the active marks H3 acetylation and H3K4 methylation in the Control iPS cell clone to levels higher than the positive control *APRT* while the FXS and one of the uFM iPS cell clones only showed an increase of the repressive mark H3K9 methylation to values above the positive repressive control *Crystalline* (Figure 2 D, E, F). Here we did observe a difference between the two uFM clones analyzed, the H3K9 mark was not enriched in clone 2 (Figure 2 D, E, F). Next, we investigated the effects of differentiation into neural progenitor cells (NPCs, see Figure S6 for staining with marker Sox2) on *FMR1* expression and methylation. NPCs derived from the FXS and uFM iPS cells showed lack of *FMR1* expression and a methylated *FMR1* promoter in contrast to the NPCs derived from the Control iPSC clone which showed clear *FMR1* expression and an unmethylated promoter region (Figure 4A and B). These findings indicate that the reprogramming process leads to methylation of the expanded CGG repeat sequence in *FMR1*, resulting in stable shut down of *FMR1* gene expression.

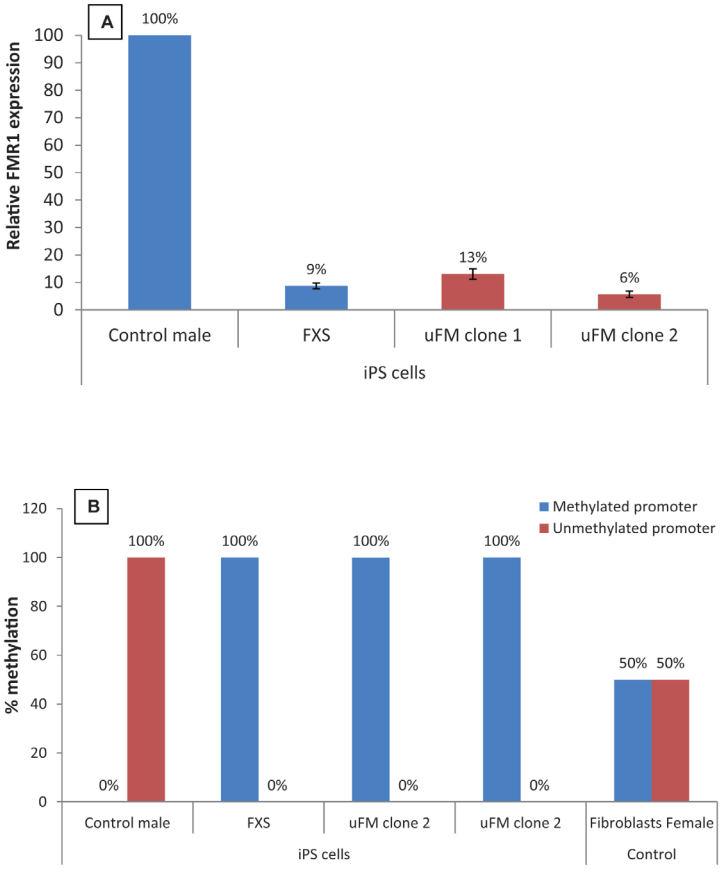


Figure 3. Methylation status and *FMR1* expression levels in the induced pluripotent stem cells. **(A)** RT-qPCR data showing *FMR1* transcript levels in induced pluripotent stem cells (iPSCs) of the male control line, fragile X line (FXS) and the unmethylated full mutation clones (uFM clone 1 and clone 2) normalized to *CLK2* expression. Values are mean ± SEM relative to appropriate male control line (n=2-3 separate measurements). **(B)** Methylation status of a region of the *FMR1* promoter in iPSCs of the male control line, fragile X line (FXS) and the unmethylated full mutation clones (uFM clone 1 and clone 2). Values are ratios relative to a female fibroblast control line, for which the ratio was set to 50% for both primer sets in the methylation assay (n=2-3 separate measurements).

DISCUSSION

We undertook this study in an attempt to unravel the epigenetic mechanisms involved in the silencing of the *FMR1* gene in fragile X syndrome by the use of a fibroblast line carrying an unmethylated full mutation. There have been several attempts to study epigenetic silencing in fragile X syndrome. Eiges *et al*.(2007), have shown that FXS human embryonic stem cells (hESCs) still express FMRP at a level similar to that in unaffected hESCs, while the FMRP level decreases as the hESCs were differentiated. Based on these results it was expected that by reprogramming FXS fibroblasts into pluripotent stem cells, the hypermethylated state of the

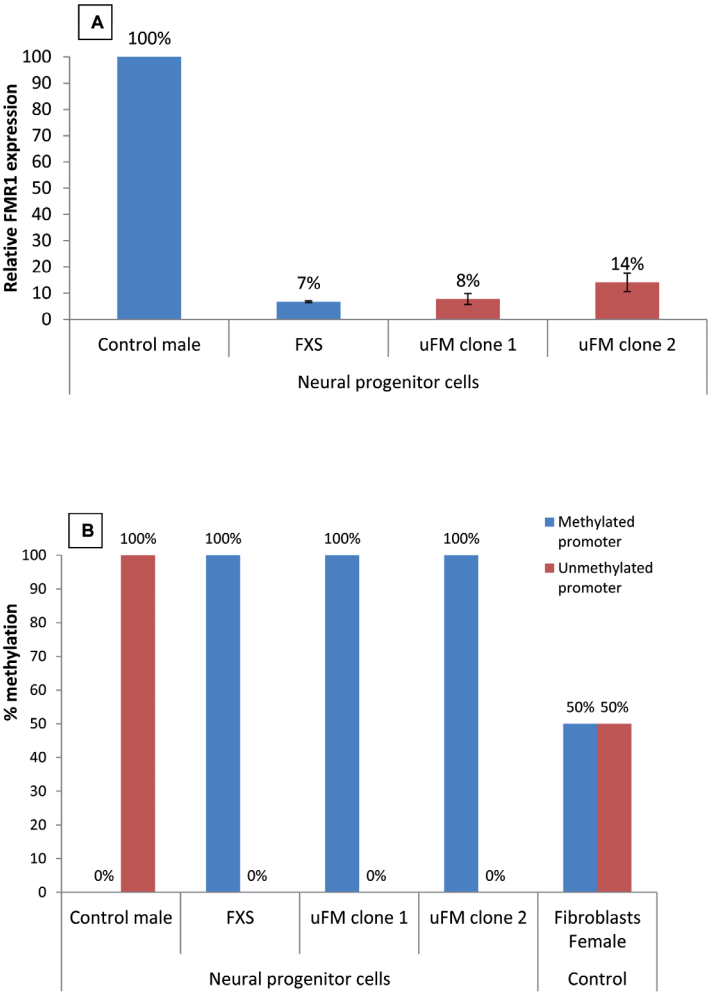


Figure 4. Methylation status and *FMR1* expression levels in the neural progenitor cells.
A) RT-qPCR data showing *FMR1* transcript levels in neural progenitor cells (NPCs) of the male control line, fragile X line (FXS) and the unmethylated full mutation clones (uFM clone 1 and clone 2) normalised to *CLK2* expression. Values are mean ± SE relative to appropriate male control line (n=2 separate measurements). **B**) Methylation status of a region of the *FMR1* promoter in NPCs of the male control line, fragile X line (FXS) and the unmethylated full mutation clones (uFM clone 1 and clone 2). Values are ratios relative to a female fibroblast control line, for which the ratio was set to 50% for both primer sets (n=2-3 separate measurements).

FMR1 promoter region would be reversed. However, by now several research groups have shown that iPSCs derived from FXS patients show epigenetic marks characteristic for heterochromatin similar to the full mutation fibroblasts they originated from [11-13]. These observations could be explained by the fact that the FXS iPS cells may not have all the characteristics of early

pluripotency, but that they represent a later stage of human development [11-13, 16]. Another approach was used in studies with human fragile X lymphoblastic cells, here a fully mutated and hypermethylated *FMR1* gene became reactivated following treatment with 5-azadeoxycytidine, a hypomethylating agent. Although such treatment significantly reduced DNA methylation in some cells, it could not restore all remaining epigenetic marks to control levels [5, 6, 17, 18]. Drugs such as 4-phenylbutyrate, sodium butyrate or trichostatin A, which block the activity of histone deacetylases, did not restore *FMR1* expression to normal levels [5-7, 18]. In addition, treatment with a compound that reduces the *in vitro* expression of the FRAXA fragile site, acetyl-l-carnitine, did not restore the *FMR1* expression either [7]. Recently, 5-azadeoxycytidine treatment was also tested on fragile X iPS cells and it appeared to restore *FMR1* expression in both iPS cells and differentiated neurons, which offers possibilities to use these cells as an epigenetic model [13].

The availability of a fibroblast cell line carrying an unmethylated full mutation (uFM), gave us a new opportunity to study the epigenetic silencing mechanisms in time. We first characterized the uFM fibroblast cell line together with a normal male fibroblast control line and a FXS fibroblast cell line carrying a fully methylated *FMR1* promoter. Although increased *FMR1* mRNA levels (up to 5 times) were reported in lymphoblastoid cells of premutation carriers (55~200 unmethylated CGGs), our findings of normal to slightly increased *FMR1* mRNA levels in the uFM fibroblasts are similar to the findings of Pietrobono *et al*. (2005), who examined a lymphoblastic cell line from the same individual. The lack of DNA methylation ensures that the chromatin is less densely packed and more accessible for transcription, which explains the *FMR1* expression in this cell line. Our ChIP results differ from the original ChIP analysis of the uFM lymphoblastoid cell line [7]. We found a similar increase in H3K4 methylation; however we did not find decreased H3 acetylation levels or intermediate H3K9 levels in the uFM fibroblasts. These differences could be explained by the fact that we have analyzed a distinct cell type (fibroblasts versus lymphoblastoid cells), and by differences in the ChIP protocol (*eg* quantification methods and reference genes used). Since the uFM fibroblast line lacked methylation of the *FMR1* promoter site despite the high number of CGG repeats, we expected to find an unmethylated *FMR1* promoter and normal levels of both *FMR1* mRNA and FMRP after transformation into iPSCs. Surprisingly, we found the promoter region of *FMR1* to be hypermethylated in these two iPSC clones. Other epigenetic chromatin marks also indicated a repressed *FMR1* promoter similar to the marks observed in the fragile X iPS cell line. Only one of the two uFM clones analyzed showed enrichment of the H3K9 mark, although the other clone did not display accumulation of any active marks either. Apparently, H3K9 marks may differ between these two clones for unclear reasons. After differentiation of these iPS cells into neural progenitor cells, the *FMR1* promoter remained methylated and thus silenced.

There are three possible explanations for our findings. Firstly, it is possible that the reprogramming process resulted in iPSCs that were solely derived from methylated FM fibroblasts and not of the unmethylated cells. This assumes that the possibility that methylated FM fibroblasts were present in our culture, which according to our methylation studies seems highly unlikely. Second, there may be an unknown genetic factor present in this individual which was protective against DNA methylation during embryonic development but which

was absent in his fibroblasts or which was altered or blocked during reprogramming. In our case, the brother of this individual was also carrier of an unmethylated full mutation. Being a carrier of an unmethylated full mutation is already a very rare phenomenon, but the fact that two children escaped methylation in one family clearly points towards the involvement of a maternal-paternal genetic component or environmental factors. Finally, the reprogramming process might activate genes that induce de novo methylation of the *FMR1* promoter. Although the *FMR1* gene in this individual escaped methylation during embryonic development, the full mutation in his fibroblasts might be recognized by epigenetic remodelers *eg* by histone and/or DNA methyltransferases that are not recruited in embryonic development. This would also explain the unmethylated full mutation observed in human embryonic FXS stem cells because these cells never went through this reprogramming process. In conclusion, standard reprogramming of somatic uFM fibroblasts into pluripotent stem cells by using 4 transcription factors, did not lead to de-methylation of the expanded CGG repeat, and even induced methylation of an unmethylated template. Very recently, Gafni *et al*. (2013) suggested that a more naïve ground state pluripotent stem cell in which epigenetic memory is completely erased could be obtained by an unique combination of cytokines and small molecule inhibitors [16]. This finding indicates that the de novo methylation of the *FMR1* promoter that we obtain in our uFM iPSCs may represent an intermediate state that can be reverted by further de-differentiation into naïve iPSCs. Most likely, these naïve pluripotent stem cells will offer new possibilities in understanding the mechanism behind the silencing of the *FMR1* gene in fragile X syndrome.

ACKNOWLEDGEMENTS

The authors thank Dr. A. Schambach for kindly sharing the iPSC reprogramming vector. This work was supported by the Netherlands Organisation for Health Research and Development (RW; ZonMw; 912-07-022), E-Rare program entitled "Cure FXS" (RW; no. EU/FISPS09102673), the Prinses Beatrix Spierfonds (WWMP and EvdW, project number: W.OR13-21), by a fellowship of the Hersenstichting Nederland (FMSdV) and the NeuroBasic-PharmaPhenomics consortium (SAK). All authors declare no financial conflict of interest.

REFERENCES

1. Pearson, C.E., K. Nichol Edamura, and J.D. Cleary, *Repeat instability: mechanisms of dynamic mutations*. Nat Rev Genet, 2005. **6**(10): p. 729-42.
2. Verkerk, A.J., et al., *Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome*. Cell, 1991. **65**(5): p. 905-14.
3. Willemsen, R., et al., *Timing of the absence of FMR1 expression in full mutation chorionic villi*. Hum Genet, 2002. **110**(6): p. 601-5.
4. Sutcliffe, J.S., et al., *DNA methylation represses FMR-1 transcription in fragile X syndrome*. Hum Mol Genet, 1992. **1**(6): p. 397-400.
5. Coffee, B., et al., *Acetylated histones are associated with FMR1 in normal but not fragile X-syndrome cells*. Nat Genet, 1999. **22**(1): p. 98-101.
6. Coffee, B., et al., *Histone modifications depict an aberrantly heterochromatinized FMR1 gene in fragile x syndrome*. Am J Hum Genet, 2002. **71**(4): p. 923-32.
7. Tabolacci, E., et al., *Differential epigenetic modifications in the FMR1 gene of the fragile X syndrome after reactivating pharmacological treatments*. Eur J Hum Genet, 2005. **13**(5): p. 641-8.
8. Pietrobono, R., et al., *Molecular dissection of the events leading to inactivation of the FMR1 gene*. Hum Mol Genet, 2005. **14**(2): p. 267-77.
9. Eiges, R., et al., *Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived from Preimplantation Genetically Diagnosed Embryos*. Cell Stem Cell, 2007. **1**(5): p. 568-577.
10. Gerhardt, J., et al., *The DNA replication program is altered at the FMR1 locus in fragile X embryonic stem cells*. Mol Cell, 2014. **53**(1): p. 19-31
11. Urbach, A., et al., *Differential modeling of fragile X syndrome by human embryonic stem cells and induced pluripotent stem cells*. Cell Stem Cell, 2010. **6**(5): p. 407-11.
12. Sheridan, S.D., et al., *Epigenetic characterization of the FMR1 gene and aberrant neurodevelopment in human induced pluripotent stem cell models of fragile x syndrome*. PLoS ONE, 2011. **6**(10): p. e26203.
13. Bar-Nur, O., I. Caspi, and N. Benvenisty, *Molecular analysis of FMR1 reactivation in fragile-X induced pluripotent stem cells and their neuronal derivatives*. J Mol Cell Biol, 2012. **4**(3): p. 180-3.
14. Smeets, H., et al., *Normal phenotype in two brothers with a full FMR1 mutation*. Hum Mol Genet, 1995. **4**(11): p. 2103-2108.
15. Takahashi, K., et al., *Induction of pluripotent stem cells from fibroblast cultures*. Nat Protoc, 2007. **2**(12): p. 3081-9.
16. Gafni, O., et al., *Derivation of novel human ground state naive pluripotent stem cells*. Nature, 2013. **504**(7479): p. 282-6.
17. Chiurazzi, P., et al., *In vitro reactivation of the FMR1 gene involved in fragile X syndrome*. Hum Mol Genet, 1998. **7**(1): p. 109-113.
18. Chiurazzi, P., et al., *Synergistic effect of histone hyperacetylation and DNA demethylation in the reactivation of the FMR1 gene*. Hum Mol Genet, 1999. **8**(12): p. 2317-2323.
19. Warlich, E., et al., *Lentiviral vector design and imaging approaches to visualize the early stages of cellular reprogramming*. Mol Ther, 2011. **19**(4): p. 782-9.
20. de Brouwer, A.P., H. van Bokhoven, and H. Kremer, *Comparison of 12 reference genes for normalization of gene expression levels in Epstein-Barr virus-transformed lymphoblastoid cell lines and fibroblasts*. Mol Diagn Ther, 2006. **10**(3): p. 197-204.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

CELL CULTURE

The rare fibroblast cell line established from a normal male carrying an unmethylated full mutation first described by Smeets *et al.* (1999) (uFM) was used. This line has been subcloned, so that a homogenous population of cells that carry a fully extended repeat was obtained. Fibroblasts from a clinically diagnosed male fragile X syndrome patient (14 years, FXS), and an unrelated unaffected male (3 years, Control) and female control fibroblast line (9 years) were all obtained from the cell repository of the department of Clinical Genetics, Erasmus MC, Rotterdam. The fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-Invitrogen) containing 10% fetal calf serum and 1% penicillin/streptomycin.

iPS CELL GENERATION

Reprogramming of human primary skin fibroblasts was performed as described previously [19]. Briefly, fibroblasts were infected with a single, multicystronic lentiviral vector encoding OCT4, SOX2, KLF4, and MYC and cultured on γ-irradiated mouse embryonic feeder (MEF) cells until iPSC colonies could be picked [19]. The iPS cells were cultured in standard ES cell culture medium containing DMEM/F12 (Gibco-Invitrogen) supplemented with 20% knock-out serum replacement (Gibco-Invitrogen), 2mM L-glutamine, 1: 100 dilution of penicillin/streptomycin/glutamine, 1:100 dilution of MEM-non-essential aminoacids (PAA Laboratories GmbH), 0.1mM β-mercaptoethanol, and 10 ng/ml bFGF (Invitrogen) filtered through a 0.22μm filter (Corning). Cells were grown in 6-wells plates on γ-irradiated MEFs and passaged weekly using collagenase IV (1 mg/ml, Invitrogen).

DIFFERENTIATION OF THE iPS CELLS

In-vitro differentiation of embryonic bodies

To form embryonic bodies (EBs), iPSC colonies from 2 wells per line were broken up by collagenase IV treatment and transferred to ultra-low attachment 6-wells plates (Corning). Floating EBs were then cultured in iPSC medium without bFGF for a minimum of 6 days with supplemented SB431542 for ectoderm conditions only. The EBs designated for endoderm were then transferred to gelatin coated 12-wells plates containing the following medium: RPMI 1640 (Gibco-Invitrogen), supplemented with 20% FBS, 1: 100 dilution of penicillin/streptomycin/glutamine and alpha-thioglycerol (0.4mM). Mesoderm differentiation from the EBs was inducted in gelatin coated 12-wells plates with DMEM low glucose medium supplemented with 15% FBS, 1: 100 dilution of penicillin/streptomycin/glutamine and 1:100 dilution of MEM-non-essential amino acids. The formation of ectoderm was induced in matrigel coated plates with the following medium: neurobasal medium (Gibco) and DMEM/F12 (v/v 50/50) supplemented with 1: 100 dilution of penicillin/streptomycin/glutamine and 1:100 dilution of MEM-non-essential aminoacids, 0.02% BSA (Gibco), 1:200 N2 (Gibco) and 1:100 B27 (Gibco). After two weeks in culture the cells were fixed with formalin and immunostainings were performed.

Neural differentiation

Human iPS cells were differentiated according to Brennand *et al*. (2011), with modifications. Briefly, iPS colonies were dissociated from MEFs with collagenase (100 U/ml) and transferred to non-adherent plates in hES cell medium on a shaker in an incubator at 37ºC/5% CO2. After two days, embryonic bodies (EBs) were placed in neural induction medium (DMEM/F12, 1x N2, 2 µg/ml heparin, penicillin/streptomycin) and cultured for another four days in suspension. EBs were gently dissociated and plated onto laminin coated dishes in NPC medium (DMEM/F12, 1x N2, 1x B27-RA, 1 µg/ml laminin and 20 ng/ml FGF2, penicillin/streptomycin). After one week, NPCs were dissociated with collagenase (100 U/ml), replated, and used for staining and methylation analysis after 3-5 passages. All cell culture reagents were obtained from Invitrogen.

Karyotype analysis

For karyotype analysis, cells in a well of a 6-wells plate were treated with colcemid (100 ng/ml) for 1 hour. Then cells were harvested with trypsin, treated with hypotonic solution and fixed. Metaphases were spread onto glass slides and stained with DAPI (Dako). Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature. At least 10 metaphases were analyzed per cell line.

Alkaline phosphatase staining and immunocytochemistry

Staining for alkaline phosphatase was carried out using the Alkaline Phosphatase kit (Sigma-Aldrich) according to the manufacturer's instructions. For immunocytochemistry, iPS cells or differentiated iPS cells were washed with PBS once, fixed with 4% formalin solution for 5 min and washed again with PBS. Cells were then incubated with 50mM glycine for 5 min, washed with PBS and permeabilized with 0.5% Triton X-100 for 5 min (only for Oct4 and Nanog). After blocking for 45 min at room temperature with 0.1% PBS-Tween containing 2% fetal bovine serum (Invitrogen), primary antibody staining was performed for 1 hour in room temperature with antibodies diluted in blocking solution. Cells were then washed and incubated with the appropriate secondary Cy3 or Alexa Fluor A555 antibody (1:200, Jackson Immunoresearch Laboratories or Invitrogen) for 45 min. Afterwards, cells were washed with twice 0.1% PBS-Tween, with a nuclear staining step in between (Hoechst or DAPI). Cells were covered with Mowiol and a glass slide. The antibodies used for pluripotency stainings or neural marker stainings were goat anti human OCT3/4 (1:100, Santa Cruz Biotechnology), goat anti human NANOG (1:50, R&D Systems), mouse anti human Tra-1-60, Tra-1-80, and SSEA4 (1:100 Santa Cruz Biotechnology) and rabbit anti Sox2 (1:1000 Millipore). Antibodies used for *in vitro* differentiation stainings were anti human smooth muscle actin (1:50, DAKO), rabbit anti human alpha-fetoprotein (1:200, Dako), mouse anti human β-tubulin III (TujI) (1:200, Sigma-Aldrich).

RNA isolation and FMR1 expression analysis

RNA was isolated using the RNAeasy kit (Qiagen), and 1 µg of RNA was reverse transcribed using iScript (BioRad). Real-time PCR was carried out in triplicate using Kappa mix and a 7300 Real-time PCR system (Applied Biosystems). The following forward and reverse primers were used to measure *FMR1* expression: 5'-GGTGGTTAGCTAAAGTGAGGA-3' and 5'-GTGGCAGGTTTGTTGGGATTA-3'. *CLK2* was used as reference gene with forward primer

5'-CCTACAACCTAGAGAAGAAGCGAG-3' and reverse primer 5'-CACTGCCAAAGTCTACCACC-3' [20]. *FMR1* expression was normalized to *CLK2* expression and data was presented as an average value from 2 to 3 independent measurements. The expression values of the male control and the female control cell lines were combined and their average relative fold enrichment was set to 100%.

DNA isolation, CGG repeat length and methylation analysis

In order to isolate total genomic DNA, cell were treated with lysis buffer containing 100mM NaCl, 10mM Tris, 15mM EDTA, 0,5% SDS, and 5% Proteinase K. After overnight incubation at 55°C, DNA was extracted and precipitated using a standard protocol containing saturated salt solution and ethanol.

CGG repeat size was determined in a PCR reaction using the primers 5'-CGGAG GCGCCGCTGCCAGG-3' and 5'-TGCGGGCGCTCGAGGCCCAG-3' with the Expand high fidelity PCR kit (Roche) supplemented with 2.5 M betaine. PCR was performed with 35 cycles of 35 seconds denaturing at 98°C, 35 seconds of annealing at 55°C, and 5 minutes elongation at 72°C. PCR products were analyzed with standard agarose gel electrophoresis.

Genomic DNA was modified by bisulfite treatment according to the EpiTect Bisulfite Kit. The diluted converted DNA was then measured using quantitative PCR with primers designed specifically for a region of *FMR1* promoter containing 53 methylation sites. Primers sets contained the methylated and the unmethylated DNA sequence of this region after bisulfite conversion, thus respectively F 5'-GGTCGAAAGATAGACGCGC-3', R 5'-AAACAA TGCGACCTATCACCG-3'; and F 5'-TGTTGGTTTGTTGTTTGTTTAGA-3', R 5'-AACATAATTTCAATATTTACACCC-3' and for the unmethylated bisulfite converted reference gene *CLK2*: F 5'- CGGTTGATTTTGGGTGAAGT-3' and R 5'-TCCCGACTAAAATCCCACAA-3'. All reactions were carried out in triplicate using SYBR Green ROX mix and a 7300 Real-time PCR system. For each sample, the values for the methylated and the unmethylated sequences were normalized to *CLK2* expression. Experiments were only analyzed when the Ct values of the female control sample were below 30 for both primer sets. The normalized values of the female control cell line were then set to 50%, because of random X inactivation in female cells. The normalized data was presented as an average ratio from at least three independent measurements.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed according to the Upstate ChIP protocol with some small modifications. In short, approximately 2.5 million cells were crosslinked with 1% formaldehyde for 5 minutes at room temperature. After quenching the reaction with 125 mM glycine, cells were subsequently suspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1) containing proteinase inhibitor (Roche, Complete). Chromatin was then sonicated using the Bioruptor (Diagonide) to create 200bp-1000bp DNA fragments. All chromatin was pre-cleared by treatment with salmon sperm agarose beads (Millipore) for 0.5 hour at room temperature. Immunoprecipitation was performed overnight using 7.5 µg anti-acetylated histone H3 (Millipore), anti-dimethyl histone H3K4 (Millipore), anti-trimethyl histone H3K9 (Millipore), or anti-IgG antibody (Millipore) in dilution buffer. Next, crosslinking was reversed by incubation with 0.2M NaCl at 65ºC and DNA was purified using a PCR clean-up kit (Mobio). Finally, eluted

DNA fragments were used for quantitative PCR analysis using primers for the *FMR1* promoter region F 5'-AACTGGGATAACCGGATGCAT-3' and R 5'- GGCCAGAACGCCCATTTC-3' as well as appropriate positive and negative controls namely *APRT* F 5'-GCCTTGACTCGCACTTTT-3', and R 5'-TAGGCGCCATCGATTTTA-3' and *Crystalline* F 5'-CCGTGGTACCAAAGCTGA-3', and R 5'-AGCCGGCTGGGGTAGAA-3'. The Ct values of both histone modifications were first normalized for the non-specific IgG antibody treatment and then for the amount of input DNA. Data was then presented in relative fold enrichment after further normalization to the *APRT* gene for H3 acetylation and H3K4 methylation and *Crystalline* for H3K9 methylation. Data from at least two separate experiments were averaged and both reference genes were previously used by Urbach *et al*. (2010) and Bar-Nur *et al*. (2012).

SUPPLEMENTAL FIGURES

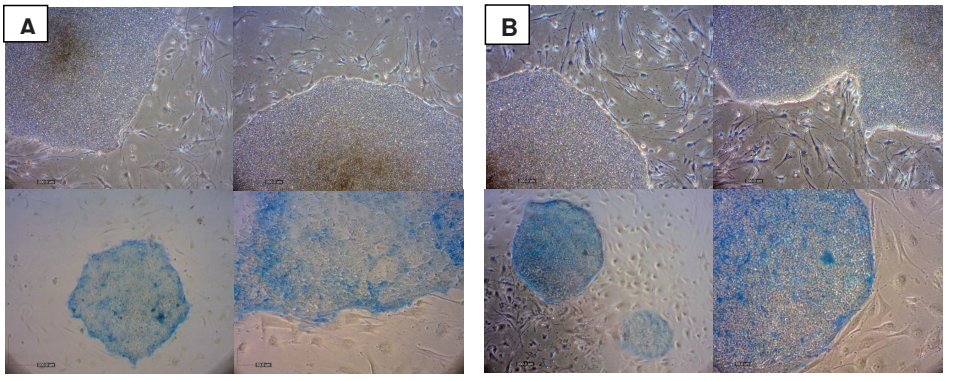


Figure S1. Morphology of the induced pluripotent stem cells.
Morphology after passage 3 (upper panel) and alkaline phosphatase staining at passage 6 (lower panel) in **A**) the male control iPSC line and **B**) the fragile X iPSC line

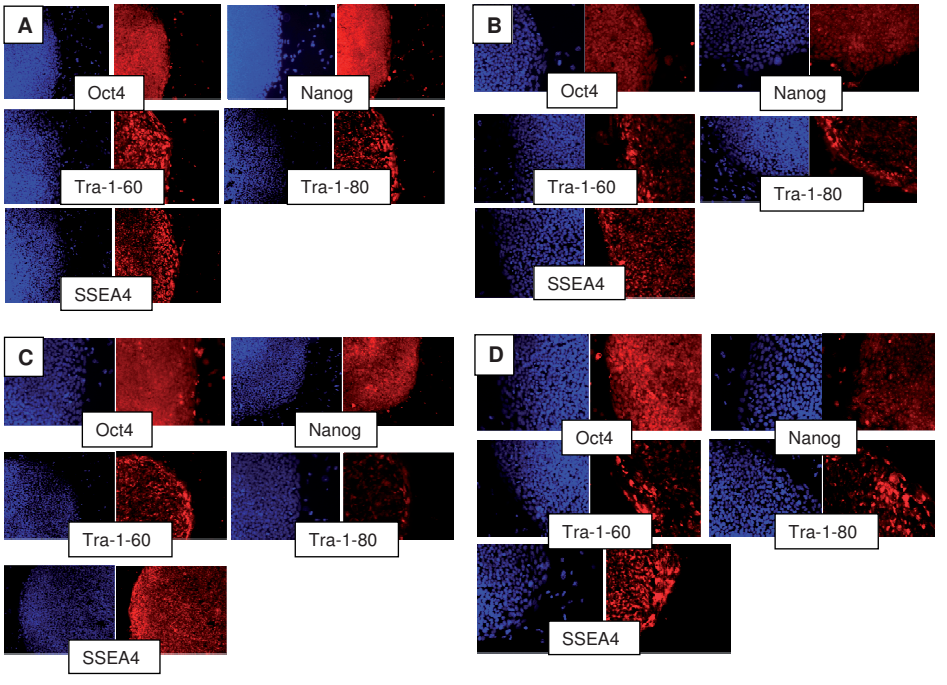


Figure S2. Expression of pluripotency markers by iPSCs.
From left to right and top to bottom you can see Oct4, Nanog, Tra-1-60, Tra-1-80 and SSEA4 (all in red) expression in the control line (A), fragile X cell line (B) and the uFM clones (C and D, clone 1 and clone 2 respectively). For each marker a nuclear Hoechst staining (in blue) is displayed as well.

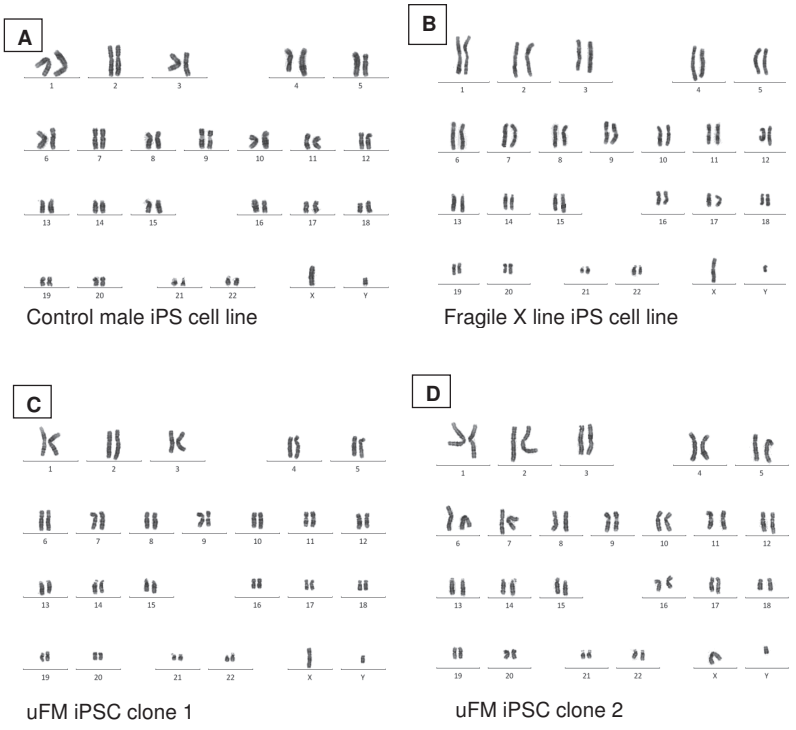


Figure S3. Representative image of karyotype analysis of all iPSC clones.
The control male iPSC line (**A**), the fragile X cell line (**B**) and the uFM iPSC clones (**C** and **D**) showed a normal karyotype (46, XY) in at least 10 spreads.

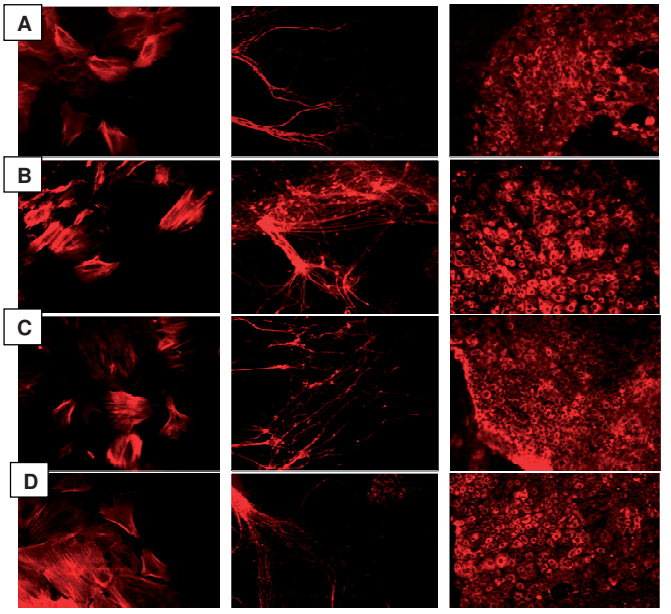


Figure S4. *In vitro* differentiation of iPS cells into different germ layers leads to clear expression of the mesodermal marker smooth muscle actin, the ectodermal marker Tuj1 and the endodermal marker alpha-fetoprotein by all iPS cell clones.
The mesodermal marker smooth muscle actin, the ectodermal marker Tuj1 and the endodermal marker alpha-fetoprotein (all in red) were expressed after *in vitro* differentiation by **A**) control line, **B)** fragile X cell line, **C)** uFM clone 1 and **D)** uFM clone 2.

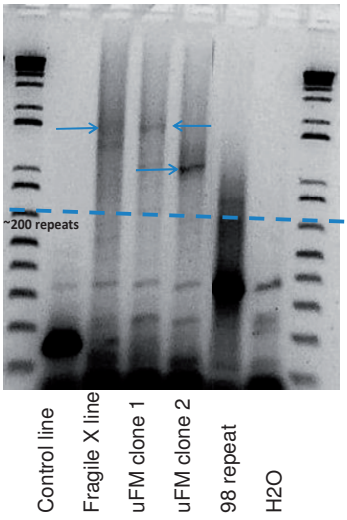


Figure S5. Repeat length analysis after reprogramming into iPS cells.
Products of the CGG repeat PCR were run on an agarose gel. The control iPSC line shows repeat lengths under 55, while the remaining iPS cell clones show repeat lengths above 200.

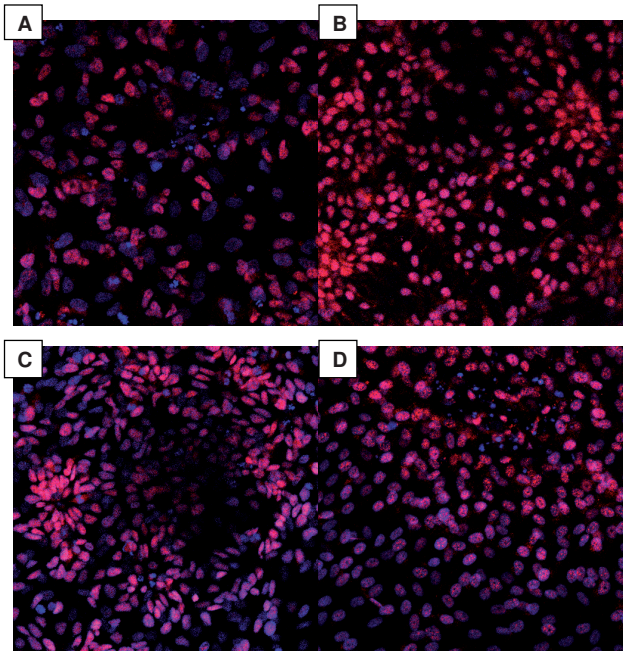


Figure S6. SOX2 expression by neural progenitor cells.
Expression of SOX2 in red by neural progenitor cells of the control line (**A**), fragile X cell line (**B**) and the uFM clones (**C** and **D,** clone 1 and clone 2 respectively). In each image a nuclear DAPI staining (in blue) is displayed as well.

CHAPTER 3

CHRONIC ADMINISTRATION OF AFQ056/MAVOGLURANT RESTORES SOCIAL BEHAVIOR IN *Fmr1* KO MICE

Ilse Gantois, Andreea S. Pop, Celine E.F. de Esch, Ronald A.M. Buijsen, Tine Pooters, Baltazar Gomez-Mancilla, Fabrizio Gasparini, Ben A. Oostra, Rudi D'Hooge and Rob Willemsen

Behavioural Brain Research, 2013 Feb 15;239:72-9.

ABSTRACT

Fragile X syndrome is caused by lack of *FMR1* protein (FMRP) leading to severe symptoms, including intellectual disability, hyperactivity and autistic-like behavior. FMRP is an RNA binding protein involved in the regulation of translation of specific target mRNAs upon stimulation of metabotropic glutamate receptor 5 (mGluR5) at the synapse. Absence of FMRP leads to enhanced activity of mGluR5 signal transduction pathways. Many conflicting results have been reported regarding social behavior deficits in *Fmr1* knockout mice, and little is known about the involvement of mGluR5 pathways on social behavior. In this study, a three-chambered task was used to determine sociability and preference for social novelty in *Fmr1* knockout mice. Disruption of *Fmr1* functioning resulted in enhanced interaction with stranger mouse during sociability while no significant changes were observed during preference for social novelty assay. Chronic administration of a specific mGluR5 antagonist, AFQ056/Mavoglurant, was able to restore sociability behavior of *Fmr1* knockout mice to levels of wild type littermates. These results support the importance of mGluR5 signaling pathways on social interaction behavior and that AFQ056/Mavoglurant might be useful as potential therapeutic intervention to rescue various behavioral aspects of the fragile X phenotype.

Keywords: Fragile X syndrome; FMR1; Mouse model; Metabotropic glutamate receptor 5; Social behavior; AFQ056/Mavoglurant

1. INTRODUCTION

Fragile X syndrome (FXS) is a widespread hereditary neurological disorder characterized by intellectual disability, physical abnormalities, epileptic seizures, anxiety and high incidences of autistic-like features [1]. The disease is caused by absence of fragile X mental retardation protein (FMRP) due to abnormal methylation of the fragile X mental retardation 1 gene (*FMR1*) as a result of an expanded CGG trinucleotide repeat (>200 CGGs) in the 5' untranslated region [2-4]. FMRP is highly expressed in neurons of the brain and is an RNA-binding protein involved in controlling mRNA translation at the synapse [5].

Activation of group 1 metabotropic glutamate receptors (mGluRs), especially mGluR5, leads to local translation of specific mRNAs at the synapse, including *FMR1* itself [6, 7]. During this process, FMRP acts as a translational repressor and lack of FMRP results in excessive translation of several target mRNAs involved in synaptic plasticity, including Map1b, SAPAP4, PSD95, CaMKII, Arc and PIKE-S [8-11]. Consequently excessive AMPA receptor internalization in the postsynaptic membrane occurs. Loss of these receptors is proposed to be responsible for several phenotypes including abnormal spine density and spine morphology and changes in electrophysiology (both long-term potentiation and long-term depression, LTP and LTD, respectively). The morphology and density of spines on dendrites of hippocampal and cerebral cortex neurons are abnormal in FXS [12-14].

The *Fmr1* knockout (KO) mouse, which does not express Fmrp, exhibits several of the neurobehavioral symptoms/features observed in humans with FXS, including hyperactivity, epileptic seizures and cognitive deficits [15]. Moreover, the *Fmr1* KO mouse exhibits abnormal spine morphology and density on dendrites and enhanced group 1 mGluR LTD [16]. FXS presents with a distinct behavioral phenotype which overlaps significantly with that of autism [1, 17-20] such as avoidance gaze and bodily contact, tactile defensiveness and repetitive and preseverative behaviors such as hand flapping or hand-biting. However, the behavior of persons with FXS is somewhat different from the one of people with autism since FXS persons show more social interest in terms of communication and dialogue, and they clearly seek social interaction [18-20]. In addition, several of the social behavioral deficits are exhibited in *Fmr1* KO mice, including social dominance, interaction and recognition [21-26]. Variable results have been reported depending on mouse background and experimental protocols.

Current research is focused on identification of specific therapeutic targets to treat FXS [27]. Genetic down-regulation of mGluR5 expression in *Fmr1* KO mice rescued several of the FXS phenotypes [28, 29]. Consequently, to reduce the excessive activity of mGluR5 and internalization of AMPA receptors, selective mGluR5 antagonists may offer effective treatment of the symptoms of FXS [27]. In *Fmr1* KO mice, administration of 2-methyl-6-phenylethynyl-pyridine hydrochloride (MPEP), a prototypic mGluR5 antagonist, rescued audiogenic seizures, AMPA internalization, prepulse inhibition (PPI) and immature spine morphology [7, 30-32]. Unfortunately MPEP is not mGluR5 specific and has been shown to inhibit NMDA receptors as well [33, 34] indicating that more specific mGluR5 antagonist drugs might be needed for therapeutic intervention to reverse specific symptoms of FXS. Administration of AFQ056/Mavoglurant, a selective mGluR5 antagonist, in *Fmr1* KO mice resulted in the rescue of PPI, associative motor learning and avoidance defects and spinal dysmorphia [35, 36]. Moreover, in

FXS patients with full methylation of the *FMR1* promoter, AFQ056/Mavoglurant treatment did improve behavioral attributes [37].

Using a three-chambered task to test for sociability and preference for social novelty (SPSN), our findings show that (a) *Fmr1* KO mice display enhanced social interaction behavior during the sociability assay of the SPSN task and (b) this aberrant behavior is mGluR5 signaling pathway dependent and can be rescued by administering the selective mGluR5 antagonist AFQ056/Mavoglurant.

2 MATERIALS AND METHODS

2.1 Animals

Generation of male *Fmr1* KO mice and WT littermates was established as described previously [38]. The mice were > 10 x backcrossed in C57BL/6J background. Animals used for experiments were adult males. All animals were group housed if possible (3-4 animals in cage; fighting males were separated) and held in animal rooms with 12 h light- 12 h dark-cycle (lights on at 8.00 h), room temperature of 20 °C and 50% air humidity. Food and water was available *ad libitum* at all times. Experiments were performed during light phase (morning) and animals were always transported to the experimental room at least 30 min before the start of the experiment to acclimate.

A first group of male mice (*Fmr1* KO: n = 11; WT littermates: n = 11; untreated group) was transported to Laboratory of Biological Psychology at the age of approximately 9 weeks (1 WT animal was separate housed, other animals group housed), and behavioral testing started at the age of 12 - 13 weeks. A second group of male mice (*Fmr1* KO: n = 11; WT littermates: n = 11; AFQ056/Mavoglurant treated group) was transported to Laboratory of Biological Psychology at the age of approximately 12 weeks, received chronic administration of AFQ056/Mavoglurant via food pellets during 3 weeks before being tested in behavioral SPSN set up.

2.2 Ethical aspect

All protocols were reviewed and approved by the animal ethics committee of University of Leuven and the animal welfare committee of Erasmus MC and were performed in accordance with the European Community Council Directive (86/609/EEC).

2.3 Sociability/preference for social novelty task (SPSN)

The SPSN task was a modified version of a protocol described by Nadler and colleagues [39, 48]. Setup was made of transparent Plexiglass and consisted of 3 chambers. The central chamber (36 x 28 x 30 cm) was divided from a left and right chamber (29 x 28 x 30 cm) by sliding doors (w x h: 6 x 8 cm). Left and right chamber contained cylindrical wire cups (height x diameter: 11 x 12 cm) that could contain stranger mice. Stranger mice used during the test were adult, male animals of a C57BL/6J background (Elevage Janvier, Le-Genest-Saint-Isle, France). Two cameras mounted above the setup (60 cm) transferred images to a computer with ANY-maze™ Video Tracking System software (Stoelting Co., IL, USA).

Testing comprised three assays: acclimation assay, sociability assay and preference for social novelty assay. During the acclimation assay, the test mouse was placed in the central compartment for 5 min without access to the left and right chamber. During the sociability

assay, a stranger mouse (STR1) was placed into the wire cup randomly in either the left or right chamber. The other chamber contained an empty wire cup. Sliding doors were opened and the test mouse could freely explore all three chambers. After 10 min, the test mouse was guided into the central compartment and sliding doors were closed. A second stranger mouse (STR2) was placed in the remaining wire cup while STR1 stayed in its wire cage. The divider doors were opened for a 10 min exploration of the 3 chambers.

Preferential exploration of STR1 over the empty cup (sociability assay) and STR2 over STR1 (preference for social novelty assay) were recorded and analyzed. Explorative social behavior towards stranger mice was scored using ANY-maze™ Video Tracking System software (Stoelting Co., IL, USA). Time spent in each chamber, number of entries and total distance travelled as well as velocity were calculated. Manually scored behavior (time spent sniffing the wire cups) was defined as the time the test animal was in direct nose contact with the wire cup, and was scored in a blinded manner.

Stranger mice were 16 male C57BL/6J mice (adult age) that were group-housed (4 per cage) and that had served as stranger mice in other SPSN experiments. They were used in counterbalanced way and only once a day. STR1 and STR2 mice always came from different home cages. Mice were tested in the morning, during the light phase.

2.4 Drug treatment

AFQ056/Mavoglurant is a selective mGluR5 antagonist, with a non-competitive inhibitory mode of action. AFQ056/Mavoglurant is currently in clinical development and undergoing a number of clinical trials. PK experiments in mice showed that AFQ056/Mavoglurant has short plasmatic and brain half-life (0.2 h i.v. administration) with no detectable level 24 h after oral administration of 30 mg/kg (limit of quantification 5 nM (plasma), 15 nM (brain) [40]. To determine PK values in mice fed with food pellets containing AFQ056/Mavoglurant (150mg/kg; Bio Services BV, postbox 29, 5400 AA Uden), 3 WT mice were fed AFQ056/Mavoglurant-food pellets for 4 days and sacrificed to measure the blood/brain ratio of AFQ056/Mavoglurant. Based on an average intake of 3 gram food pellets per day and a body weight of approximately 25 gram a dose of 18mg/kg/day is established. The AFQ056/Mavoglurant concentration was determined by liquid chromatography separation followed by mass spectrometry (LC-MS). Control mice received normal food. SD from the PK measurements in plasma were 95 nM (± 90; individual values: 31; 56; 199) and in brain were 244 nM (± 200; individual values: 89; 162; 479). The results indicated the average brain to blood ratio for the AFQ056/Mavoglurant concentration to be 2.7. This means that AFQ056/Mavoglurant passes the blood brain barrier and is effectively delivered to the brain. For chronic administration of AFQ056/Mavoglurant, mice (KO and WT, n = 22) received food pellets containing AFQ056/Mavoglurant and were tested in SPSN essay after 3 weeks of administration.

2.5 Statistical analysis

Data were analyzed with Sigmastat 4 (Aspire Software International, Ashburne, VA, USA) and SPSS (IBM, NY, USA) for Windows statistical software. Effects were evaluated with repeated measures analysis of variance (ANOVA) with genotype (KO and WT) and/or treatment (untreated and AFQ056/Mavoglurant treated) as between-subjects factors and stranger side (empty and STR1 or STR1 and STR2) and time (time bins 1-5; when looking at sniffing time over

different time points) as within-subjects factors. Tukey's studentized range test (α-level set at 0.05) was used for *post hoc* evaluation. Data are represented as mean ± standard error of the mean (S.E.M.).

3. RESULTS

3.1 Chronic administration of AFQ056/Mavoglurant rescued sociability in Fmr1 KO mice

Sociability was assessed with social approach assay in SPSN task. During this essay a stranger mouse (STR1) was placed into the wire cup in one of the chambers while the other chamber contained as novel object an empty wire cup (empty).

3.1.1 Sniffing time during sociability assay

Measures of direct sniffing at both wire cups were taken during the assay, which is a measurement of direct interaction [39, 41]. Both untreated and AFQ056/Mavoglurant treated WT and Fmr1 KO mice demonstrated preference for sniffing at STR1 mouse over the novel object (empty) (Figure 1A).

Untreated WT and *Fmr1* KO mice (Figure 1A, left panel) showed significant higher sniffing at STR1 mouse [two-way repeated measures ANOVA: main effect of stranger side, $F(1,20) = 58.383$, $P < 0.001$]. Also a main effect of genotype was determined [$F(1,20) = 5.373$, $P = 0.031$] where KO mice spent more time in proximity to STR1 than WT mice ($P = 0.006$). Interaction between genotype x stranger side approached significance [$F(1,20) = 3.235$, $P = 0.087$]. Chronic administration of AFQ056/Mavoglurant indicated for both KO and WT mice (Figure 1A, right panel) higher sniffing time at STR1 compared with empty cage with significant main effect of stranger side [two-way repeated measures ANOVA: $F(1,20) = 43.652$, $P < 0.001$], while there was no significant main effect of genotype [$F(1,20) = 0.024$, $P = 0.878$] or genotype x stranger side interaction [$F(1,20) = 0.061$, $P = 0.808$].

Chronic administration of AFQ056/Mavoglurant in WT mice had no significant effect on sociability compared with untreated WT mice (Figure1A, white bars) [repeated measures ANOVA, main effect of treatment: $F(1,20) = 1.882$; $P = 0.185$; treatment x stranger side interaction: $F(1,20) = 0.171$; $P = 0.684$]. This indicated that measurements in both untreated and treated groups are reliable.

A repeated measures ANOVA comparing all groups resulted in a significant main effect of treatment [$F(1,40) = 14.995$, $P < 0.001$] and stranger side [$F(1,40) = 101.815$, $P < 0.001$] and approached significance for genotype [$F(1,40) = 3.799$, $P = 0.058$] and stranger side x treatment interaction [$F(1,40) = 3.693$, $P = 0.062$].

In contrast to untreated and treated WT mice, repeated measures ANOVA indicated that there was a significant interaction between AFQ056/Mavoglurant treatment and stranger side for sniffing time in *Fmr1* KO mice (Figure 1A, black bars) [$F(1,20) = 7.024$, $P = 0.015$]. Treatment with AFQ056/Mavoglurant reduced sniffing in KO mice at STR1 ($P < 0.001$) while no difference due to treatment was observed upon sniffing interaction with empty. While untreated KO mice spent more time in proximity to STR1 than WT mice ($P = 0.006$), chronic treatment of KO animals with AFQ056/Mavoglurant (Figure 1A, right panel black bars) reduced sniffing time of KO mice at STR1 to levels of WT animals (Figure 1A, white bars) [$F(1,20) = 1.833$, $P = 0.191$].

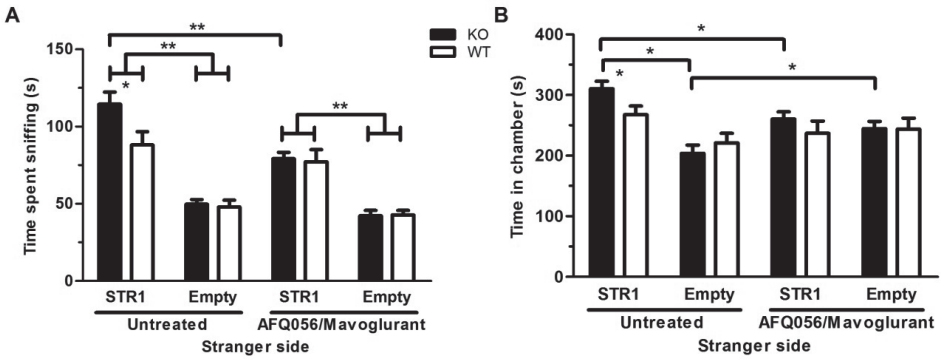


Figure 1. Sociability behavior in untreated and chronic AFQ056/Mavoglurant treated WT and *Fmr1* KO mice.

(A) Time spent sniffing (direct interaction approach) during sociability assay by untreated (left panel) and chronic AFQ056/Mavoglurant treated (right panel) WT and *Fmr1* KO mice. Overall, both groups, untreated WT (left panel, white bars, n = 11) and *Fmr1* KO (left panel, black bars, n = 11) mice and chronic AFQ056/Mavoglurant treated WT (right panel, white bars, n = 11) and *Fmr1* KO (right panel, black bars, n = 11) mice, spent significantly more time in proximity to STR1 compared with empty. Untreated *Fmr1* KO mice spent significantly more time in proximity to STR1 compared with untreated WT animals (P = 0.006). Chronic treatment in WT animals did not influence sniffing time compared with untreated WT animals. Chronic AFQ056/Mavoglurant treatment of *Fmr1* KO animals (right panel, black bars) significantly decreased sniffing time interaction with STR1 to levels of WT animals (white bars). (B) Time spent in STR1 and empty chamber (exploration of whole chamber) for untreated WT (left panel, white bars) and *Fmr1* KO (left panel, white bars) mice and chronic AFQ056/Mavoglurant treated WT (right panel, white bars) and *Fmr1* KO (right panel, black bars) mice during sociability assay. Although there is a significant main effect of chamber side for untreated WT and KO mice, *post hoc* test indicated that WT mice did not spent more time in STR1 chamber compared with empty chamber (P = 0.115). There was no significant difference between treated and untreated WT mice and no preference in exploration time between STR1 and empty chamber. Chronic treatment of *Fmr1* KO mice significantly decreased exploration time in STR1 chamber and increased exploration time in empty chamber compared with untreated KO mice (P = 0.012; P = 0.035). Data are expressed as mean ± S.E.M. *P < 0.05, **P < 0.001.

3.1.2 Time in chamber during sociability assay

Total time in chamber represented exploration of the whole chamber (containing STR1 or empty) during sociability assay (Figure 1B).

Repeated measures ANOVA for exploration time in both chambers of untreated *Fmr1* KO and WT animals revealed a significant genotype and chamber side effect [main effect of genotype, $F(1,20) = 12.806$, P = 0.002; main effect of chamber side, $F(1,20) = 14.750$, P = 0.001] with no significant genotype x chamber side interaction [$F(1,20) = 2.279$, P = 0.147] (Figure 1B, left panel). Although there was a significant main effect of chamber side, *post hoc* test indicated that WT mice did not spent more time in STR1 chamber compared with empty chamber (P = 0.115). KO mice spent more time in STR1 chamber compared with empty chamber (P = 0.001) and more time in STR1 chamber compared with WT mice (P = 0.047).

Chronic administration of AFQ056/Mavoglurant indicated for both KO and WT mice (Figure 1B, right panel) no preference for STR1 over empty chamber [main effect of chamber

side: F(1,20) = 0.042, P = 0.841], but there was a significant main effect of genotype [F(1,20) = 17.419, P < 0.001), probably due to higher variation in WT group for STR1 chamber exploration.

Both untreated and AFQ056/Mavoglurant treated WT mice (Figure 1B, white bars) showed similar exploration time in both chambers [main effect of chamber side: F(1,20) = 0.665, P = 0.425] and no significant change in exploration time due to treatment [main effect of treatment: F(1,20) = 1.307, P = 0.266]. These results are different from sniffing time measurements which clearly indicated higher interaction of both WT groups with STR1 than empty (Figure 1A, white bars).

An overall repeated measures ANOVA of both untreated and AFQ056/Mavoglurant treated WT and *Fmr1* KO mice for exploration time in both chambers (Figure 1B) revealed a significant main effect of chamber side [F (1,40) = 7.258, P < 0.01] and genotype [F (1,40) = 29.256, P < 0.001] and a significant chamber side x treatment interaction [F (1,40) = 5.705, P = 0.022].

Chronic AFQ056/Mavoglurant treatment of *Fmr1* KO mice significantly decreased exploration time in the chamber containing STR1 (P = 0.012) compared with untreated KO mice (Figure 1B, black bars) and increased exploration in empty chamber (P = 0.035), leading to absence of exploration preference between STR1 and empty chamber (Figure 1B, right panel black bars).

The absence of exploration preference in WT animals (both treated and untreated) for STR1 chamber compared with empty chamber (Figure 1B, white bars) made it difficult to compare KO and WT groups. Comparing time in chamber with sniffing time measurements (results section 3.1.1) suggested that sniffing time is a more sensitive measurement to study direct social interaction during sociability assay, as described previously [39, 41].

3.1.3 Sniffing time over time bins of 2 minutes during sociability assay

Enhanced sniffing time at STR1 in untreated *Fmr1* KO and decrease in sniffing time at STR1 due to chronic administration of AFQ056/Mavoglurant in KO mice was further confirmed by looking more closely into social approach over different time points (time bins 1-5, each time bin representing 2 min; Figure 2).

Comparing all groups (untreated and AFQ056/Mavoglurant treated WT and *Fmr1* KO mice; Figure 2) determined a significant time x stranger side x genotype x treatment interaction [F (4,37) = 2.951, P = 0.022] and significant main effects of stranger side [F (1,40) = 101.903, P < 0.001] and treatment [P (1,40) = 14.989, P < 0.001] and approached significance for genotype [P (1,40) = 3.806, P = 0.058].

Untreated *Fmr1* KO animals (Figure 1B) showed clearly a preference for STR1 over empty cage from the beginning of the test and over most of the time course, while untreated WT animals (Figure 1A) showed a preference for STR1 from 4 minutes onwards. This was confirmed by repeated measures ANOVA which indicated a significant time x stranger side x genotype interaction [F(4,80) = 2.938, P = 0.025]. This interaction signified a difference in preference over time for STR1 for each genotype; untreated WT mice sniffing time at STR1 increased over time, while untreated KO animals showed high sniffing at STR1 over the whole time course. This enhanced sniffing at STR1 might be due to decreased social anxiety.

Chronic AFQ056/Mavoglurant treated *Fmr1* KO mice showed a significant reduction over time points of STR1 compared with untreated KO mice [effect of stranger side x treatment:

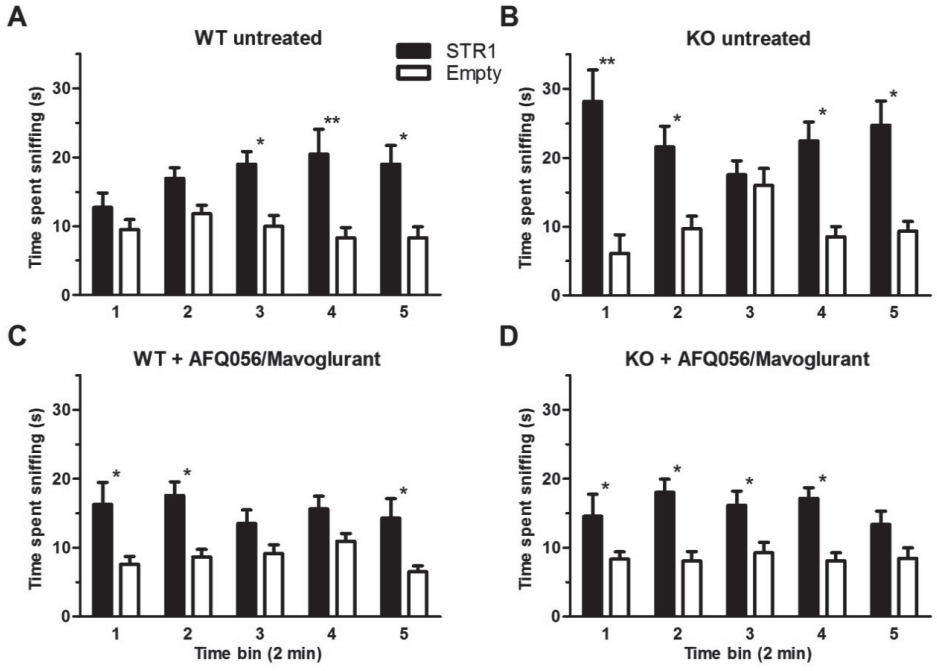


Figure 2. Sniffing time over 2 min time bins during sociability assay in untreated and AFQ056/Mavoglurant treated *Fmr1* KO and WT mice.

(A) Untreated WT mice increased sniffing duration at STR1 over time with a preference for STR1 from 4 min onwards. (B) Untreated KO mice demonstrated significant enhanced sniffing interaction with STR1 over most time points compared with untreated WT mice. (C) Chronic AFQ056/Mavoglurant treated WT mice did not show any statistically significant sniffing time difference with untreated WT, while (D) treated KO animals displayed a reduction of sniffing time at STR1 over the time course in comparison with untreated KO animals and a similar social approach behavior with WT groups. Data are expressed as mean ± S.E.M. *P < 0.05, **P < 0.001.

F(1,20) = 6.985, P = 0.016] (Figure 2D). This reduction in sniffing time at STR1 in *Fmr1* KO mice due to chronic AFQ056/Mavoglurant treatment was also observed in sniffing time over the total time of the sociability assay (Figure 1B). Repeated measures ANOVA indicated no significant difference between chronic treated WT and KO (Figure 2C and D) mice over the time course, with both groups showing a preference for STR1 over empty from beginning of the assay and over most of the time points.

3.1.4 Number of entries to each chamber, total distance travelled and velocity during sociability assay

Number of entries in each chamber is mainly a measurement of activity and exploration behavior. There were no significant effects of *Fmr1* genotype and treatment on number of entries into STR1 and empty chamber during the sociability assay [repeated measures ANOVA; main effect of genotype: F(1,40) = 0.117, P = 0.734; main effect of treatment: F(1,40) = 0.428, P = 0.517; three- and two-way interactions were not statistical significant] (data not shown).

Therefore, the differences in social interaction were not caused by altered exploration behavior in untreated and AFQ056/Mavoglurant treated *Fmr1* KO mice.

Total distance travelled and velocity during sociability assay revealed a significant main effect of treatment [distance: F(1,40) = 6.059, P = 0.018; velocity: F(1,40) = 6.011, P = 0.019] but was not statistically significant for genotype and three- and two-way interactions (data not shown). *Post hoc* tests determined that AFQ056/Mavoglurant treated WT mice covered less distance (P = 0.01) and moved slower (P = 0.01) than untreated WT mice, but no significant difference was observed with untreated and treated KO mice. This indicated that chronic AFQ056/Mavoglurant treatment had an effect on WT animals, but this difference did not have an effect on number of entries or direct interaction measurements like sniffing time where no difference was observed between untreated and treated WT animals (results section 3.1.1).

3.2 Preference for social novelty assay did not reveal any major differences between untreated and AFQ056/Mavoglurant treated WT and Fmr1 KO mice

Preference for social novelty was tested measuring social approach towards a second novel stranger in comparison with the first stranger mouse. A second unfamiliar mouse (STR2) was placed in the wire cup that had been empty during the sociability assay. For this assay, only sniffing time measurements will be discussed which is a more sensitive measurement than total time in chamber.

3.2.1 Sniffing time measurement during preference for social novelty assay

Both untreated and AFQ056/Mavoglurant treated WT and *Fmr1* KO mice demonstrated significant main effect of stranger side (Figure 3A).

Comparing untreated WT (Figure 3A, left panel, white bars) and KO mice (Figure 3A, left panel, black bars) indicated a main effect of stranger side [repeated measures ANOVA: F(1,20) = 14.221, P= 0.001], but no effect of genotype [F(1,20) = 0.213, P= 0.649] regarding preference for social novelty. Although there was no significant genotype x stranger side interaction effect [F(1,20) = 2.076, P= 0.165], *post hoc* test indicated that untreated WT mice spent more time sniffing at STR2 than STR1 (P = 0.002), while untreated KO mice did show reduced sniffing preference between STR1 and STR2 (P = 0.115). Chronic administration of AFQ056/Mavoglurant in WT (Figure 3A, right panel, white bars) and *Fmr1* KO mice (Figure 3A, right panel, black bars) revealed a main effect of stranger side [two-way repeated measures ANOVA: F(1,20) = 8,303, P < 0.009], while there was no significant main effect of genotype [F(1,20) = 1.433, P = 0.245] or genotype x stranger side interaction [F(1,20) = 0.693, P = 0.415]. *Post hoc* test indicated that treated WT mice did not show any sniffing preference between STR1 and STR2 (P = 0.163) while treated KO mice spent more time sniffing at STR2 than STR1 (P = 0.016).

A repeated measures ANOVA comparing all groups, untreated and AFQ056/Mavoglurant treated WT and *Fmr1* KO animals, resulted in a significant main effect of stranger side [F (1,40) = 21.502, P < 0.001] and treatment [F (1,40) = 5.589, P = 0.023]. Chronic administration of AFQ056/Mavoglurant in WT mice (Figure 3A, white bars) caused a significant main effect of stranger side [F(1,20) = 8,521, P = 0.008]. While untreated WT mice displayed a sniffing preference for STR2 over STR1, we did not observe this preference after chronic AFQ056/Mavoglurant treatment. For *Fmr1* KO mice, chronic AFQ056/Mavoglurant treatment promoted a main effect of treatment

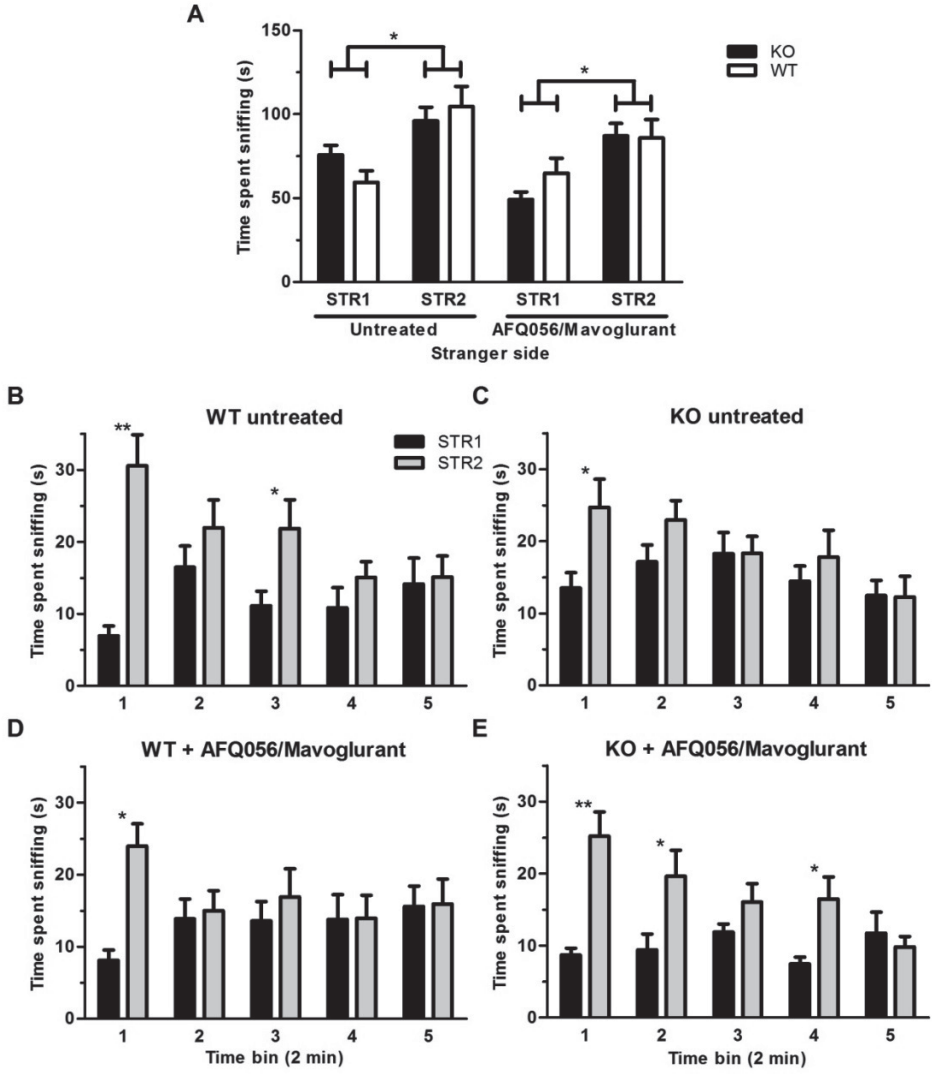


Figure 3. Preference for social novelty in untreated and AFQ056/Mavoglurant treated WT and *Fmr1* KO mice.

(A) Untreated WT (left panel, white bars, n = 11) and *Fmr1* KO (left panel, black bars, n = 11) mice spent more time in proximity to STR2 (sniffing time) compared with STR1 (significant main effect of stranger side). AFQ056/Mavoglurant treatment in KO mice (right panel, black bars, n = 11) decreased sniffing time interaction with STR1 compared with untreated KO mice. Treated WT mice (right panel, white bars, n = 11) spent less time in proximity to STR2 than untreated WT animals. There was an overall significant main effect of stranger side and treatment. (B) Untreated WT mice showed preference for STR2 at different time points during the assay which decreased along time. (C) Untreated KO mice showed preference for STR2 only during the first 2 min of the assay and lost preference for the novel mouse at later time points. (D) AFQ056/Mavoglurant treatment in WT mice reduced preference for STR2 to first 2 min of test while (E) AFQ056/Mavoglurant treatment in *Fmr1* KO mice suggested an increased interaction preference for STR2, which continued over most time points (0-4 min and 6-8 min). Data are expressed as mean ± S.E.M. *P < 0.05, **P < 0.001.

and stranger side [treatment: F(1,20) = 8.628, P = 0.008; stranger side: F(1,20) = 16.479, P < 0.001] (Figure 3A black bars). *Post hoc* test indicated that chronic AFQ056/Mavoglurant treatment in KO mice increased the sniffing time at STR2 compared with STR1 (P = 0.001), but this could not be statistical confirmed since we did not find any significant interaction effect.

3.2.2 Sniffing time over time bins of 2 minutes during preference for social novelty assay

Sniffing time over 2 minute time bins indicated that sniffing decreased over time for all groups (Figure 3B-E). Repeated measures ANOVA comparing all groups (untreated and treated) showed a significant time and stranger side interaction effect [F(4,37) = 9.305, P < 0.001].

AFQ056/Mavoglurant treated *Fmr1* KO mice (Figure 3E) showed high interaction with STR2 over most time points (during first 4 minutes and between 6-8 minutes), while untreated KO mice (Figure 3C) only showed sniffing time preference for STR2 over the first 2 minutes of the assay [main effect of treatment: F(1,20) = 8.604, P = 0.008]. In contrast, interaction preference for STR2 in AFQ056/Mavoglurant treated WT mice was reduced to the first 2 minutes of the assay, while this was not the case in untreated WT mice (Figure 3B and D).

These data indicated a reduction in preference for social novelty in mice lacking *Fmr1* gene product, with only an interaction preference for STR2 during the first 2 minutes of the assay, which could be enhanced by chronic treatment of AFQ056/Mavoglurant. Due to absence of significant interaction effects, we could not statistically confirm these observations.

3.2.3. Number of entries in each chamber, total distance travelled and velocity during preference for social novelty assay

There were no significant effects of *Fmr1* genotype and treatment on number of entries into STR1 and STR2 chamber during the preference for social novelty assay [repeated measures ANOVA; main effect of genotype: F(1,40) = 0.051, P = 0.822; main effect of treatment: F(1,40) = 0.033, P = 0.857; three- and two-way interactions were not statistical significant] (data not shown). Also no significant difference was observed in total distance travelled and velocity between all tested groups during preference for social novelty assay (data not shown).

4. DISCUSSION

In this study we found that lack of *Fmr1* expression impairs social behavior in *Fmr1* KO mice. Specifically, we demonstrated increased social approach to the stranger mouse during the sociability assay of the sociability/preference for social novelty (SPSN) task. Chronic administration of mGluR5 antagonist AFQ056/Mavoglurant was able to restore this social deficit. AFQ056/Mavoglurant administration in *Fmr1* KO mice also indicated an enhanced preference for the novel mouse over the familiar mouse in comparison to untreated *Fmr1* KO mice during the social preference for novelty assay.

SPSN is a behavioral task used to study several neuropsychiatric disorders including fragile X syndrome, autism and schizophrenia [23, 25, 42-46]. Its two assays measure different components of social behavior [39, 47]. The first part of the task included an assay for sociability where preference for social approach between an unfamiliar partner (STR1), and a novel object (empty) was determined. As described previously and confirmed by our results, sniffing time is

a better measurement for quantification of social interaction compared with time in chamber, which is more an explorative measurement [39, 48]. *Fmr1* KO mice showed enhanced social approach with STR1 during the sociability assay compared with WT littermates. Enhanced social approach in KO mice was not caused by differences in activity and exploratory behavior since there was no significant difference in total entries in STR1 and empty chamber. It is highly unlikely that reduced general anxiety caused this difference since several reports indicated that *Fmr1* KO mice on C57BL/6J background did not show anxiety [43, 49]. Enhanced active social approach in *Fmr1* KO mice compared with WT mice was described previously (with C57BL/6 background) in a direct social interaction test, indicating increased sniffing of the partner [22, 25, 29]. This enhanced social approach, described in our task, was not previously reported by other studies, possibly due to different background of KO mice or differences in the SPSN protocol [23, 24, 26, 43]. Similar to our findings, abnormal increased sociability has also been described in a mouse model of Rett syndrome [50, 51].

The sociability part of the SPSN task has been described as being the most sensitive to study autism-like symptoms in mice [52]. Abnormal social behavior is frequently observed in subjects with FXS and is also a major component of autism [1]. Although several social impairments in FXS persons are associated with autism, Hall *et al*, 2010 suggested a difference between FXS and autism, where subjects with FXS seek social interactions. Steinhausen *et al*, 2002 described FXS subjects to be more empathetic in comparison with other individuals with intellectual disability. This difference between FXS and autism, might explain the enhanced sniffing we observed in *Fmr1* KO mice during the sociability assay, which is opposite to social interaction in autistic mouse models [45]. Furthermore, impulsivity or aberrant social behavior (very anxious but they do seek social interaction to others, more empathetic) was also observed and described in patients with FXS, a behavioral profile that could possibly be reflected here in the observed enhanced social approach [20, 53].

The second part of the SPSN task included an assay studying preference for social novelty to provide a secondary measure of social approach based on discrimination between two partners (STR1 and the more novel STR2). During the social novelty assay, KO animals did indicate reduced preference for STR2, but no significant main effect of genotype was measured. When we measured sniffing over the time course of the assay, KO animals showed a preference for STR2 only during the first 2 minutes, and lost any preference during the following 8 min. Reduction for social novelty was also previously reported in *Fmr1* KO animals [24, 26, 44]. These studies did not further look into the time course of the test, so we cannot directly compare KO behavior over time to our findings. Impairments in social novelty preference were reported in mouse models of autism [42, 43, 45].

Impairment in mGluR5 signaling is described in both FXS subjects and *Fmr1* KO mice, leading to higher activity of the receptor and enhanced internalization of AMPA receptors [6]. Both, FMRP and mGluR5 are expressed in many brain regions and circuits that might be involved in the regulation of social behaviors [54]. A few studies have described rescue of social behavior of *Fmr1* KO mice in the SPSN task following chronic treatment with lithium, a mood stabilizer of which the function is mediated by several pathways [44, 55]. A recent study of [56] indicated the importance of mGluR5 signaling in social behaviors, where social interaction deficits could

be altered and rescued in a mouse model of autism after administration of a selective negative allosteric modulator of mGluR5. To determine if the social deficits observed in *Fmr1* KO mice in the SPSN task were caused by enhanced mGluR5 activity, we administered a specific mGluR5 antagonist, AFQ056/Mavoglurant, and studied the effects in both *Fmr1* KO and WT littermates. Three weeks of chronic administration of AFQ056/Mavoglurant restored social behavior in *Fmr1* KO mice during sociability assay to WT mice levels, with similar levels of preference for STR1 over empty. We observed that chronic administration of AFQ056/Mavoglurant caused a significant main effect of treatment regarding total distance travelled and velocity during the sociability assay. Administration of other mGluR5 antagonists was previously described to change behavior in WT animals as well [31, 57]. Apparently, a critical balance in mGluR5 signaling exists in WT mice. Auerbach *et al*, 2011 described that changes in the optimal range of mGluR5 protein synthesis can cause behavioral impairments, as we observed here, where administration of AFQ056/Mavoglurant alters distance and velocity in WT mice [58].

During preference for social novelty assay treated *Fmr1* KO mice displayed a decreased preference for STR1, the familiar mouse, in comparison with non-treated KO mice, but no significant interaction effect could further confirm this change in preference. Since there was initially no significant difference in this assay due to absence of *Fmr1*, we cannot make any assumptions regarding this assay of the task.

Our data suggest that the observed disturbance in social behavior due to the absence of Fmrp is a result of enhanced mGluR5 activity. We demonstrated here that the sociability behavioral deficit due to absence of Fmrp was reversed by a specific mGluR5 antagonist. A recent study of Michalon *et al*, 2012 reported that chronic administration of mGluR5 antagonist CTEP rescued a range of phenotypes in *Fmr1* KO, including dendritic spine density and LTD, but we cannot compare their findings to our study, since no social behavior tasks were included [59]. Next to our SPSN results, treatment of AFQ056/Mavoglurant in *Fmr1* KO mice was also able to rescue a deficit in inhibition of the startle response after a prepulse (PPI) to WT levels, and reversed abnormalities in dendritic spine morphology using cultured *Fmr1* KO hippocampal neurons [35]. Moreover, AFQ056/Mavoglurant administration in male FXS patients could also improve behavior in a specific subgroup of patients with FXS [37].

CONCLUSION

In conclusion, we determined that *Fmr1* expression regulates certain specific social behaviors. Absence of Fmrp leads to enhanced mGluR5 signaling causing social behavioral impairments in *Fmr1* KO mice such as increased social approach with unfamiliar mouse. Chronic administration of AFQ056/Mavoglurant, a specific mGluR5 inhibitor, did rescue sociability behavior in KO mice. Different social behavior was described between FXS subjects and subjects with idiopathic autism, which might be in line with our findings suggesting a distinctive social behavior in *Fmr1* KO mouse [18-20]. Our results suggest that AFQ056/Mavoglurant might be a new treatment for impaired specific social behaviors of FXS. Currently, several clinical trials have been conducted or are ongoing using a variety of existing and new drugs, including mGluR5 antagonists, $GABA_B$-receptor agonists, positive allosteric modulator of $GABA_A$-receptors, minocycline and lithium [27]. Their results are encouraging, however, there is a critical need for objective

and quantifiable outcome measures as well as for putative biomarkers that will support the development and evaluation of therapeutic interventions in FXS. The SPSN task can be used to assess efficacy of therapeutic intervention in both pre-clinical and clinical studies using *Fmr1* KO mice or an observation lab, respectively.

ACKNOWLEDGEMENTS

This work was supported by Research Foundation (FWO) Flanders (30005) (IG), The Netherlands Organisation for Health Research and Development (ZonMw) 912-07-022 (RW), E-Rare program entitled "Cure FXS" (no. EU/FIS PS09102673) (RW) and FRAXA Research Organisation (RW).

REFERENCES

1. Hagerman, R.J., *The physical and behavioural phenotype*, in *Fragile-X syndrome: diagnosis, treatment and research*, R.J. Hagerman and P. Hagerman, Editors. 2002: The Johns Hopkins University Press, Baltimore. p. 3-109.
2. Oberlé, I., et al., *Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome*. Science, 1991. **252**(5010): p. 1097-1102.
3. Pieretti, M., et al., *Absence of expression of the FMR-1 gene in fragile X syndrome*. Cell, 1991. **66**(4): p. 817-822.
4. Verkerk, A.J., et al., *Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome*. Cell, 1991. **65**(5): p. 905-914.
5. Willemsen, R., J. Levenga, and B. Oostra, *CGG repeat in the FMR1 gene: size matters*. Clin Genet, 2011. **80**(3): p. 214-25.
6. Bear, M.F., K.M. Huber, and S.T. Warren, *The mGluR theory of fragile X mental retardation*. Trends Neurosci, 2004. **27**(7): p. 370-7.
7. Nakamoto, M., et al., *Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors*. Proc Natl Acad Sci U S A, 2007. **104**(39): p. 15537-42.
8. Lu, R., et al., *The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development*. Proc Natl Acad Sci U S A, 2004. **101**: p. 15201-15206.
9. Narayanan, U., et al., *S6K1 phosphorylates and regulates FMRP with the neuronal protein synthesis-dependent mTOR signaling cascade*. J Biol Chem, 2008. **283**: p. 18478-18482.
10. Dictenberg, J.B., et al., *A direct role for FMRP in activity-dependent dendritic mRNA transport links filopodial-spine morphogenesis to fragile X syndrome*. Dev Cell, 2008. **14**(6): p. 926-39.
11. Sharma, A., et al., *Dysregulation of mTOR signaling in fragile X syndrome*. J Neurosci, 2010. **30**(2): p. 694-702.
12. Kaufmann, W.E. and H.W. Moser, *Dendritic anomalies in disorders associated with mental retardation*. Cereb Cortex, 2000. **10**(10): p. 981-91.
13. Irwin, S.A., et al., *Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: A quantitative examination*. Am J Med Genet, 2001. **98**(2): p. 161-167.
14. Levenga, J., et al., *Subregion-specific dendritic spine abnormalities in the hippocampus of Fmr1 KO mice*. Neurobiol Learn Mem, 2011. **95**: p. 467-472.
15. Bakker, C.E., et al., *Fmr1 knockout mice: A model to study fragile X mental retardation*. Cell, 1994. **78**: p. 23-33.
16. Huber, K.M., et al., *Altered synaptic plasticity in a mouse model of fragile X mental retardation*. Proc Natl Acad Sci U S A, 2002. **99**: p. 7746-50.
17. Hagerman, R.J., et al., *An analysis of autism in fifty males with the fragile X syndrome*. Am J Med Genet, 1986. **23**(1-2): p. 359-74.
18. Steinhausen, H.C., et al., *Behavioral phenotypes in four mental retardation syndromes: fetal alcohol syndrome, Prader-Willi syndrome, fragile X syndrome, and tuberosis sclerosis*. Am J Med Genet, 2002. **111**(4): p. 381-7.
19. Hall, S.S., et al., *Autism in Fragile X Syndrome: A Category Mistake?* J Am Acad Child Adolesc Psychiatry, 2010. **49**(9): p. 921-933.
20. Tranfaglia, M.R., *The Psychiatric Presentation of Fragile X: Evolution of the Diagnosis and Treatment of the Psychiatric Comorbidities of Fragile X Syndrome*. Dev Neurosci, 2011. **33**: p. 337-348.
21. Mineur, Y.S., L.X. Huynh, and W.E. Crusio, *Social behavior deficits in the Fmr1 mutant mouse*. Behav Brain Res, 2005. **168**: p. 172-175.

22. Spencer, C.M., et al., *Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome*. Genes Brain Behav, 2005. **4**(7): p. 420-30.
23. McNaughton, C.H., et al., *Evidence for social anxiety and impaired social cognition in a mouse model of fragile X syndrome*. Behav Neurosci, 2008. **122**(2): p. 293-300.
24. Liu, Z.H. and C.B. Smith, *Dissociation of social and nonsocial anxiety in a mouse model of fragile X syndrome*. Neurosci Lett, 2009. **454**(1): p. 62-6.
25. Spencer, C.M., et al., *Modifying behavioral phenotypes in Fmr1KO mice: genetic background differences reveal autistic-like responses*. Autism Res, 2011. **4**(1): p. 40-56.
26. Pietropaolo, S., et al., *Genetic-background modulation of core and variable autistic-like symptoms in FMR1 knock-out mice*. PLoS ONE, 2011. **6**(2): p. e17073.
27. Levenga, J., et al., *Potential therapeutic interventions for fragile X syndrome*. Trends Mol Med, 2010. **16**: p. 516-527.
28. Dolen, G., et al., *Correction of Fragile X Syndrome in Mice*. Neuron, 2007. **56**(6): p. 955-962.
29. Thomas, A.M., et al., *Genetic reduction of group 1 metabotropic glutamate receptors alters select behaviors in a mouse model for fragile X syndrome*. Behav Brain Res, 2011. **223**: p. 310-321.
30. Yan, Q.J., et al., *Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP*. Neuropharmacology, 2005. **49**: p. 1053-1066.
31. De Vrij, F.M.S., et al., *Rescue of behavioral phenotype and neuronal protrusion morphology in FMR1 KO mice*. Neurobiol Dis, 2008. **31**: p. 127-132.
32. Thomas, A.M., et al., *Group I metabotropic glutamate receptor antagonists alter select behaviors in a mouse model for fragile X syndrome*. Psychopharmacology (Berl), 2011. **19**: p. 47-58.
33. O'Leary, D.M., et al., *Selective mGluR5 antagonists MPEP and SIB-1893 decrease NMDA or glutamate-mediated neuronal toxicity through actions that reflect NMDA receptor antagonism*. Br J Pharmacol, 2000. **131**(7): p. 1429-37.
34. Lea, P.M.t., V.A. Movsesyan, and A.I. Faden, *Neuroprotective activity of the mGluR5 antagonists MPEP and MTEP against acute excitotoxicity differs and does not reflect actions at mGluR5 receptors*. Br J Pharmacol, 2005. **145**(4): p. 527-34.
35. Levenga, J., et al., *AFQ056, a new mGluR5 antagonist for treatment of fragile X syndrome*. Neurobiol Dis, 2011. **42**: p. 311-317.
36. Vinueza Veloz, M.F., et al., *The effect of an mGluR5 inhibitor on procedural memory and avoidance discrimination impairments in Fmr1 KO mice*. Genes Brain Behav, 2012. **11**: p. 325-331.
37. Jacquemont, S., et al., *Epigenetic Modification of the FMR1 Gene in Fragile X Syndrome Is Associated with Differential Response to the mGluR5 Antagonist AFQ056*. Sci Transl Med, 2011. **3**(64): p. 64ra1.
38. Mientjes, E.J., et al., *The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo*. Neurobiol Dis, 2006. **21**: p. 549-555.
39. Nadler, J.J., et al., *Automated apparatus for quantitation of social approach behaviors in mice*. Genes Brain Behav, 2004. **3**(5): p. 303-14.
40. Levenga, J., et al., *AFQ056, a new mGluR5 antagonist for treatment of fragile X syndrome*. Neurobiol Dis, 2011. **42**(3): p. 311-7.
41. Moy, S.S., et al., *Social approach in genetically engineered mouse lines relevant to autism*. Genes Brain Behav, 2009. **8**(2): p. 129-42.
42. McFarlane, H.G., et al., *Autism-like behavioral phenotypes in BTBR T+tf/J mice*. Genes Brain Behav, 2008. **7**(2): p. 152-63.
43. Moy, S.S., et al., *Social Approach in Genetically-Engineered Mouse Lines Relevant to Autism*. Genes Brain Behav, 2009. **8**: p. 129-142.
44. Mines, M.A., et al., *GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of fragile X syndrome and autism*. PLoS ONE, 2010. **5**(3): p. e9706.
45. Peca, J., et al., *Shank3 mutant mice display autistic-like behaviours and striatal dysfunction*. Nature, 2011. **472**(7344): p. 437-42.
46. Silverman, J.L., et al., *Low Stress Reactivity and Neuroendocrine Factors in the Btbr T(+)Tf/J Mouse Model of Autism*. Neuroscience, 2010. **171**(4): p. 1197-1208.
47. Moy, S.S., et al., *Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains*. Behav Brain Res, 2007. **176**(1): p. 4-20.
48. Naert, A., Z. Callaerts-Vegh, and R. D'Hooge, *Nocturnal hyperactivity, increased social novelty preference and delayed extinction of fear responses in post-weaning socially isolated mice*. Brain Research Bulletin, 2011. **85**(6): p. 354-362.
49. Mineur, Y.S., et al., *Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse*. Hippocampus, 2002. **12**(1): p. 39-46.
50. Chao, H.T., et al., *Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes*. Nature, 2010. **468**(7321): p. 263-269.

51. Schaevitz, L.R., et al., *Cognitive and social functions and growth factors in a mouse model of Rett syndrome*. Physiology & Behavior, 2010. **100**(3): p. 255-263.
52. Yang, M., J.L. Silverman, and J.N. Crawley, *Automated three-chambered social approach task for mice*. Curr Protoc Neurosci, 2011. **Chapter 8**: p. Unit 8 26.
53. Fisch, G.S., *What is associated with the fragile X syndrome?*. Am J Med Genet, 1993. **48**(2): p. 112-21.
54. Insel, T.R. and R.D. Fernald, *How the brain processes social information: searching for the social brain*. Annu Rev Neurosci, 2004. **27**: p. 697-722.
55. Liu, Z.H., D.M. Chuang, and C.B. Smith, *Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome*. Int J Neuropsychopharmacol, 2010: p. 1-13.
56. Silverman, J.L., et al., *Negative Allosteric Modulation of the mGluR5 Receptor Reduces Repetitive Behaviors and Rescues Social Deficits in Mouse Models of Autism*. Sci Transl Med, 2012. **4**(131): p. 131ra51.
57. Burket, J.A., et al., *Complex effects of mGluR5 antagonism on sociability and stereotypic behaviors in mice: possible implications for the pharmacotherapy of autism spectrum disorders*. Brain Res Bull, 2011. **86**(3-4): p. 152-8.
58. Auerbach, B.D., E.K. Osterweil, and M.F. Bear, *Mutations causing syndromic autism define an axis of synaptic pathophysiology.* Nature, 2011. **480**: p. 63-68.
59. Michalon, A., et al., *Chronic Pharmacological mGlu5 Inhibition Corrects Fragile X in Adult Mice*. Neuron, 2012. **74**(1): p. 49-56.

CHAPTER 4

ALTERED SOCIAL INTERACTION IN FRAGILE X MICE

CEF de Esch[a], WE van den Berg[b], RAM Buijsen[a], IA Jaafar[a], IM Nieuwenhuizen-Bakker[a], F. Gasparini[c], SA Kushner[b*], R Willemsen[a*]

[a]Department of Clinical Genetics, [b]Department of Neurobiological Psychiatry, Erasmus Medical Centre, Rotterdam, the Netherlands. [c] Novartis Institutes for BioMedical Research Basel, Switzerland.
*Both authors contributed equally

Manuscript in preparation.

ABSTRACT

Fragile X syndrome is the most common monogenetic form of autism. Although the *Fmr1* knockout mouse model recapitulates many aspects of the human FXS condition, the establishment of robust social behavioral phenotypes suitable for drug screening has been difficult. Here, we describe a novel social behavioral paradigm (Automated Tube Test, ATT) for which *Fmr1* knockout mice demonstrate a highly reliable and robust phenotype. Moreover, we confirm the validity of the mGluR5 hypothesis of fragile X syndrome, by demonstrating a rescue of *Fmr1* knockout mice using both genetic and pharmacological interventions independently. Together, our results demonstrate that the Automated Tube Test is a reliable outcome measure for social behavior in preclinical research for FXS.

INTRODUCTION

Fragile X syndrome (FXS) is a monogenic developmental disorder with a prevalence of 1 in 4,000 males and 1 in 8,000 females [1]. The complex neurological phenotype observed in FXS results from a lack of fragile X mental retardation protein (FMRP) from early development [2]. FMRP is an RNA-binding protein involved in synaptic connectivity and function by repressing local mRNA translation [3-6]. Studies demonstrating that FMRP regulates group 1 metabotropic glutamate receptor (mGluR) induced protein synthesis, have resulted in the widely-held hypothesis that absence of FMRP causes exaggerated mGluR5 signaling, leading to many of the hallmark neurological phenotypes of FXS [7].

FXS patients show intellectual disability ranging from moderate to severe, physical abnormalities including macro-orchidism and mild facial abnormalities, as well as behavioral alterations such as hyperactivity, aggressiveness, anxiety and sensory hyper-responsiveness [8, 9]. Furthermore, approximately one-half to two-thirds of male FXS patients exhibit the full diagnostic criteria of autism spectrum disorder (ASD), including impaired social interaction, communication deficits and repetitive or stereotyped behavior [10-14]. FXS patients commonly receive symptom-based treatments including compounds for attention deficits, selective serotonin reuptake inhibitors for anxiety, and atypical neuroleptics for aggression, since compounds specifically designed to counteract the underlying neuronal defects have not yet shown to ameliorate functioning in FXS [15, 16].

The evaluation of and the search for new therapeutic compounds in both human patients and the fragile X mouse model (*Fmr1* KO) have been hindered by the wide variability in the behavioral manifestations of FXS, the difficulty of identifying reliable outcome measures with sufficiently high sensitivity and specificity, and the lack of reliable biomarkers. The *Fmr1* KO mouse exhibits many of the hallmark characteristics found in FXS patients, such as macroorchidism, hyperactivity and cognitive deficits. In particular, substantial effort has been made over the past several years to investigate the ASD symptoms of FXS, for which a reliable autistic-like behavioral assay in the mouse model would be highly important. Previous studies have indeed shown that adult male *Fmr1* KO mice display abnormalities in social interaction and interest tests, as well as signs of social anxiety and the presence of repetitive behaviors [17-23]. However the wide variety of genetic backgrounds and test procedures between different laboratories have led to substantial variability in the reported behavioral phenotypes [24-28](Kooy, Dhooge et al. 1996; Dobkin, Rabe et al. 2000; Bakker and Oostra 2003; Errijgers and Kooy 2004; Spencer, Alekseyenko et al. 2011). In this study, we implemented a novel behavioral assay (Automated Tube Test, ATT) to characterize the social dominance behavior of adult male *Fmr1* KO mice. In addition, we investigated the role of mGluR5 signaling, by applying genetic (mGluR5+/-) and pharmacological (AFQ056/Mavoglurant) rescue strategies. Moreover, we confirmed the use of ERK1/2 and phosphorylated ERK1/2 levels in cortical synaptoneurosomes of our experimental animals as a potential biomarker.

MATERIALS AND METHODS

Animals

Male *Fmr1* KO mice (*Fmr1* KO(2)) and wild type (WT) littermates were generated as previously described and backcrossed to C57BL/6J for more than 10 generations [29]. For mGluR5 experiments, we focused on reducing the mGluR5 signaling by 50% since a homozygous deletion is known to impair brain function [30, 31]. Males heterozygous for *mGluR5* (*Grm5*$^{+/-}$, C57BL/6 background) were bred with females heterozygous for *Fmr1*$^{+/-}$, generating male offspring with four different genotypes: *Fmr1*$^{+/y}$/*Grm5*$^{+/+}$ (WT), *Fmr1*$^{-/y}$/*Grm5*$^{+/+}$ (*Fmr1* KO), *Fmr1*$^{+/y}$/*Grm5*$^{+/-}$ (*Grm5*$^{+/-}$), and *Fmr1*$^{-/y}$/*Grm5*$^{+/-}$ (*Fmr1* KO *Grm5*$^{+/-}$) [30]. Male offspring were weaned during the 4th postnatal week. Mice were housed either individually or in couples, and some of the experiments were replicated, for an overview see Table 1. Behavioral testing began in the 12th postnatal week and we used at least four but preferably six couples for each experiment. In the majority of the experiments animals were weighed at the start and the end of the training period and at the end of the tournaments. Animals were genotyped again after the experiments were finished to confirm the genotype. Animals were maintained under standard laboratory conditions with *ad libitum* access to food and water. Experiments were performed with the prior approval of the Netherlands Animal Ethical Committee.

Automated Tube Test (ATT)

The ATT protocol was performed similarly as previously described (van den Berg and Kushner). We used the same automated ventilated transparent fiberglass tube with an opaque center door and a length of 50 cm, which is connected at both ends to identical fiberglass boxes through automated opaque doors (Benedictus, the Netherlands). The inside diameter of the tube (2.5 cm) does not permit the animals to reverse direction once they are inside the tube. All mice first participated in a 5-day training protocol involving two habituation trials on Day 1, followed by 6 training trials per day on Days 2-5. During the Day 1 habituation trials, mice are given 2 habituation trials in which they are permitted to freely explore the tube until they travel from the starting box through the tube into the goal box, up to a maximum of 180 seconds per trial after which they were returned to their homecage. From the second day onwards, mice were placed inside a pseudo-randomly designated starting box and expected to walk through the tube to the goal box. Mice that remained in their starting box for more than 5 seconds would receive an air puff as a stimulation to advance into the tube. Once the mouse reached within 4 cm of the center door, it was opened automatically after a random variable delay of 1-3 seconds. When the mouse had not travelled through the tube within the 30 second time limit, he was gently pushed through the tube with a poking device to make sure that he would end up in the goal box on the other side. Training days were followed by two days of rest, after which a tournament protocol was initiated. On tournament days, each mouse received two training trials, one hour before participating in matches between pairs of mice. At the start of a match, one mouse was placed in each of the two boxes, after which the box doors were opened simultaneously, allowing the mice to enter the tube. Mice that remained in their respective starting box for more than 5 seconds received an air puff to encourage them to enter the tube. During matches, the center door opened only when

Table 1. Housing conditions, number of animals and tournament periods for each tube experiment.

Experiment	Total number of animals	Number of matches	Housing
Validation of set-up[1]			
WT	N=24	2 experiments, 30 matches per experiment	Couples: animal 'A' with animal 'B'
Fmr1 KO	N=10	20 matches	Couples: animal 'A' with animal 'B'
Establishment of a phenotype			
WT - *Fmr1* KO	N=22	2 experiments, 20 or 30 matches per experiment	Couples: WT with *Fmr1* KO
WT - *Fmr1* KO	N=21	2 experiments, 25 or 30 matches per experiment	Single
WT - *Fmr1* KO	N=12	36 matches	Couples: WT with WT or *Fmr1* KO with *Fmr1* KO
Involvement of the mGluR5 receptor			
Fmr1 KO-*Fmr1* KO *Grm5* +/-	N=12	30 matches	Couples: *Fmr1* KO with *Fmr1* KO *Grm5* +/-
WT - *Fmr1* KO *Grm5* +/-	N=20	2 experiments, 12 or 30 matches per experiment	Couples: WT with *Fmr1* KO *Grm5* +/-
WT - *Grm5* +/-	N=12	30 matches	Couples: WT with *Grm5* +/-
Pharmacological rescue			
WT (placebo) - *Fmr1* KO AFQ056/Mavoglurant	N=14	49 matches	Single (to prevent injuries)
Fmr1 KO (placebo) - *Fmr1* KO AFQ056/Mavoglurant	N=14*	49 matches	Single (to prevent injuries)

[1]In each cage the animals were labelled 'A' or 'B' matches were performed only between 'A' and a non-cage mate 'B'. *This group accidently contained one WT animal.

both mice were both within 4 cm of the door. A match was considered completed as soon as one mouse had retreated back into its own starting box with all four feet ('the looser'). After every training trial and tournament match, the interior of the tube was cleaned with 70% ethanol. Matches were performed with the experimenter blind to the genotype of the mice, and according to a randomly coded scheme by which matches were only performed between mice from different homecages, and between genotypes or treatment groups. On every tournament day, the same set of matches was performed, but always with a randomized order for which the starting position and inter-match interval of each mouse were explicitly balanced.

Drug treatment

Pellets containing AFQ056/Mavoglurant (Novartis, Basel) or placebo were obtained from Innovative Research of America (Sarasota, USA). Pellets were implanted subcutaneously in the flank of isoflurane (2.5%)-anesthetized animals at 8 to 9 weeks of age (4 groups, n=7 per group, see Table 1). AFQ056 pellets were designed to release a total of 0.08 mg AFQ056/Mavoglurant per day for a maximum of 60 days. For mice with an average weight of 25 grams, this dose corresponds to a daily IP injection with a standard dose of AFQ056/ Mavoglurant (3 mg AFQ per kg body weight).

Synaptoneurosome preparation

Synaptoneurosomes (SNs) were isolated according to Till *et al*, 2012 with minor modifications, one to four weeks after finishing the tube tournaments [32]. In short, the frontal parts of freshly isolated cortices were gently homogenized with a Teflon-glass homogenizer (10mM HEPES, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, protease inhibitors (Roche)). After centrifugation (1 min, 4ºC, 2000*g*), the homogenate was filtered through two layers of polypropylene mesh (pore size: 100µm, Millipore) followed by a Durapore filter (pore size: 5µm, Millipore). The filtrate was centrifuged (10 min, 4ºC, 1000*g*) and the SNs pellets were lysed in 1% SDS. Samples were boiled in Laemmli buffer after measurement of the protein concentrations by the BCA protein assay (Pierce). Thirty microgram protein per pooled sample was resolved by SDS–PAGE and transferred to a nitrocellulose membrane at least twice. Membranes were first blocked with 5% milk in PBS-Tween 20 (0.1%) for 60 min and then incubated with PBS-Tween 0.1% buffer and primary antibody overnight. After washing, the membranes were incubated with the appropriate fluorescent-conjugated secondary antibodies (Invitrogen) and ultimately imaged on an Odyssey infrared imaging system (Li-COR Biosciences). The primary antibodies used were as follows: ERK 1/2 (1:2000; p44/42 MAPK, Cell Signal), phospho-ERK (1:2000; Phospho-p44/42 MAPK, Cell Signal), eIF4E (1:1000, Cell signal), GluR1 (1:1000, Millipore), Akt (1:1000, Cell signal) and tubulin (1:2000, Sigma). To prove synaptoneurosomal enrichment, synapsin (1:2000, Synaptic Systems) and actin (1:1000, Sigma) levels were determined in a homogenate and SN fraction of the frontal cortex of an adult mouse (Supplemental Figure 6).

Statistical analysis

All tube data were analyzed with the statistical software package SPSS (Chicago, USA). Data from experiments that were replicated were combined before analysis. Individual match data was compared against the binomial distribution to determine whether the scores were significantly different from an outcome expected by chance, *ie* a 50%-50% win-lose outcome. All training data were analyzed using a repeated measures ANOVA with genotype and day of training as factors. Western blot samples were analyzed with the Odyssey 3.0 software. The expression level of each protein of interest was normalized to tubulin, except synapsin which was normalized against actin. After normalization, the samples were analyzed using a one sample t-test.

RESULTS

Establishment of an Fmr1 KO phenotype in the tube

When an *Fmr1* KO animal was housed together with a WT animal, the *Fmr1* KO animals showed strong social dominant behavior in the tube test. The *Fmr1* KO animals won on average 68% of the matches from wild types from a different cage on the first day, 76% on the second day and this then increased up to an average of 82% for the remaining tournament days (n=11/group; two experiments were averaged; P<0.001) (Figure 1A). In an additional experiment were the animals were housed individually to control for prior fighting experience, the *Fmr1* KO mice showed an even stronger behavioral phenotype. On the first two days, they won respectively 65% and 90% of the matches (Figure 1B). After two days of tournaments they won on average 95% of the matches (n=10 or 11/group; two experiments were averaged; P<0.05 or P<0.001). The matches performed between WT and *Fmr1* KO littermates housed in couples with a similar

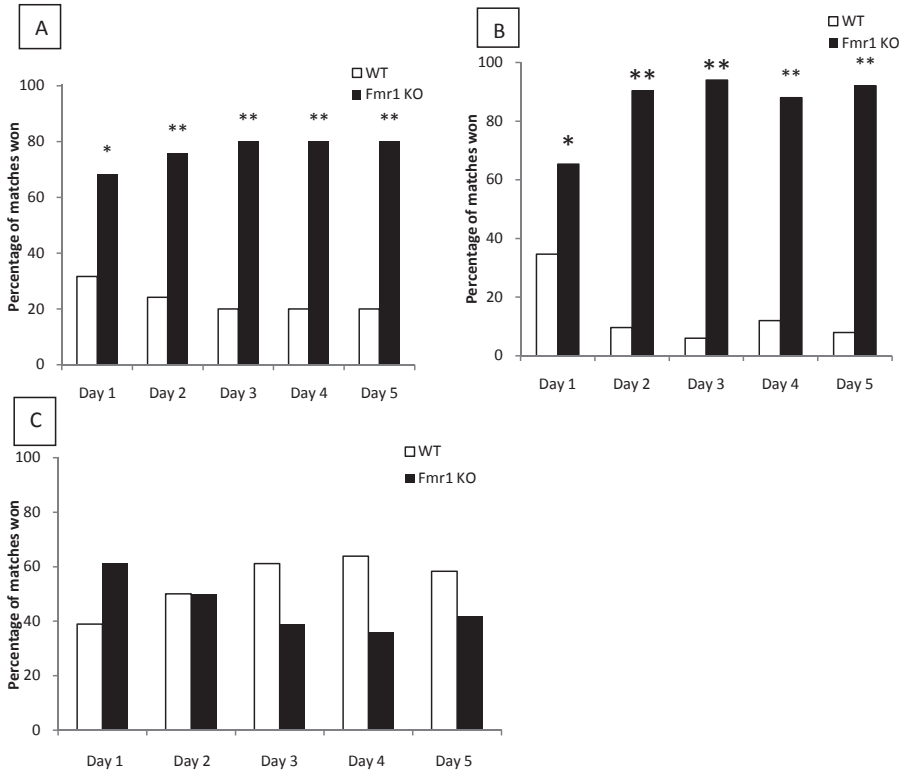


Figure 1. Average percentage of matches won by WT and *Fmr1* KO animals in tube tournaments after different housing conditions.

A: *Fmr1* KO animals housed with a WT littermate showed socially dominant behavior in the tube test. Data was averaged from two independent experiments (n=11/genotype) (*P<0.05, **P<0.001). **B**: Individually-housed *Fmr1* KO animals also showed social dominant behavior. Data was averaged from two independent experiments (WT=11 *Fmr1* KO=10) (*P<0.05, **P<0.001). **C**: *Fmr1* KO animals housed in couples with a similar genotype did not show a behavioral phenotype in the tube test (n=6/genotype) (P>0.05).

genotype, thus either two WT or two *Fmr1* KO animals in a cage, did not show a difference in social dominance behavior between both genotypes (see Figure 1C).

In order to confirm that the above results did not occur simply by chance, we performed two control experiments, the first one with only WT littermates and the second only with *Fmr1* KO littermates. In both control experiments the animals were housed in couples and in each cage an animal was randomly labelled either 'A' or 'B'. We only performed matches between the 'A' animals and 'B' animals from different cages. Since both groups consist of animals of the same genotype we expected a 50% win and 50% loose outcome for the 'A' animals in both experiments. Indeed, the experiment with WT animals as well as with *Fmr1* KO animals resulted in an outcome, which did not significantly differ, from 50%-50% win-lose for the 'A' animals (Supplemental Figure 1A and B). This confirms that the ATT is a reliable measure of detecting significant differences in behavior between different groups of animals. To test whether the outcome of the matches was influenced by external factors during the experiments, we determined the stability over time for each tube experiment. The percentage of matches with the same outcome as the first day was above 65% for each remaining tournament day in the experiments. When comparing the outcomes to the second day of each tournament, the minimum stability increased to 73%.

The effect of the training was clearly visible in the latency to enter the tube, which significantly decreased over time within all groups (repeated measures ANOVA). Further analysis showed that on the 4th and 5th training day the single housed *Fmr1* KO animals needed significantly less time to enter the tube (repeated measures ANOVA followed by a T-test, $P<0.05$) (Supplemental Figure 2). However, no differences were observed in the weight of mice from different genotypes (Supplemental Figure 5). Notably however, and independent of genotype, mice consistently lost weight during the training and tournaments (Supplemental Figure 5).

Involvement of mGlu5 receptor signaling in the tube

Since aberrant mGluR5 signaling is believed to play a key role in the pathology of fragile X syndrome, we investigated whether a genetic reduction of the mGluR5 signaling would normalize the social dominance behavior of the *Fmr1* KO mice. It is well known that mice housed in groups suffer from less stress, thus we decided to house the animals in couples between genotypes for the following experiments. *Fmr1* KO animals heterozygous for the mGlu5 receptor were generated and matches were performed between these animals and their WT or *Fmr1* KO littermates (Figure 2A and 2B). *Fmr1* KO *Grm5*+/- behaved socially different from *Fmr1* KO animals, since they lost the majority of the matches from their *Fmr1* KO littermates. They only won a maximum of 23% of the matches (n=6/group; $P<0.001$) (Figure 2A). However, *Fmr1* KO *Grm5*+/- animals still won significantly more matches than their WT littermates, on the first two days they won 74% and on the remaining days on average 93% (n=10/group, 2 experiments were averaged, $P<0.001$). (Figure 2B). To investigate the reduced mGluR5 signaling independently from the *Fmr1* deletion, WT animals were housed with littermates heterozygous for the mGlu5 receptor (*Grm5* +/-). After performing the matches between the two genotypes, it was clear that WT animals won significantly more matches than their *Grm5* +/- opponents. On the first day they won 80%, and this percentage remained more or less stable over time (n=6/group; $P<0.001$) (Figure 2C).

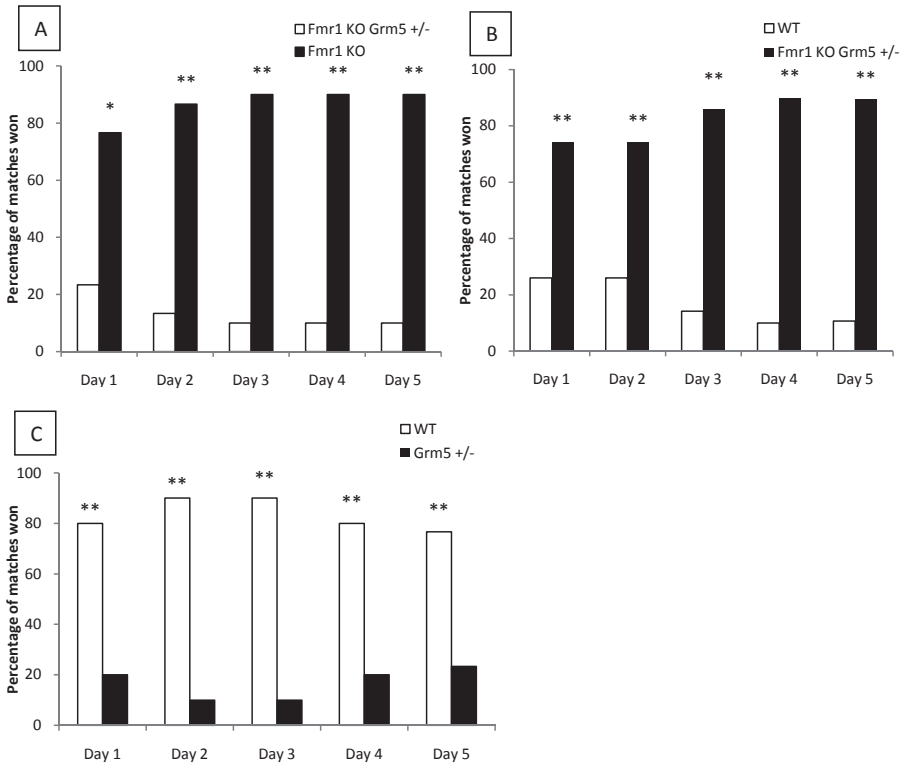


Figure 2. Involvement of mGlu5 receptor signaling in social dominance behavior.
A: Average percentage of matches won by *Fmr1* KO animals against *Fmr1* KO Gmr5 +/- littermates (n=6/genotype) (*P<0.05, **P<0.001). **B**: Average percentage of matches won by *Fmr1* KO *Gmr5* +/- animals against WTs. Data was averaged from two independent experiments (n=10/genotype) (**P≤0.001). **C**: Average percentage of matches won by WT animals in tournament against Grm5 +/- littermates (n=6/genotype). The WT animals won the majority of the matches (**P<0.001).

Analysis of the training trials revealed no significant differences between genotypes or treatment groups in latency to enter the tube (Supplemental Figure 3). However, the effect of the training was clearly visible in the latency to enter the tube, which significantly decreased over time within all groups (repeated measures ANOVA). We only measured weights of the experiment between *Fmr1* KO and *Fmr1* KO *Grm5* +/- animals and we did not observe significant differences between both groups. Notably however, and independent of genotype, mice consistently lost weight during the training and tournaments (Supplemental Figure 5).

Pharmacological rescue (AFQ056/Mavoglurant) of social dominance behavior

A pellet containing AFQ056/Mavoglurant or placebo was implanted subcutaneously in single housed WT and *Fmr1* KO littermates, four weeks before commencing the tube training. This resulted in a continuous release of AFQ056/Mavoglurant into the bloodstream of the treated

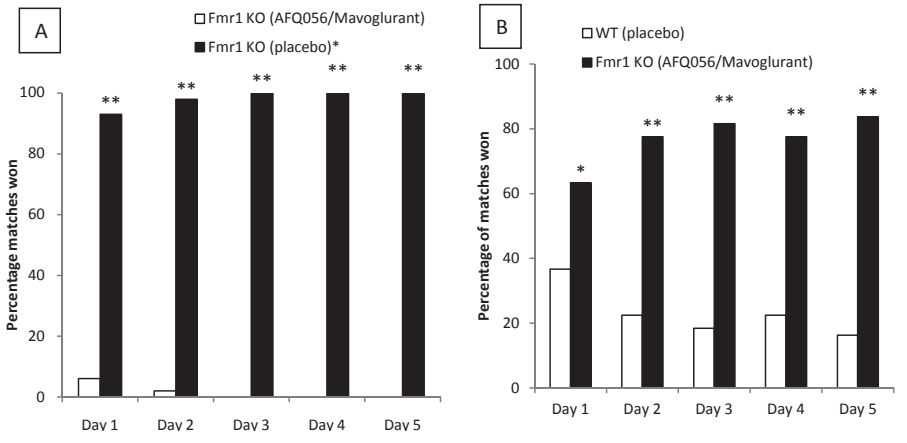


Figure 3. Average percentage of matches won by WT and *Fmr1* KO animals in tube tournaments after placebo or AFQ056/Mavoglurant treatment.

A: *Fmr1* KO animals under AFQ056/Mavoglurant treatment lost the majority of matches from *Fmr1* KO animals under placebo treatment. *Please note that the results of one animal in the *Fmr1* KO placebo group were left out due to a mistake in genotyping (n=6/7 per group) (**P<0.001). **B**: *Fmr1* KO animals under AFQ056/Mavoglurant treatment won the majority of matches from WT animals under placebo treatment (n=7/group) (*P<0.05, **P<0.001).

animals. After performing the tube experiment with *Fmr1* KO animals under placebo or AFQ056/Mavoglurant treatment, one animal in the placebo group turned out to be a wild type animal. This animal performed matches against the AFQ056/Mavoglurant treated *Fmr1* KO animals during all tournaments, but we left out the results of these matches in the figures. Other data gathered from this mouse was also left out in the remaining supplemental results. When the group of *Fmr1* KO animals under AFQ056/Mavoglurant treatment performed matches against *Fmr1* KO animals with a placebo pellet, they lost significantly (Figure 3A).

The *Fmr1* KO mice with a placebo pellet won on 94% of the matches the first day and this increased up to 100% after two days of tournaments (n=7/6 per group; P<0.001) (Figure 3A). Tube tournaments between WT animals with a placebo pellet and *Fmr1* KO littermates under AFQ056/Mavoglurant treatment were performed as well. The *Fmr1* KO animals under AFQ056/Mavoglurant treatment showed socially dominant behavior, they won the majority of matches from their WT opponents (Figure 3B). The percentage of matches increased from 63% on the first day to an average of 82% on the remaining tournament days (n=7/group; P<0.001).

Analysis of the training trials revealed no significant differences between genotypes or treatment groups in latency to enter the tube (Supplemental Figure 4). However, the effect of the training was clearly visible in the latency to enter the tube, which significantly decreased over time within all groups (repeated measures ANOVA). Notably however, and independent of genotype or treatment, mice consistently lost weight during the training and tournaments (Supplemental Figure 5).

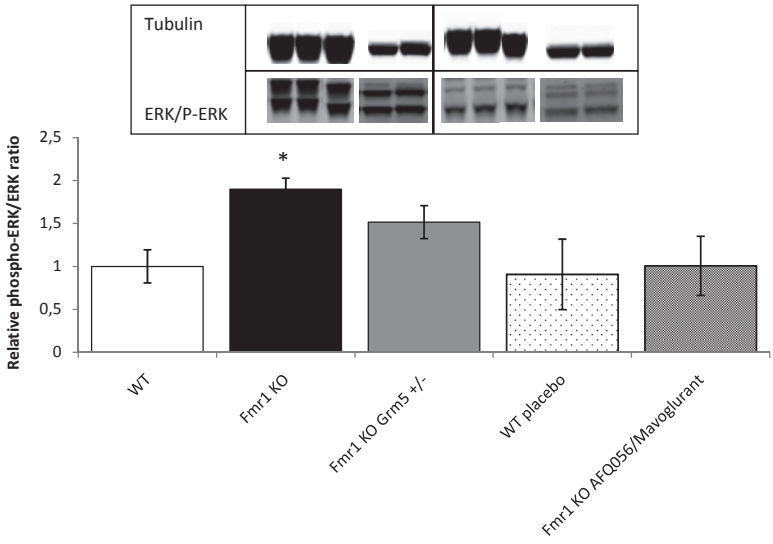


Figure 4. Increased proportion of activated ERK in cortical synaptoneurosomes from *Fmr1* KO mice. Representative immunoblots of cortical SNs from WT, *Fmr1* KO and *Fmr1* KO *Grm5* +/- animals as well as the micropellet-treated animals WT (placebo) and *Fmr1* KO(AFQ056/Mavoglurant) (from left to right). Quantification of the Western blots show the average (+/- SEM) phosphorylated-ERK/total ERK ratios for the different genotypes relative to the WT average which was set to 1. The p-ERK/ERK ratio is significantly higher in *Fmr1* KO samples than in WTs or WTs with a placebo pellet (t-test, $p<0.05$). The pERK/ERK ratio is not significantly different between WTs in the WT placebo, *Fmr1* KO HetmGluR5+/- or *Fmr1* KO AFQ056/Mavoglurant samples. For WT samples we averaged 8 measurements of pooled samples (n=4 animals), for *Fmr1* KO 16 measurements (n=6 animals), 6 measurements for *Fmr1* KO *Grm5* +/- (n=3 animals), 7 measurements for WT(placebo) (n=5 animals), 7 measurements for *Fmr1* KO(AFQ056/Mavoglurant) (n=6 animals).

Biochemical outcome marker

Quantitative Western blotting was performed with synaptoneurosomes isolated from the frontal cortices of experimental animals in the search of a biochemical read-out of the altered mGlur5 signaling in *Fmr1* KO mice. Analysis of synapsin levels confirmed the enrichment of synaptoneurosomes in our samples (Supplemental Figure 6). We analyzed expression levels of several signaling proteins including eIF4E, GluR1 and Akt, however we did not found a significant difference between WT and *Fmr1* KO mice in the expression levels of these proteins (Supplemental Figure 7). However, we did observe a significant increase in the proportion of phosphorylated and thus active ERK1/2 (pERK1/2) in *Fmr1* KO samples compared to wild type samples (Figure 4). In samples of animals with a genetically reduced mGluR5 signaling, this difference disappeared and the ratio pERK1/2-ERK1/2 was reduced to levels between WT and *Fmr1* KO samples. In addition, the pERK1/2-ERK1/2 ratio in WT samples with a placebo pellet did not significantly differ from the ratio in WT samples and in samples from *Fmr1* KO mice under AFQ056/Mavoglurant micropellet treatment, the pERK1/2-ERK1/2 ratio was not significantly different from WT animals with or without a placebo pellet.

DISCUSSION

Our results demonstrate a robust social behavioral phenotype of the fragile X mice in the ATT. Although fragile X mice have been previously tested in a similar apparatus, this is the first study that investigates the social dominance behavior in an automated environment using a highly distinct behavioral protocol [22, 33].

The tube test of social dominance was originally developed as a measure for social hierarchies in mice [34]. For mice and most other animal species, a stable social hierarchy assures more steady access to food, shelter, mates and breeding sites. To establish a social ranking, mice typically show aggressive behavior like tail rattling, clawing or biting. Once a hierarchy is established, it often remains stable over time, thereby minimizing energy losses due to fights between group members [35, 36]. However, it is not clear whether the tube test is directly linked to aggressiveness. In a study by [37], mutant animals showed aggressive behavior in the tube test, however we have never observed any signs of aggressive behavior, consistent with the previous report using the ATT in a CB6F1 genetic background (van den Berg and Kushner). Most classical anxiety test do not indicate any significant differences between *Fmr1* KO mice and WT mice, however some studies report *Fmr1* KO mice to be less anxious which could explain the observed phenotype in the tube test ((for a review see [17]). This is in contrast with human fragile X patients who usually show increased social anxiety, however they also often display a strong social interest, hyperactivity, impulsivity and aggressive behavior [38-40]. This specific combination of behavioral features might relate to the social dominance behavior by *Fmr1* KO mice observed in the automated tube test.

The behavioral phenotype heavily depended on previous social experiences since *Fmr1* KO animals housed together with *Fmr1* KO animals did not show dominant behavior over WT animals in the tube. The social hierarchy that is naturally established by each couple in a cage could explain this. In an experiment carried out with only WT couples and *Fmr1* KO couples, there will be an equal number of dominant WT animals and *Fmr1* KO animals. Performing matches between these mice, will most likely result in the observed 50%-50% results. We also observed a winner-loser effect: during the first few days of the tournaments, the winners become more confident and start to win more matches, while the losers settle in their role as losers. This is also be explained by the paradigm of minimizing the loss of energy during confrontations [41].

The mGluR5 signaling pathway is likely to be involved in this type of social behavior since WT animals with a reduced mGluR5 signaling already showed more submissive behavior. Previous studies have shown either a partial or a full rescue of fragile X phenotypes by reducing the mGluR5 signaling by 50% (*Fmr1* KO *Grm5* +/-) in *Fmr1* KO mice [42, 43]. In our study, a 50% genetic reduction of mGluR5 in *Fmr1* KO animals from conception led to altered behavior, as these animals became submissive to *Fmr1* KO animals. Importantly however, this behavioral rescue is not 100% complete, since *Fmr1* KO *Grm5+/-* animals still win significantly more matches than WT animals.

In addition, we measured the effects of AFQ056/Mavoglurant treatment, which began in the 9th postnatal week. At this age, the mouse brain is developmentally mature, but retains significant plasticity. Rescue of a behavioral phenotype by treatment at this age would imply that the behavioral phenotype results from an ongoing imbalance in synaptic signaling, in

contrast to a terminally altered signaling or early developmental abnormality. The social behavior of AFQ056/Mavoglurant-treated *Fmr1* KO mice significantly differed from the behavior of placebo-treated *Fmr1* KO mice. However, this rescue was also incomplete since their behavior significantly differed from WT animals treated with a placebo. Nonetheless, these results lead to the conclusion that in *Fmr1* KO mice, other signaling pathways are likely involved in establishing social dominance behavior.

The behavioral assay itself has its benefits; human interference is minimized and in combination with the extensive training protocol, mice quickly get accustomed to the tube apparatus and the handlings. Perhaps the most important benefit of the tube test is that only two outcomes are possible, namely winner or loser which leads to objective and straightforward results. After analyzing the results of the AFQ056/Mavoglurant experiment with *Fmr1* KO animals, one animal in the placebo group turned out to be a wild type animal. This animal surprisingly won his matches against the *Fmr1* KO animals under AFQ056/Mavoglurant treatment despite being a wild type. Since the *Fmr1* KO animals with AFQ056/Mavoglurant treatment were used to losing the majority of their matches, this leads to the conclusion that the outcome is not only determined by the winner but also by animals who loose.

This study confirms the use of the pERK1/2-ERK1/2 ratio as biochemical read-out for the altered mGluR5 signaling in *Fmr1* KO mice [44-47]. The proportion of active ERK1/2, returned to WT levels in cortices of *Fmr1* KO *Grm5*+/- animals. However, the values were not significantly lower than the values found for SNs of *Fmr1* KO mice. This could be the result of the variability between the measurements or an additional reflection of the partial rescue. It must be kept in mind that several other groups have not observed a difference in pERK/ERK ratio between WT and *Fmr1* KO animals in their samples [48-51]. However, the sample collection and isolating methods used vary widely, which makes it difficult to compare the results between studies.

From our tube experiments, it is clear that *Fmr1* KO mice establish dominance over WT littermates in the ATT. Although, we have not used any other measure of dominance such as a barber assay or a urine-marking assay in this study to rule out other factors such as learning, persistence or sensorimotor capacity, a recent study has validated the tube test as a measure of social dominance [52]. More importantly, by comparing different types of ranking assays, Wang *et al*, 2011 even suggests that social dominance is a distinct trait of animal behavior, which does not rely on sensorimotor or learning skills. Wang *et al*, 2011 show social dominance behavior in mice is regulated by the strength of glutamate-mediated synaptic transmission in the medial prefrontal cortex (mPFC). However, the mPFC is not the only brain structure involved in animal hierarchy, the amygdala and lateral septum have also been shown to be key players [53]. Moreover, since olfactory discrimination is normal in *Fmr1* KO animals [54] and because of the lack of differences in training data between WT and *Fmr1* KO animals, we feel comfortable in the conclusion that the observed phenotypes are likely related directly to social behavior, consistent with the high incidence of ASD in the human FXS patient population. Our results suggest that the ATT assay can serve as an important model for investigating the *Fmr1* KO mouse to determine which underlying networks and brain structures are involved in establishing and maintaining social dominance behavior.

In conclusion, the ATT is a robust social behavioral test for the *Fmr1* KO model, thereby opening the door to a reliable preclinical outcome measure for translational studies of fragile X syndrome.

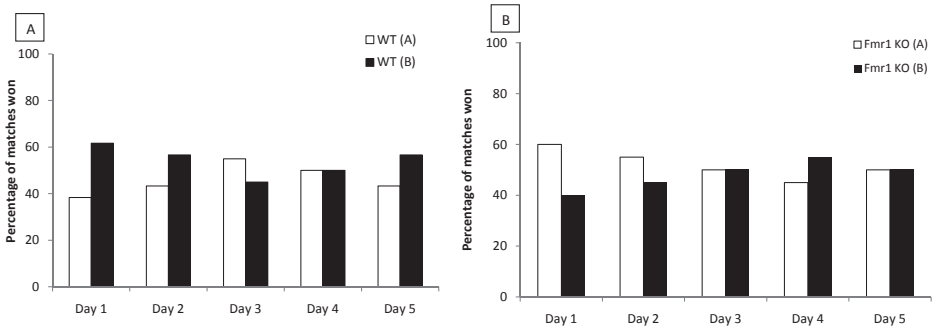
REFERENCES

1. Turner, G., et al., *Prevalence of fragile X syndrome*. Am J Med Genet, 1996. **64**(1): p. 196-197.
2. Oostra, B.A. and R. Willemsen, *A fragile balance: FMR1 expression levels*. Hum Mol Genet, 2003. **12 Suppl 2**: p. R249-57.
3. Verkerk, A.J., et al., *Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome*. Cell, 1991. **65**(5): p. 905-914.
4. Greenough, W.T., et al., *Synaptic regulation of protein synthesis and the fragile X protein*. Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7101-6.
5. Bagni, C. and E. Klann, *Molecular functions of the mammalian fragile X mental retardation protein: Insights into mental retardation and synaptic plasticity*, in *The Autisms*, C.M. Powell and L.M. Monteggia, Editors. 2012. p. 126-139.
6. Darnell, J.C. and E. Klann, *The translation of translational control by FMRP: therapeutic targets for FXS*. Nat Neurosci, 2013. **16**(11): p. 1530-6.
7. Bear, M.F., K.M. Huber, and S.T. Warren, *The mGluR theory of fragile X mental retardation*. Trends Neurosci, 2004. **27**(7): p. 370-7.
8. Hagerman, R.J., *The physical and behavioural phenotype*, in *Fragile-X syndrome: diagnosis, treatment and research*, R.J. Hagerman and P. Hagerman, Editors. 2002: The Johns Hopkins University Press, Baltimore. p. 3-109.
9. Lachiewicz, A.M., et al., *Aberrant behaviors of young boys with fragile X syndrome*. Am J Ment Retard, 1994. **98**(5): p. 567-79.
10. Hatton, D.D., et al., *Autistic behavior in children with fragile X syndrome: Prevalence, stability, and the impact of FMRP*. Am J Med Genet A, 2006. **140A**: p. 1804-1813.
11. Rogers, S.J., E.A. Wehner, and R. Hagerman, *The Behavioral Phenotype in Fragile X: Symptoms of Autism in Very Young Children with Fragile X Syndrome, Idiopathic Autism, and Other Developmental Disorders*. J Dev Behav Pediatr, 2001. **22**(6): p. 409-417.
12. Bailey, D.B., Jr., et al., *Autistic behavior, FMR1 protein, and developmental trajectories in young males with fragile X syndrome*. J Autism Dev Disord, 2001. **31**(2): p. 165-74.
13. Brock, M. and D. Hatton, *Distinguishing features of autism in boys with fragile X syndrome*. J Intellect Disabil Res, 2010. **54**: p. 894-905.
14. Sabaratnam, M., et al., *Autistic-like behaviour profile and psychiatric morbidity in Fragile X Syndrome: a prospective ten-year follow-up study*. Eur Child Adolesc Psychiatry, 2003. **12**(4): p. 172-7.
15. Berry-Kravis, E., et al., *Clinic-based retrospective analysis of psychopharmacology for behavior in fragile x syndrome*. Int J Pediatr, 2012. **2012**: p. 843016.
16. Berry-Kravis, E. and K. Potanos, *Psychopharmacology in fragile X syndrome-Present and future*. Ment Retard Dev Disabil Res Rev, 2004. **10**(1): p. 42-8.
17. Bernardet, M. and W.E. Crusio, *Fmr1 KO Mice as a Possible Model of Autistic Features*. ScientificWorldJournal, 2006. **6**: p. 1164-76.
18. Liu, Z.H., D.M. Chuang, and C.B. Smith, *Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome*. Int J Neuropsychopharmacol, 2010: p. 1-13.
19. Mines, M.A., et al., *GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of fragile X syndrome and autism*. PLoS ONE, 2010. **5**(3): p. e9706.
20. Pietropaolo, S., et al., *Genetic-background modulation of core and variable autistic-like symptoms in FMR1 knock-out mice*. PLoS ONE, 2011. **6**(2): p. e17073.
21. Bhattacharya, A., et al., *Genetic Removal of p70 S6 Kinase 1 Corrects Molecular, Synaptic, and Behavioral Phenotypes in Fragile X Syndrome Mice*. Neuron, 2012. **76**(2): p. 325-37.
22. Goebel-Goody, S.M., et al., *Genetic manipulation of STEP reverses behavioral abnormalities in a fragile X syndrome mouse model*. Genes Brain Behav, 2012. **11**: p. 586-600.
23. Gantois, I., et al., *Chronic administration of AFQ056/Mavoglurant restores social behaviour in Fmr1 knockout mice*. Behav Brain Res, 2013. **239** p. 72-79.
24. Kooy, R.F., et al., *Transgenic mouse model for the fragile X syndrome*. Am J Med Genet, 1996. **64**(2): p. 241-245.
25. Errijgers, V. and R.F. Kooy, *Genetic modifiers in mice: the example of the fragile X mouse model*. Cytogenet Genome Res, 2004. **105**(2-4): p. 448-54.
26. Dobkin, C., et al., *Fmr1 knockout mouse has a distinctive strain-specific learning impairment*. Neuroscience, 2000. **100**: p. 423-429.
27. Bakker, C.E. and B.A. Oostra, *Understanding fragile X syndrome: insights from animal models*. Cytogenet Genome Res, 2003. **100**(1-4): p. 111-23.
28. Spencer, C.M., et al., *Modifying behavioral phenotypes in Fmr1KO mice: genetic background*

differences reveal autistic-like responses. Autism Res, 2011. **4**(1): p. 40-56.

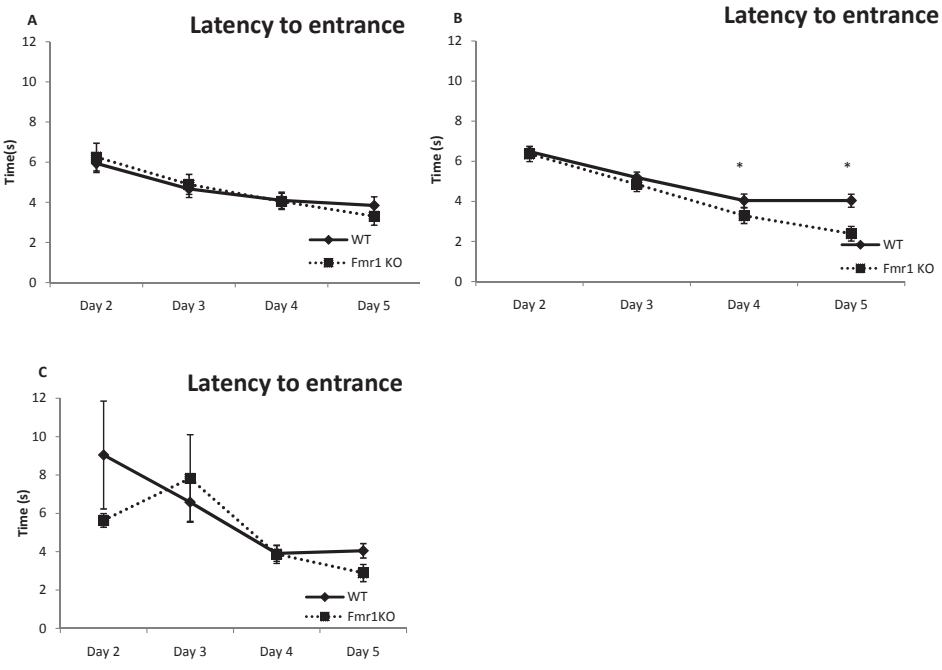
29. Mientjes, E.J., et al., *The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo*. Neurobiol Dis, 2006. **21**: p. 549-555.
30. Lu, Y.M., et al., *Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP*. J Neurosci, 1997. **17**(13): p. 5196-205.
31. Jia, Z., et al., *Selective abolition of the NMDA component of long-term potentiation in mice lacking mGluR5*. Learn Mem, 1998. **5**(4-5): p. 331-43.
32. Till, S.M., et al., *Altered maturation of the primary somatosensory cortex in a mouse model of fragile X syndrome*. Hum Mol Genet, 2012. **21**(10): p. 2143-2156.
33. Spencer, C.M., et al., *Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome*. Genes Brain Behav, 2005. **4**(7): p. 420-30.
34. Lindzey, G., D.T. Lykken, and H.D. Winston, *Confusion, conviction, and control groups*. J Abnorm Soc Psychol, 1961. **63**: p. 221-2.
35. Sapolsky, R.M., *The influence of social hierarchy on primate health*. Science, 2005. **308**(5722): p. 648-52.
36. Yeh, S.R., R.A. Fricke, and D.H. Edwards, *The effect of social experience on serotonergic modulation of the escape circuit of crayfish*. Science, 1996. **271**(5247): p. 366-9.
37. Rodriguiz, R.M., et al., *Aberrant responses in social interaction of dopamine transporter knockout mice*. Behav Brain Res, 2004. **148**(1-2): p. 185-98.
38. Gross, C., E.M. Berry-Kravis, and G.J. Bassell, *Therapeutic strategies in fragile X syndrome: dysregulated mGluR signaling and beyond*. Neuropsychopharmacology, 2012. **37**(1): p. 178-95.
39. Tranfaglia, M.R., *The Psychiatric Presentation of Fragile X: Evolution of the Diagnosis and Treatment of the Psychiatric Comorbidities of Fragile X Syndrome*. Dev Neurosci, 2011. **33**: p. 337-348.
40. Garber, K.B., J. Visootsak, and S.T. Warren, *Fragile X syndrome*. Eur J Hum Genet, 2008. **16**: p. 666-672.
41. Hsu, Y., R.L. Earley, and L.L. Wolf, *Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes*. Biol Rev Camb Philos Soc, 2006. **81**(1): p. 33-74.
42. Dolen, G., et al., *Correction of Fragile X Syndrome in Mice*. Neuron, 2007. **56**(6): p. 955-962.
43. Thomas, A.M., et al., *Genetic reduction of group 1 metabotropic glutamate receptors alters select behaviors in a mouse model for fragile X syndrome*. Behav Brain Res, 2011. **223**: p. 310-321.
44. Wang, X., et al., *Activation of the extracellular signal-regulated kinase pathway contributes to the behavioral deficit of fragile x-syndrome*. J Neurochem, 2012. **121**(4): p. 672-9.
45. Hou, L., et al., *Dynamic Translational and Proteasomal Regulation of Fragile X Mental Retardation Protein Controls mGluR-Dependent Long-Term Depression*. Neuron, 2006. **51**(4): p. 441-54.
46. Price, T.J., et al., *Decreased nociceptive sensitization in mice lacking the fragile X mental retardation protein: role of mGluR1/5 and mTOR*. J Neurosci, 2007. **27**(51): p. 13958-67.
47. Weng, N., et al., *Early-phase ERK activation as a biomarker for metabolic status in fragile X syndrome*. Am J Med Genet B Neuropsychiatr Genet, 2008. **147B**: p. 1253-1257.
48. Hu, H., et al., *Ras signaling mechanisms underlying impaired GluR1-dependent plasticity associated with fragile X syndrome*. J Neurosci, 2008. **28**(31): p. 7847-62.
49. Gross, C., et al., *Excess phosphoinositide 3-kinase subunit synthesis and activity as a novel therapeutic target in fragile x syndrome*. J Neurosci, 2010. **30**(32): p. 10624-38.
50. Ronesi, J.A. and K.M. Huber, *Metabotropic glutamate receptors and fragile x mental retardation protein: partners in translational regulation at the synapse*. Sci Signal, 2008. **1**(5): p. pe6.
51. Osterweil, E.K., et al., *Hypersensitivity to mGluR5 and ERK1/2 Leads to Excessive Protein Synthesis in the Hippocampus of a Mouse Model of Fragile X Syndrome*. J Neurosci, 2010. **30**(46): p. 15616-27.
52. Wang, F., et al., *Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex*. Science, 2011. **334**(6056): p. 693-7.
53. Timmer, M., et al., *Evidence for a role of oxytocin receptors in the long-term establishment of dominance hierarchies*. Neuropsychopharmacology, 2011. **36**(11): p. 2349-56.
54. Larson, J., et al., *Olfactory discrimination learning in mice lacking the fragile X mental retardation protein*. Neurobiol Learn Mem, 2008. **90**(1): p. 90-102.

SUPPLEMENTARY FIGURES



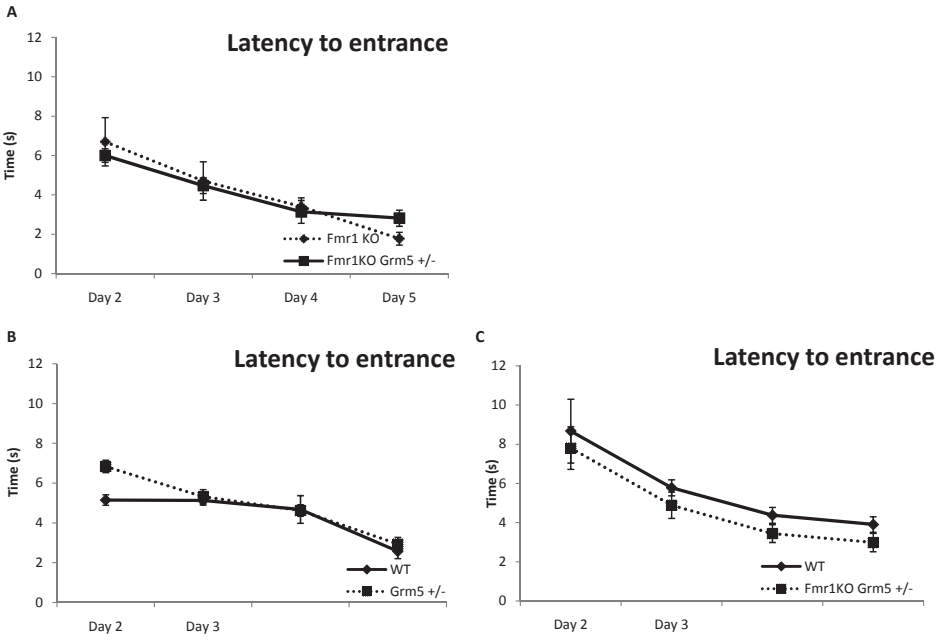
Supplemental Figure 1. Matches between animals from a similar genotype which were housed in couples results in a 50%-50% outcome.

A: A group of WT animals were housed in couples and each animal in a cage was labelled either 'A' or 'B'. Matches performed between animal 'A' and a non-cage mate 'B' resulted in a 50%-50% outcome. Data were averaged from two independent experiments (n=12/group, $P \geq 0.05$). **B:** A group of *Fmr1* KO animals were housed in couples and each animal in a cage was labelled either 'A' or 'B'. Matches performed between animal 'A' and a non-cage mate 'B' resulted in a 50%-50% outcome (n=5/group, $P > 0.05$).

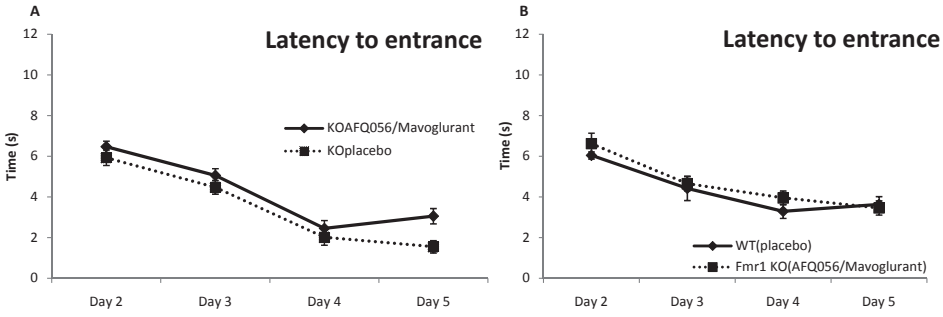


Supplemental Figure 2. Overview of training data of WT and Fmr1 KO animals housed single or in couples

A: Latency to enter the tube by WT and Fmr1 KO animals housed together in couples was displayed for each training day. Data was averaged from two independent experiments (time in seconds, +-SEM; n=11/genotype). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,390)=18.887$, $p<0.001$)) **B:** Latency to enter the tube by single housed WT and Fmr1 KO animals was displayed for each training day. Data was averaged from two independent experiments (time in seconds, +-SEM; (WT=11 Fmr1 KO=10)). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,372)=64.076$, $p<0.001$) Repeated measures ANOVA also indicated a significant interaction of time and genotype($F(1,372)=3.892$, $p<0.01$. On Day 4 and 5, the Fmr1 KO animals needed significantly less time to enter the tube (T-test, $P<0.05$). **C:** Latency to enter the tube by WT and Fmr1 KO animals housed in couples with a similar genotype was displayed for each training day (time in seconds, +-SEM; n=6/genotype). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,210)=4.645$, $p<0.05$)).

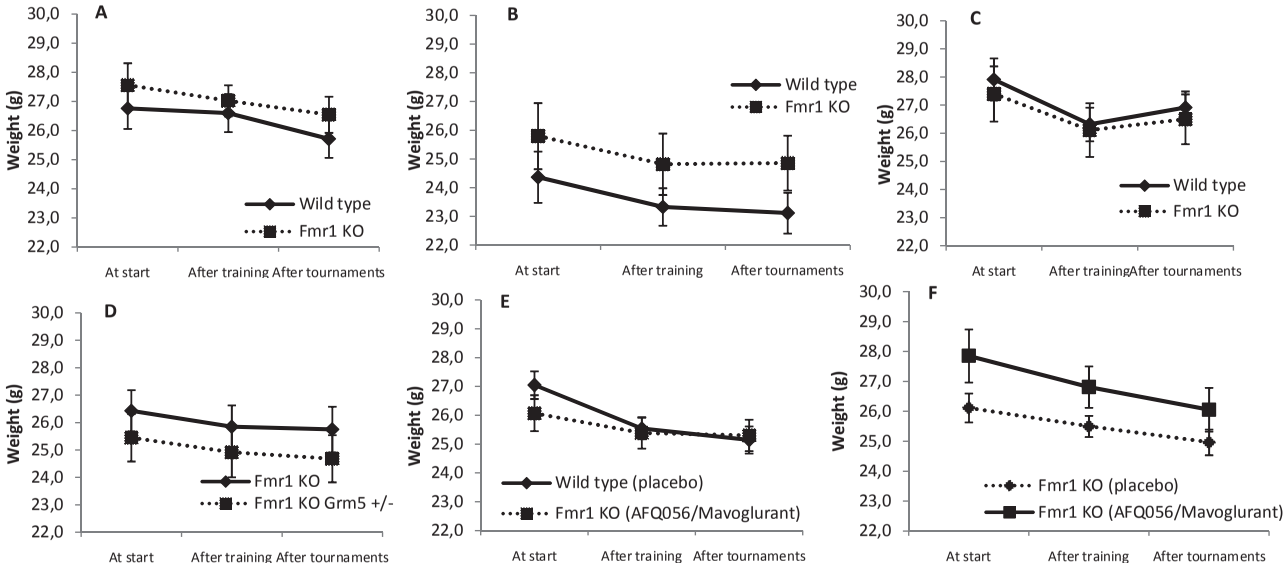


Supplemental Figure 3. Overview of training data of WT and *Grm5* +/- animals.
A: Latency to enter the tube by *Fmr1* KO and *Fmr1* KO *Grm5* +/- animals was displayed for each training day (time in seconds, +-SEM; n=6/ genotype). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,210)=15.016$, $p<0.001$)). **B:** Latency to enter the tube by WT and *Fmr1* KO *Grm5* +/- animals was displayed for each training day. Data was averaged from two independent experiments (time in seconds, +-SEM; n=10/genotype). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,390)=22.712$, $p<0.001$ **C:** Latency to enter the tube by WT and *Grm5* +/- animals was displayed for each training day (time in seconds, +-SEM; n=6/ genotype). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,210)=26.116$, $p<0.001$)).

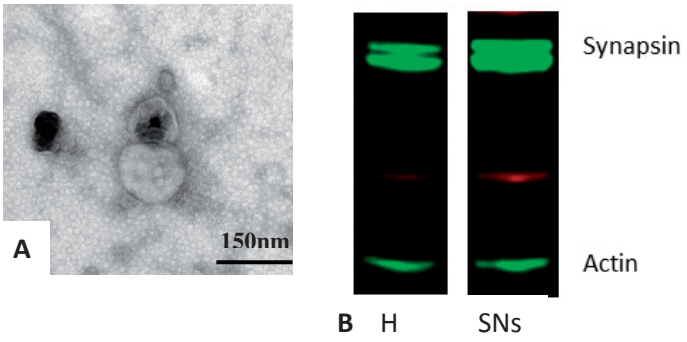


Supplemental Figure 4. Overview of training data of WT(placebo) and *Fmr1* KO(AFQ056/ Mavoglurant) animals.

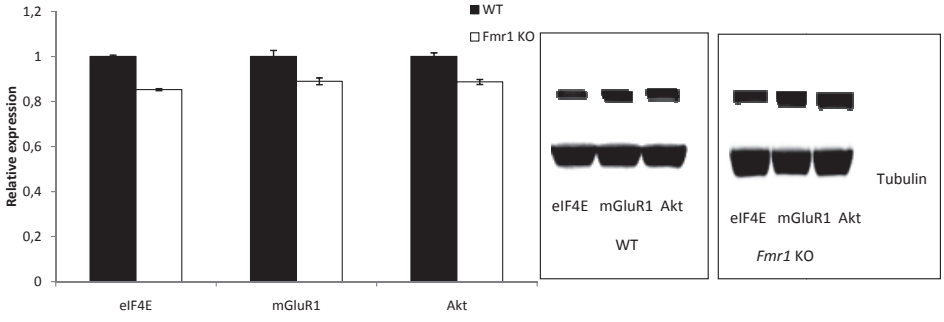
A: Latency to enter the tube by *Fmr1* KO(placebo) and *Fmr1* KO(AFQ056/ Mavoglurant) animals was displayed for each training day (time in seconds, +-SEM; n=6/7 per group). Please note that the results of one animal in the *Fmr1* KO placebo group were left out due to a mistake in genotyping (n=6/7 per group). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,228)=71.856$, $p<0.001$. **B**: Latency to enter the tube by WT(placebo) and *Fmr1* KO(AFQ056/ Mavoglurant) animals was displayed for each training day (time in seconds, +-SEM; n=7/ group). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,246)=22.758$, $p<0.001$)).



Supplemental Figure 5. Weights of several groups of animals at different time points during the tube experiments.
A: Weights (+/-SEM) of *Fmr1* KO animals housed with a WT littermate. Data was averaged from two independent experiments but not all weights were available for each training day. Repeated measures ANOVA indicated a significant effect of time on weight in both groups ($F(1,16)= 39.041$, $p<0.001$)) **B:** Weights (+/-SEM) of individually-housed wild type and *Fmr1* KO animals (WT n=6; KO n=5). Repeated measures ANOVA indicated a significant effect of time on weight in both groups ($F(1,18)= 7.971$, $p<0.01$)). **C:** Weights (+/-SEM) of *Fmr1* KO animals and wild type animals housed in couples with a similar genotype (n=6/group). Repeated measures ANOVA indicated a significant effect of time on weight in both groups ($F(1,20)=85.242$, $p<0.001$)) **D:** Weights (+/-SEM) of *Fmr1* KO animals and *Fmr1* KO *Grm5* +/- (n=6/group). Repeated measures ANOVA indicated a significant effect of time on weight in both groups ($F(1,20)=16.079$, $p<0.001$)) **E:** Weights (+/-SEM) of wild type (placebo) animals and *Fmr1* KO (AFQ056/Mavoglurant) animals (n=7/group). Repeated measures ANOVA indicated a significant effect of time on weight in both groups ($F(1,24)= 92.253$, $p<0.001$)) as well as a significant interaction of genotype and time ($F(1, 24)=15.456$, $p<0.001$). **F:** Weights (+/-SEM) of *Fmr1* KO (placebo) animals and *Fmr1* KO (AFQ056/Mavoglurant) animals (n=6/7/group). Repeated measures ANOVA indicated a significant effect of time on weight in both groups ($F(1,22)= 34.566$, $p<0.001$)).



Supplemental Figure 6. Synaptoneurosomal enrichment in samples from the frontal cortex.
A: Electron-microscopic image of synaptoneurosomal fraction (uranyl acetate staining) of the frontal cortex of an adult mouse. **B**: Western blot showing synapsin levels in homogenate (H) and synaptoneurosomes (SNs) prepared from the frontal cortex of an adult mouse. A 1.8 fold increase in synapsin was found in the SNs fraction compared to the homogenate.



Supplemental Figure 7. Analysis of the westernblots on synaptoneurosomes from WT and *Fmr1* KO animals
Quantification of the Western blots show the average (+/- SEM) fluorescence eIF4E, GluR1 and Akt for the *Fmr1* KO samples relative to the WT average which was set to 1. No significant differences were observed in the expression levels between WT and *Fmr1* KO animals. For eIF4E and mGluR1 we each averaged 10 measurements (n=5/genotype), for Akt we averaged 5 measurements (n=5/genotype).

CHAPTER 5

ALTERED SOCIAL INTERACTION AFTER REGION OR CELL-TYPE SPECIFIC DELETION OF *Fmr1* EXPRESSION IN MICE

CEF de Esch[a], S Zeidler[a], WE van den Berg[b], RAM Buijsen[a], IA Jaafar[a], E. Severijnen[a], SA Kushner[b*], R Willemsen[a*]

[a]Department of Clinical Genetics, [b]Department of Neurobiological Psychiatry, Erasmus Medical Centre, Rotterdam, the Netherlands.
*Both authors contributed equally

Manuscript in preparation.

ABSTRACT

The automated tube test has shown to be a reliable outcome measure for the altered social dominance behavior of fragile X mice. However, the underlying cellular mechanisms involved in this complex autistic-like behavior remain elusive. Here we used *Emx1-Cre* and *Gad2-Cre* driver lines to specifically knock out *Fmr1* expression in glutamatergic and GABAergic neurons, respectively. When adults, these animals were subjected to the automated tube test. Our results showed that a lack of Fmrp in the glutamatergic neurons results in socially submissive behavior. In contrast, deletion of *Fmr1* expression in GABAergic neurons resulted in socially dominant behavior, mimicking the global knockout phenotype. These results suggest that the excitation/inhibition balance is involved in regulating social behavior.

INTRODUCTION

Patients with fragile X syndrome (FXS) experience moderate to severe intellectual disability, physical abnormalities, as well as distinct behavioral alterations such as epileptic seizures, anxiety, hyperactivity, impaired social interaction and communication deficits similar to those seen in autism spectrum disorder (ASD) [1-3]. FXS results from silencing of the *FMR1* gene due to the presence of a large expansion (> 200 copies) of a naturally occurring CGG repeat in the 5'-UTR of the gene. The *FMR1* protein product, fragile X mental retardation protein (FMRP), is an RNA-binding protein involved in synaptic functioning by repressing mRNA translation at dendritic spines [4-7]. In FXS, several signaling pathways have been shown to be affected by the lack of FMRP expression and the de-regulated synaptic protein synthesis.

In the fragile X mouse model (*Fmr1* KO) compelling evidence points to an altered excitatory group 1 metabotropic glutamate receptor (mGluR) signaling, which is proposed to underlie many of the fragile X phenotypes such as the immature dendritic spine phenotype, the reduced synaptic plasticity and susceptibility to audiogenic seizures [5, 8-10]. However, this is not the only signaling pathway implicated in FXS, the *Fmr1* KO mouse also displays reduced expression of several components of the major inhibitory neurotransmitter system, the γ-aminobutyric acid (GABA) pathway. Several subunits of the $GABA_A$ receptor, and proteins and enzymes involved in synthesis and degradation of GABA were shown to be reduced in the *Fmr1* KO mouse [11-15]. $GABA_A$ receptors are implicated in anxiety, epilepsy and learning and memory, and treatment with GABAergic drugs such as diazepam or ganaxolone has shown to rescue the occurrence of epileptic seizures in fragile X mice [16, 17].

Recently, the automated tube test has been proven to be a reliable outcome measure of the autistic-like social behavior of the fragile X mouse (de Esch *et al*, 2014). However, the specific brain areas or cell types involved in this type of social dominance behavior of fragile X mice remain elusive. In this study we crossed a conditional *Fmr1* KO (*Fmr1* cKO) mouse with a tissue specific Cre-recombinase expressing mouse, to spatially control Fmrp expression in order to identify brain regions or cell types involved in regulating social dominant behavior. To investigate the involvement of the olfactory bulb, cerebral cortex, hippocampus and amygdala we used the *Emx1-Cre* driver, which is specifically expressed in forebrain glutamatergic pyramidal neurons [18]. In addition, we investigated the involvement of the GABA system by using the *Gad2-Cre* driver which is specifically expressed by inhibitory interneurons [19].

MATERIALS AND METHODS

Animals

Male *Fmr1* KO mice (*Fmr1* KO(2)), *Fmr1* conditional KO (cKO) mice and wild type (WT) littermates were generated as previously described and backcrossed to C57BL/6J for more than 10 generations [20]. Males heterozygous for the *Emx1-Cre* or the *Gad2-Cre* driver (C57BL/6 background) were bred with females heterozygous for the *Fmr1* cKO allele generating male offspring with four genotypes: *Cre*, *Cre*-specific *Fmr1* KO, *Fmr1* cKO or wild type. As control littermates for the *Emx1*- and *Gad2*-specific *Fmr1* knockout animals, WT or *Cre* positive animals were used in the tube test. Male offspring were weaned and housed in couples during the 4th

postnatal week. Behavioral testing began in the 12th postnatal week and we used at least five but preferably six couples for each experiment. All animals were genotyped again after completion of the experiments to re-confirm the genotype. Animals were maintained under standard laboratory conditions with *ad libitum* access to food and water. Experiments were performed with the prior approval of the Netherlands Animal Ethical Committee.

Automated Tube Test (ATT)

The ATT protocol was performed similarly as previously described (de Esch *et al*; van den Berg and Kushner). We used the same automated ventilated transparent fibreglass tube with an opaque center door and a length of 50 cm, which is connected at both ends to identical fibreglass boxes through automated opaque doors (Benedictus, the Netherlands). The inside diameter of the tube (2.5 cm) does not permit the animals to reverse direction once they are inside the tube. All mice first participated in a 5-day training protocol involving two habituation trials on day 1, followed by 6 training trials per day on days 2-5. During the day 1 habituation trials, mice are given 2 habituation trials in which they are permitted to freely explore the tube until they travel from the starting box through the tube into the goal box, up to a maximum of 180 seconds per trial after which they were returned to their homecage. From the second day onwards, mice were placed inside a pseudo-randomly designated starting box and expected to walk through the tube to the goal box. Mice that remained in their starting box for more than 5 seconds would receive an air puff as a stimulation to advance into the tube. Once the mouse reached within 4 cm of the center door, it was opened automatically after a random variable delay of 1-3 seconds. When the mouse had not travelled through the tube within the 30 second time limit, he was gently pushed through the tube with a poking device to make sure that he would end up in the goal box on the other side. Training days were followed by two days of rest, after which a tournament protocol was initiated. On tournament days, each mouse received two training trials, one hour before participating in matches between pairs of mice. At the start of a match, one mouse was placed in each of the two boxes, after which the box doors were opened simultaneously, allowing the mice to enter the tube. Mice that remained in their respective starting box for more than 5 seconds received an air puff to encourage them to enter the tube. During matches, the center door opened only when both mice were both within 4 cm of the door. A match was considered completed as soon as one mouse had retreated back into its own starting box with all four feet ('the looser'). After every training trial and tournament match, the interior of the tube was cleaned with 70% ethanol. Matches were performed with the experimenter blind to the genotype of the mice, and according to a randomly coded scheme by which matches were only performed between mice from different homecages, and between genotypes. On every tournament day, the same set of matches was performed, but always with a randomized order for which the starting position and inter-match interval of each mouse were explicitly balanced.

Immunohistochemical staining

Standard immunohistochemistry, including a haematoxylin and eosin staining, was performed on 6 µm slices of paraffin-embedded brains of *Emx1*-specific *Fmr1* KO and WT animals, using a peroxidase conjugated secondary antibody followed by DAB substrate (DAKO), for the detection of Fmrp (mouse T1A, 1:200). Slices (6 µm) of paraffin-embedded *Gad2*-specific *Fmr1* KO and WT

brains were simultaneously stained for Fmrp (mouse T1A, 1:200) and parvalbumin (rabbit 1:750, Swant) as a marker for inhibitory interneurons, using anti-mouse conjugated Cy3 and anti-rabbit conjugated Cy2 as secondary antibodies in addition to a nuclear staining (Hoechst).

Statistical analysis

All tube data were analyzed with the statistical software package SPSS (Chicago, USA). Individual match data was compared against the binomial distribution to determine whether the scores were significantly different from an outcome expected by chance, *ie* a 50%-50% win-lose outcome. All training data were analyzed using a repeated measures ANOVA with genotype and day of training as factors.

RESULTS

Cre expression results in region or cell-type specific deletion of Fmrp expression

Immunohistochemical staining was carried out to confirm the absence of Fmrp expression in the brain areas were the *Cre* drivers were expressed. In the *Emx1*-specific *Fmr1* KO animals, Fmrp expression was absent in the forebrain while Fmrp was still present in the remaining areas of the brain (Figure 1). In the *Gad2*-specific *Fmr1* KO animals, we used an antibody against parvalbumin (PV) to examine expression in GABAergic interneurons. Although the PV-positive neurons only represent a specific subset of the interneurons found throughout the brain, we could easily detect these interneurons spread throughout the cortex and hippocampus (Figure 2). While the vast majority of the neurons in the brain still expressed Fmrp, the PV-positive cells showed a lack of Fmrp expression which confirmed the specificity of the *Gad2-Cre* expression to interneurons (Figure 2).

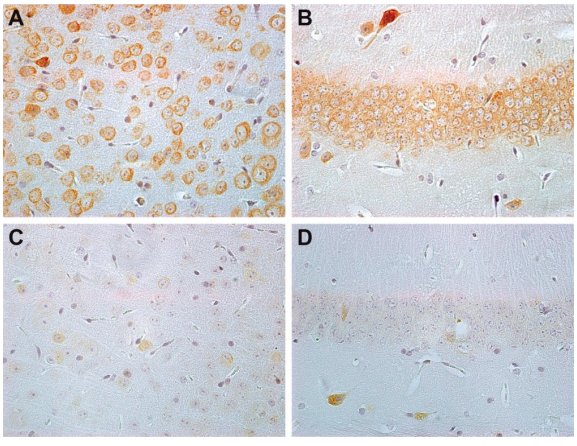


Figure 1. Immunohistochemical staining of Fmrp (brown) in the cortex and hippocampus of WT and *Emx1*- specific *Fmr1* KO animals.
A-B: WT brain showed Fmrp expression in the cortex (A) and in the hippocampus (B). **C-D**: In an *Emx1*-specific *Fmr1* KO brain, Fmrp expression was absent in the cortex and (C) and hippocampus (D).

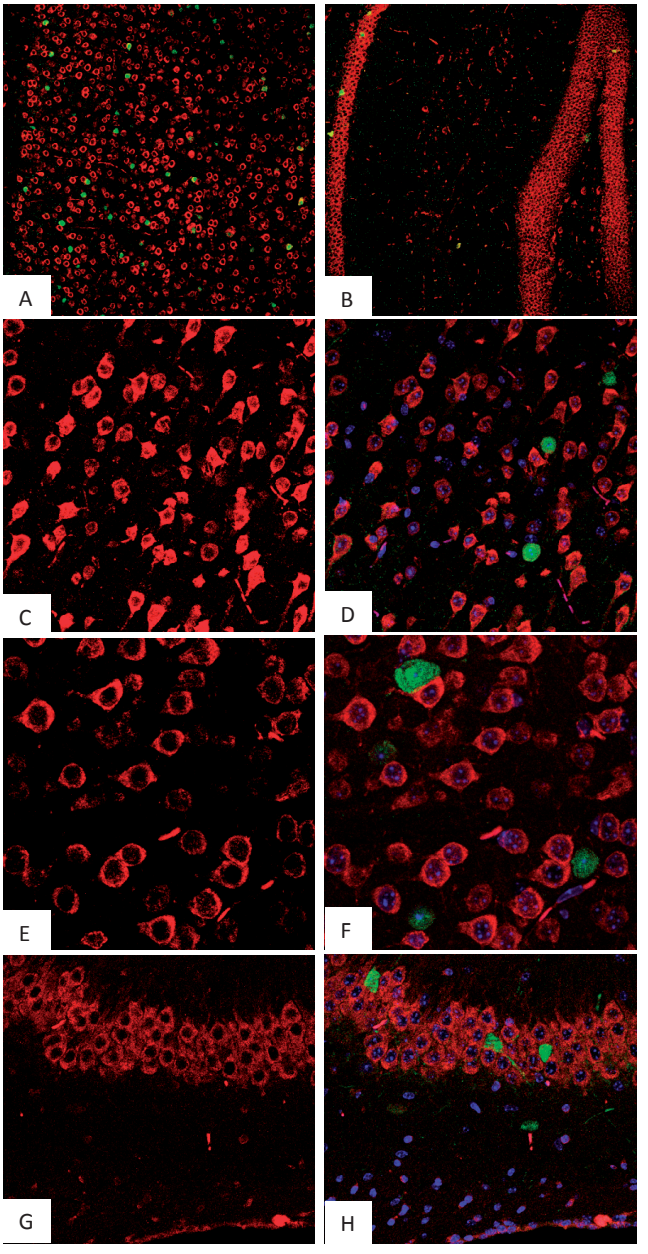


Figure 2. Immunofluorescent staining of Fmrp (red), parvalbumin (green) and the nuclues (blue) in the cortex and hippocampus of WT and *Gad2-specific Fmr1* KO animals.
A-D: WT brain showed parvalbumin (PV) positive cells (green) throughout the cortex (A) and in the hippocampus (B). In C only the Fmrp (red) staining is shown, while in D the overlay of Hoechst (blue), PV and Fmrp is shown. In WT brain, the PV positive cells expressed Fmrp. **E-H**: In a *Gad2-specific Fmr1* KO brain, the PV positive cells did not show FMRP expression in the cortex (E and F) or hippocampus (G and H). In E and G only Fmrp staining is shown while F and H shows all markers in an overlay (Hoechst, Fmrp and PV).

Lack of Fmrp in the forebrain results in socially submissive behavior in the tube test

Tournaments were carried out between *Emx1*-specific *Fmr1* KO animals and their WT littermates. Animals were housed in couples, thus a *Emx1*-specific *Fmr1* KO together with a WT animal, and matches were only performed between non-cagemates. After performing tournaments, it turned out that the WT animals won the majority of the matches (Figure 3). The WT animals won significantly more matches than their *Emx1*-specific *Fmr1* KO littermates from the second day onwards (70%), and on the remaining days they win on average 81% of the matches (n=6/group; P<0.05 or P<0.001).

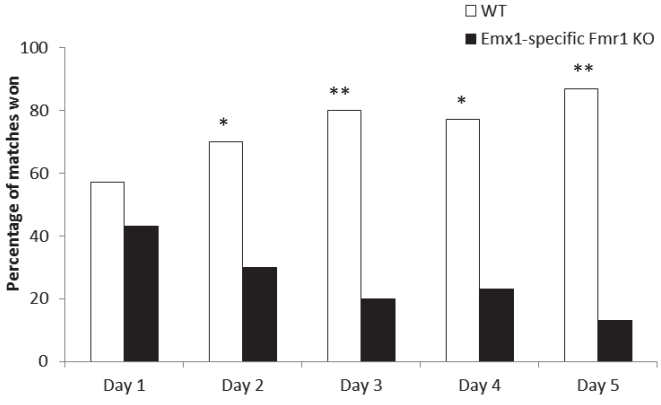


Figure 3. Average percentage of matches won by WT and *Emx1*-specific *Fmr1* KO animals in tube tournaments.

Emx1-specific *Fmr1* KO animals housed with a WT littermate showed socially submissive behavior in the tube test (n=6/group)(*P<0.05 **P<0.001).

As a control experiment we carried out tournaments between *Fmr1* cKO, thus without the *Cre* allele, and *Emx1-Cre* positive animals to make sure that both groups were not showing a social behavioral phenotype in the ATT (Figure 4). Both genotypes win on average between 35% and 65% of the matches which did not result in a significant difference between the groups.

To test whether the outcome of the matches of the two experiments mentioned above was influenced by external factors, the stability of the outcomes over time was determined. The percentage of matches with the same outcome as on the first day was 67% or higher for each continuing tournament day in the experiments. When comparing the outcomes to the second day of each tournament, the minimum stability increased to 73%. In addition, analysis of the training trials revealed no significant differences between genotypes in latency to enter the tube (Supplemental Figure 1).

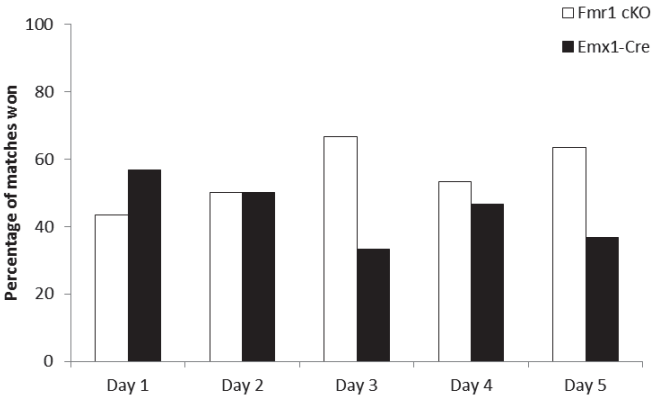


Figure 4. Average percentage of matches won by *Fmr1* cKO and *Emx1-Cre* animals in tube tournaments.
Fmr1 cKO animals and *Emx1-Cre* littermates won on average an equal amount of matches in the tube test (n=6/group, P ≥ 0.05).

Lack of Fmrp in GABAergic inhibitory neurons leads to socially dominant behavior in the tube test

Tube tournaments were carried out between animals lacking Fmrp expression in their GABAergic interneurons (*Gad2*-specific *Fmr1* KO) and as a control, their *Gad2-Cre* littermates. The animals were housed in couples and only matches between non-cagemates were performed. Figure 5 shows that the *Gad2*-specific *Fmr1* KO animals won significantly more matches than their control *Gad2-Cre* littermates. They won 75% percent of the matches on the first day and this increased to 90% on the second day. On the last days of the tournament they won 95% or 100% of the matches (n=5/group; P<0.05 or P<0.001).

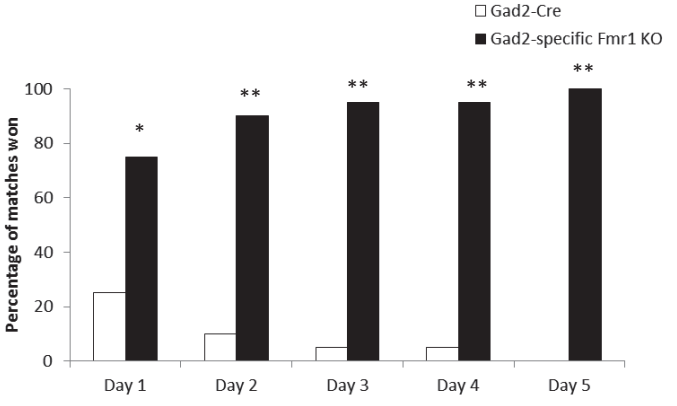


Figure 5. Average percentage of matches won by *Gad2-Cre* and *Gad2*-specific *Fmr1*KO animals in tube tournaments.
Gad2-specific *Fmr1*KO animals housed with a *Gad2-Cre* littermate showed socially dominant behavior in the tube test (n=5/group) (*P<0.05; **P<0.001).

In an additional tube experiment we tested couple-housed *Fmr1* KO and *Gad2*-specific *Fmr1* KO animals to investigate which genotype would display stronger dominant behavior. Here, the *Gad2*-specific *Fmr1* KO animals lost the majority of the matches (Figure 6). The *Fmr1* KO animals won 95% to 100% of the matches against the *Gad2*-specific *Fmr1* KO animals on all tournament days (n=5/group, P<0.001).

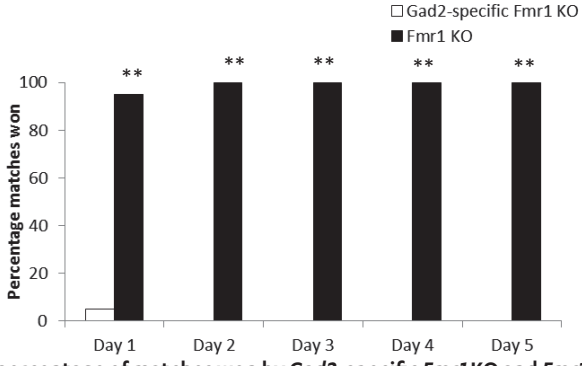


Figure 6. Average percentage of matches won by *Gad2*-specific *Fmr1* KO and *Fmr1* KO animals in tube tournaments.
Fmr1 KO animals housed with a *Gad2*-specific *Fmr1* KO animal showed socially dominant behavior in the tube test (n=5/group) (**P<0.001).

In the *Gad2*-specific *Fmr1* KO versus *Gad2-Cre* experiment the stability was minimal 75% after comparing the outcomes to the results of the first day of tournament. When compared with the second day of the tournament, the minimum stability increased to 85%. In the experiment with *Fmr1* KO animals, the minimal stability was 95% compared with the first day and this increased to a 100% when compared with the second day. The effect of the training was clearly visible since a significant interaction of time was found in the latency to enter the tube in both experiments.

DISCUSSION

In this study, we investigated the consequences of a lack of Fmrp expression in a subset of neurons, on social dominance behavior. We used the automated tube test which has proven to be a reliable outcome measure for social dominance behavior of mice. The results showed that absence of Fmrp in forebrain glutamatergic neurons, including the cortex and limbic system, did not result in altered social dominance behavior such as was observed in Fmrp null mice (de Esch *et al*, 2014). Deletion of Fmrp in the *Gad2*-expressing inhibitory neurons however did result in an altered social dominant phenotype in the tube test.

Only a few attempts have been made to investigate the consequences of a lack of Fmrp in specific brain regions or cell types using specific Cre-drivers. Deletion of *Fmr1* in the Purkinje cells of the cerebellum by the expression of an *L7-Cre* driver, resulted in an morphological spine phenotype as well as in an impaired cerebellar eye blink conditioning [21]. An inducible *Nestin-*

Cre driver was used to delete *Fmr1* in adult hippocampal neural stem cells. This led to decreased neuronal differentiation, reduced dendritic complexity and deficits in learning tasks in the conditional KO mice [22, 23]. Very recently, a *Nse-Cre* driver line was used to knock out *Fmr1* expression in a subset of differentiated neurons of the hippocampus and cortex. This resulted in dendritic alterations but not in an apparent behavioral phenotype in a social assay, anxiety assay or a learning and memory assay [24]. These results show that a brain region-specific lack of Fmrp expression may already lead to abnormal brain morphology or behavior.

Our results with the *Emx1-Cre* driver line showed that lack of Fmrp expression in the cortex, hippocampus and amygdala is not sufficient to create a fragile X behavioral phenotype in the tube test. In contrast, the *Emx1*-specific *Fmr1* KO mice showed a submissive behavior in the ATT. Synaptic strength in forebrain glutamatergic pyramidal neurons have been implicated in regulating dominance behavior and our results would confirm the hypothesis that a reduced synaptic strength in the cortex, here due to a lack of Fmrp, leads to submissive social behavior [25, 26]. However, the fact that fragile X mice display features of altered synaptic plasticity in combination with dominant behavior, implicates that additional brain regions must be involved in the establishment of social dominance behavior in FXS. Although the neuronal development and synaptic plasticity of the forebrain was most likely altered by a lack of Fmrp, more experiments are needed to fully characterize the effects of the Cre-mediated ablation on synaptic plasticity and signaling pathways in the forebrain. Since the whole brain is involved in the outcome of a behavioral test it would also be interesting to see whether one of the major hallmarks of FXS, increased protein synthesis levels, are also observed in these conditional mice.

Inhibitory interneurons produce and release γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in central nervous system. GABA is synthesized from the neurotransmitter glutamate via two isoforms of glutamic acid decarboxylases, GAD67 and GAD65, encoded by the *Gad1* and *Gad2* genes, respectively, which are co-expressed in most brain regions [27]. By the use of a *Gad2-Cre* driver line, Cre is co-expressed with *Gad2* throughout development in GABAergic neurons, which results in a reporter expression almost entirely restricted to interneurons, with a specificity of ~92% and an efficiency of ~91% [19]. GABAergic interneurons are very diverse with a distinct cytoarchitecture, electrophysiological properties and connectivity patterns [28, 29]. The heterogeneity of the interneurons has hindered the understanding of their behavior-related functioning. Our experiments with the *Gad2*-specific *Fmr1* KO animals clearly show social dominant behavior which may lead to the conclusion that the inhibitory signaling system contributes to the social dominance behavior in FXS. However, the *Gad2*-specific *Fmr1* KO animals are socially submissive to *Fmr1* KO mice which implies the involvement of additional signaling pathways in social dominance behavior.

Based on the expression of molecular markers, interneurons can be divided into subtypes including parvalbumin (PV), calretinin, calbindin or somatostatin (SST) expressing cells and others. PV and SST expressing interneurons form the two major classes of inhibitory neurons [30]. PV interneurons are suggested to control the precise timing of cortical network oscillations and believed to play a role in neurodevelopmental disorders such as schizophrenia [31-33]. SST containing interneurons are dendrite-targeting interneurons and very sensitive to excitotoxicity. SST-interneurons are believed to be involved in neurological diseases and insults

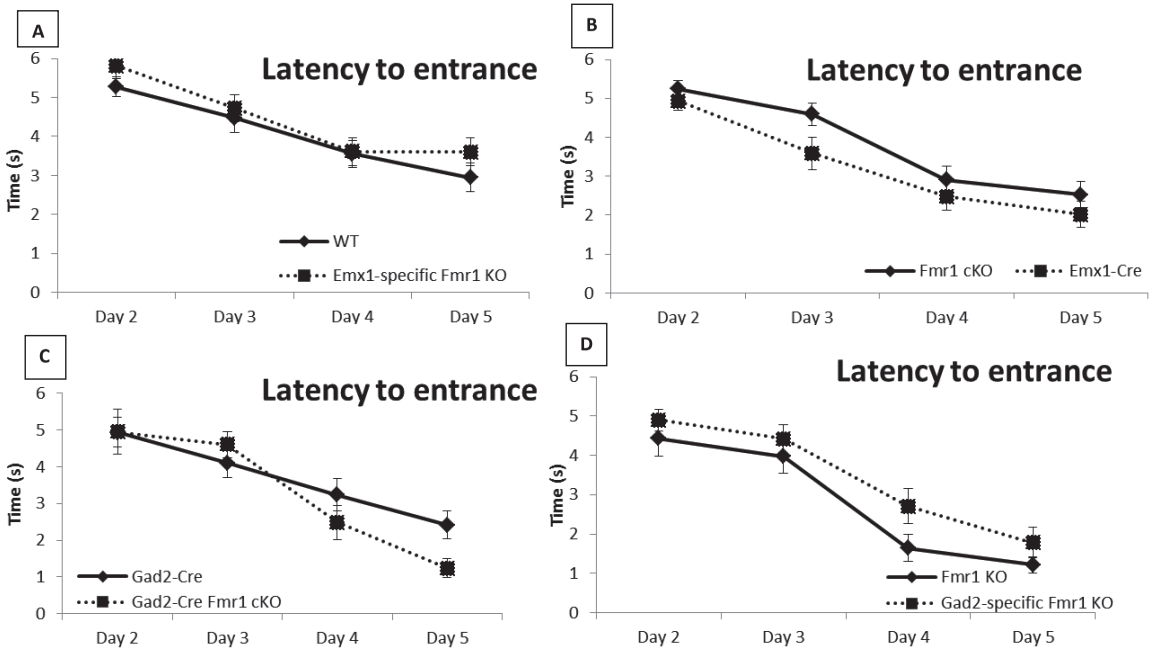
including epilepsy and Alzheimer's disease [34, 35]. It would be interesting to target these two major subtypes of interneurons by the use parvalbumin- or somatostatin-Cre specific driver lines to further explore the role of the inhibitory system in social behavior [19]. In addition, more experiments are necessary in order to investigate the effects of an interneuron specific lack of Fmrp on the GABA signaling in the brain. Although these results are still preliminary, ultimately, these may lead to a better understanding of the autistic-like social behavior observed in fragile X syndrome.

REFERENCES

1. Hagerman, R.J., *The physical and behavioural phenotype*, in *Fragile-X syndrome: diagnosis, treatment and research*, R.J. Hagerman and P. Hagerman, Editors. 2002: The Johns Hopkins University Press, Baltimore. p. 3-109.
2. Lachiewicz, A.M., et al., *Aberrant behaviors of young boys with fragile X syndrome*. Am J Ment Retard, 1994. **98**(5): p. 567-79.
3. Musumeci, S.A., et al., *Epilepsy and EEG findings in males with fragile X syndrome*. Epilepsia, 1999. **40**(8): p. 1092-9.
4. Darnell, J.C., et al., *FMRP Stalls Ribosomal Translocation on mRNAs Linked to Synaptic Function and Autism*. Cell, 2011. **146**(2): p. 247-61.
5. Huber, K.M., et al., *Altered synaptic plasticity in a mouse model of fragile X mental retardation*. Proc Natl Acad Sci U S A, 2002. **99**: p. 7746-50.
6. Osterweil, E.K., et al., *Hypersensitivity to mGluR5 and ERK1/2 Leads to Excessive Protein Synthesis in the Hippocampus of a Mouse Model of Fragile X Syndrome*. J Neurosci, 2010. **30**(46): p. 15616-27.
7. Qin, M., J. Kang, and C.B. Smith, *A null mutation for Fmr1 in female mice: effects on regional cerebral metabolic rate for glucose and relationship to behavior*. Neuroscience, 2005. **135**(3): p. 999-1009.
8. Bear, M.F., K.M. Huber, and S.T. Warren, *The mGluR theory of fragile X mental retardation*. Trends Neurosci, 2004. **27**(7): p. 370-7.
9. Hou, L., et al., *Dynamic Translational and Proteasomal Regulation of Fragile X Mental Retardation Protein Controls mGluR-Dependent Long-Term Depression*. Neuron, 2006. **51**(4): p. 441-54.
10. Chuang, S.C., et al., *Prolonged epileptiform discharges induced by altered group I metabotropic glutamate receptor-mediated synaptic responses in hippocampal slices of a fragile X mouse model*. J Neurosci, 2005. **25**(35): p. 8048-55.
11. D'Hulst, C., et al., *Decreased expression of the GABA(A) receptor in fragile X syndrome*. Brain Res, 2006. **1121**: p. 238-245.
12. D'Hulst, C., et al., *Expression of the GABAergic system in animal models for fragile X syndrome and fragile X associated tremor/ataxia syndrome (FXTAS)*. Brain Res, 2009. **1253**: p. 176-183.
13. Selby, L., C. Zhang, and Q.Q. Sun, *Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein*. Neurosci Lett, 2007. **412**: p. 227-232.
14. Centonze, D., et al., *Abnormal Striatal GABA Transmission in the Mouse Model for the Fragile X Syndrome*. Biol Psychiatry, 2007. **63**: p. 963-973.
15. Curia, G., et al., *Downregulation of Tonic GABAergic Inhibition in a Mouse Model of Fragile X Syndrome*. Cereb Cortex, 2009. **19**: p. 1515-1520.
16. Heulens, I., et al., *Pharmacological treatment of fragile X syndrome with GABAergic drugs in a knockout mouse model*. Behav Brain Res, 2012. **229**: p. 244-249.
17. D'Hulst, C. and R.F. Kooy, *The GABA(A) receptor: a novel target for treatment of fragile X?* Trends Neurosci, 2007. **30**: p. 425-431.
18. Guo, H., et al., *Specificity and efficiency of Cre-mediated recombination in Emx1-Cre knock-in mice*. Biochem Biophys Res Commun, 2000. **273**(2): p. 661-5.
19. Taniguchi, H., et al., *A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex*. Neuron, 2011. **71**(6): p. 995-1013.
20. Mientjes, E.J., et al., *The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo*. Neurobiol Dis, 2006. **21**: p. 549-555.
21. Koekkoek, S.K., et al., *Deletion of FMR1 in Purkinje Cells Enhances Parallel Fiber LTD, Enlarges Spines, and Attenuates Cerebellar Eyelid Conditioning in Fragile X Syndrome*. Neuron, 2005. **47**(3): p. 339-52.
22. Guo, W., et al., *Ablation of Fmrp in adult neural stem cells disrupts hippocampus-dependent learning*. Nat Med, 2011. **19**: p. 559-565.
23. Luo, Y., et al., *Fragile x mental retardation protein regulates proliferation and differentiation of*

adult neural stem/progenitor cells. PLoS Genet, 2010. **6**(4): p. e1000898.

24. Amiri, A., et al., *Analysis of FMR1 deletion in a subpopulation of post-mitotic neurons in mouse cortex and hippocampus*. Autism Res, 2014. **7**(1): p. 60-71.
25. Wang, F., et al., *Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex*. Science, 2011. **334**(6056): p. 693-7.
26. Timmer, M., et al., *Evidence for a role of oxytocin receptors in the long-term establishment of dominance hierarchies*. Neuropsychopharmacology, 2011. **36**(11): p. 2349-56.
27. Soghomonian, J.J. and D.L. Martin, *Two isoforms of glutamate decarboxylase: why?* Trends Pharmacol Sci, 1998. **19**(12): p. 500-5.
28. De Marco Garcia, N.V., T. Karayannis, and G. Fishell, *Neuronal activity is required for the development of specific cortical interneuron subtypes*. Nature, 2011. **472**(7343): p. 351-5.
29. Fu, C., et al., *GABAergic interneuron development and function is modulated by the Tsc1 gene*. Cereb Cortex, 2012. **22**(9): p. 2111-9.
30. Rudy, B., et al., *Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons*. Dev Neurobiol, 2011. **71**(1): p. 45-61.
31. Lewis, D.A., *The chandelier neuron in schizophrenia*. Dev Neurobiol, 2011. **71**(1): p. 118-27.
32. Smith-Hicks, C.L., *GABAergic dysfunction in pediatric neuro-developmental disorders*. Front Cell Neurosci, 2013. **7**: p. 269.
33. Gonzalez-Burgos, G. and D.A. Lewis, *NMDA receptor hypofunction, parvalbumin-positive neurons, and cortical gamma oscillations in schizophrenia*. Schizophr Bull, 2012. **38**(5): p. 950-7.
34. Tallent, M.K., *Somatostatin in the dentate gyrus*. Prog Brain Res, 2007. **163**: p. 265-84.
35. Binaschi, A., G. Bregola, and M. Simonato, *On the role of somatostatin in seizure control: clues from the hippocampus*. Rev Neurosci, 2003. **14**(3): p. 285-301.



Supplemental Figure 1. Overview of training data of all experiments.
A: Latency to enter the tube by WT and *Emx1-specific Fmr1* KO animals was displayed for each training day (time in seconds, +-SEM; n=6/group). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,210)=20.716$, $p<0.001$). **B:** Latency to enter the tube by *Fmr1* cKO and *Emx1-Cre* animals was displayed for each training day (time in seconds, +-SEM; n=6/group). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,210)=32.309$, $p<0.001$) **C:** Latency to enter the tube by *Gad2-Cre* and *Gad2*-specific *Fmr1* KO animals was displayed for each training day (time in seconds, +-SEM; n=5/group). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,174)=22.964$, $p<0.001$ **D:** Latency to enter the tube by *Fmr1* KO and *Gad2*-specific *Fmr1* KO animals was displayed for each training day (time in seconds, +-SEM; n=5/group). Repeated measures ANOVA indicated a significant effect of training day on latency to enter the tube in both groups ($F(1,174)=37.934$, $p<0.001$)).

CHAPTER 6

GENERAL DISCUSSION

Fragile X syndrome results from silencing of the *FMR1* gene, which encodes the fragile X mental retardation protein (FMRP). FMRP is an RNA binding protein that is highly expressed in neurons. Lack of FMRP from early development onwards is believed to produce the typical characteristics of FXS, including moderate to severe intellectual disability, hyperactivity, anxiety, and deficits in social interaction and communication. Evidence from the fragile X mouse model and other animal models have led to the hypothesis that the absence of FMRP does not necessarily lead to permanently altered neuronal connectivity but that many symptoms of FXS arise from modest changes in synaptic plasticity which can be corrected with targeted therapies.

Current therapies in FXS include treatment of medical problems, psychotherapy and behavioral therapy, and psychopharmacology to address attention deficits, anxiety, and aggression or mood disorders [1, 2]. However, these therapies are all symptom-based treatments and there is a critical need for agents that modify the underlying pathophysiology of the disorder. The aim of this thesis is to contribute to the development of reliable pre-clinical translational outcome measures and putative biomarkers that will aid in the development and evaluation of targeted therapeutic interventions in FXS.

HUMAN CELL MODEL FOR FXS

Studies using cultured primary neurons from the *Fmr1* KO mouse model have gathered an enormous amount of knowledge on FXS-related aberrant spine morphology and synaptic plasticity and this has encouraged the development of targeted therapies for FXS. Since studies on post-mortem brain material from human FXS patients also showed an altered spine morphology, cultured human neurons have the potential to be an excellent *in vitro* model for FXS [3-5]. Due to advancing techniques, it is now possible to generate disease-specific induced pluripotent stem cells (iPSCs) and even differentiated cell types like neurons from somatic cells such as skin fibroblasts [6].

In 2007, it became possible to create induced pluripotent stem cells from somatic cells such as fibroblasts [7]. The creation, characterization and culturing of iPSCs is time-consuming and expensive, but various protocols and specialized culturing reagents are now widely available which facilitates working with these cells. To date, several groups have shown that undifferentiated FXS iPSCs carry a methylated *FMR1* promoter in contrast to their human embryonic stem cell equivalents [8-10]. The most likely explanation for this finding is that the standard fibroblast reprogramming methods are not sufficient to create naïve ground state iPSCs, as recently demonstrated by Gafni *et al*, who found an unmethylated *FMR1* promoter in FXS iPSCs after using an unique reprograming cocktail [11]. Thus, the existing iPS protocols need to be optimized and standardized in order to be able to efficiently create disease-specific iPSCs, which resemble their human embryonic stem cell equivalents as close as possible. In addition, in FXS research a neurological cell model would be preferred and by now there are also many protocols available to differentiate iPSCs into neuronal subtypes [12-16]. However, the current characterization of human neuronal subtypes is difficult because of the limited knowledge of the temporal expression of specific genes during human brain development. In addition, it has been shown that different iPSC clones from one fibroblast cell line can have variable neuronal differentiation potentials [17]. Last but not least, the current neural differentiation strategies

are not capable of providing a large number of homogenous neural cell types in a reliable, reproducible and cost-effective way. The technique to create neurons directly out of human fibroblasts may circumvent a few of the steps in the existing iPSC creation and differentiation protocols, which might reduce the time and costs of creating human fragile X neurons [18]. However, this technology is even more inefficient in human cells and has the disadvantage to skip certain steps in neuronal development, which makes this technique less useful to create a human neuronal cell model. A few groups have differentiated FXS iPSCs into neurons, but not all groups examined the potential neurological phenotype of these cells [9, 10, 19]. FXS differentiated neurons were flatter and with fewer and shorter processes as reported by Sheridan *et al* [9]. In addition, they found an increased number of glial cells in the differentiated FXS cultures [9].

In **chapter two,** we describe a study initiated to investigate the epigenetic modifications of the *FMR1* gene in time by the use of iPS cells. By creating iPSCs from a human fibroblast line that carried a fully expanded but yet unmethylated CGG repeat, we expected to be able to study the occurrence of epigenetic modifications such as histone methylation or acetylation of the *FMR1* promoter region during differentiation. Surprisingly, the *FMR1* promoter region turned out to be already fully methylated in the undifferentiated iPS cells derived from this fibroblast line, and thus this line could not be used to investigate the silencing process of the *FMR1* gene in FXS.

In an initial attempt to unravel the silencing mechanism of the *FMR1* gene, human embryonic stem cells (hESCs) obtained through a pre-implantation genetic diagnostic (PGD) procedure were used. In these stem cells, the unmethylated *FMR1* promoter became methylated upon differentiation [20, 21]. These results are in accordance with the results found by Willemsen *et al*, where FMRP was still expressed in chorionic villi from FXS pregnancies during early embryonic development [22]. Thus, during early development the *FMR1* gene is still active and produces mRNA transcripts with expanded CGG repeats. A conclusive answer to the underlying molecular mechanisms of the epigenetic silencing of the *FMR1* gene came very recently, Colak *et al* showed in hESCs that these expanded CGG-containing transcripts mediate the silencing of the *FMR1* gene by forming an RNA-*FMR1* DNA duplex during the early stages of differentiation. However since the *FMR1* mRNA levels drop during differentiation which prevents the formation of a duplex, the mechanisms which maintain the epigenetic silencing in time remain unknown [23]. Recently, it was shown that functional neuronal cells, differentiated from FXS hESCs, demonstrated an altered early neurogenesis which ultimately lead to a poor neuronal maturation and a high number of glia cells in the FXS cultures [19]. These results showed a striking phenotype but the poor functioning of these FXS neurons does not contribute to the development of a robust model for drug screening. In addition, FXS hESCs are difficult to obtain because of ethical and practical reasons, which make them not suitable for the use as a human cell model for FXS.

In conclusion, to obtain a robust human cell model for FXS it is critical that effective, efficient and robust reprogramming and differentiation methods are developed, as well as methods that minimize variation between iPSC clones derived from the same donor. In addition, the differentiated human FXS neurons need to be characterized very carefully preferably by comparison with neurons derived from a healthy relative, in order to discover a disease

phenotype. For the characterization, measures of excitatory glutamate synapse numbers, GABA expression levels, electrophysiological functioning and spine morphology are potential candidates. If these human FXS neurons indeed show a representative morphological and/or functional phenotype then they could serve as an excellent human cell model for altered synaptic plasticity, perhaps not only for FXS but also for strongly related cognitive disorders without a specific single gene defect such as autism spectrum disorder.

SOCIAL BEHAVIOR AS A TRANSLATIONAL OUTCOME MEASURE

Due to the strong parallel between FXS and autism and the fact that laboratory mice are social animals showing social approach behavior and interactions with familiar and unknown mice, current research has been dedicated to assess the autistic-like behavioral phenotype of fragile X mice. In order to reach our aim, namely to establish a behavioral test in which the fragile X mice show a robust and reproducible phenotype that can be used in the evaluation of pre-clinical therapeutic treatments, we examined the social behavior of our FXS mice using the sociability and preference for social novelty (SPSN) test and the automated tube test (ATT) (**chapters three, four and five**). Both our behavioral tests revealed the aberrant social behavior of our fragile X mice and to validate our tests, we made use of one of the well-established features of FXS, namely the aberrant mGluR5-signaling pathway [24]. A genetic reduction of the mGluR5 signaling by 50% has shown to rescue many of the FXS phenotypes in mice [25, 26]. In addition, several mGluR5 antagonists, including MPEP, Fenobam, AFQ056/Mavoglurant and CTEP, have shown to rescue many of the synaptic and behavioral phenotypes of the *Fmr1* KO mouse as well [27-32]. In our tests we observed a partial or a complete correction of the aberrant social behavior after reducing the mGluR5 signaling, which leads to the conclusion that our behavioral measures are valid assays to measure the aberrant social behavior of fragile X mice.

As shown in **chapter three**, our fragile X mice showed aberrant sociability also referred to as social approach behavior, in the three-chambered set-up for social behavior [32]. Sociability can be measured using various set-ups, for example by the use of a cage with a partition or by the use of an open field [33, 34]. However, the three-chambered test differs significantly from these measures on the following aspects: the stranger mice are not able to move away from the test mouse, whisker contact is possible between the test mouse and the stranger mouse, and finally yet importantly, test mice are allowed to explore three chambers, including a chamber without a mouse. These differences make it difficult to compare our results to the other type of social behavioral assays, however by now the three-chambered set-up has gained preference over the other measures and has become a widely used social behavioral assay in autism research [35-39] and others.

When confronted with a stranger mouse or an empty cup in the sociability phase, the fragile X mice showed an increased social interaction with the stranger mouse compared to WT mice, as indicated by a significantly higher sniffing time. Many other measures, *eg* time in chamber, number of entries or time spent per entry were also automatically recorded during the test, but we analyzed the time sniffing the stranger mouse or empty cup since it is the most direct quantification of social interaction [37, 40]. However, other studies using a similar three-chambered set-up, have found conflicting results in *Fmr1* KO mice including decreased

sociability, increased sociability or no deficits at all [41-46]. These different outcomes can be explained by a differences in background strains (C57Bl/6J, FVB/NJ), naivety and age of mice examined (2-4 months, 10-12 months), time of testing (light or dark phase), stranger mice used (genotype, naivety, aggressiveness) and differences in protocol (housing of mice, habituation) [46-48]. In the second phase, the preference for social novelty phase, a second stranger mouse replaced the empty cup and the fragile X mice showed an increased preference for the novel mouse. However, this behavior was comparable to the behavior of WT mice, indicating a normal preference for social novelty by *Fmr1* KO mice. Similar results have been described before although one group consistently found a significantly decreased interaction with a novel stranger mouse by *Fmr1* KO mice compared to WT mice, however this could also be explained by the methodological differences mentioned before [42, 43]. In addition, the preference for social novelty phase is based on social recognition or memory and is therefore perhaps less suitable for measuring social interaction behavior by itself [38]. In addition to our results, mouse models of autism have also been tested in the three-chambered set-up and, in general, a decreased sociability is reported, which makes it difficult to translate our results directly to autistic-like behavior in mice [49-51].

In **chapter four and five,** we used the automated tube test (ATT) to measure social dominance behavior of our fragile X mice. The tube test is a standard classical test in which two mice are positioned nose-to nose in the middle of a tube, in order to define the most dominant mouse namely the one that will move forward and that will ultimately end up on the other side of the tube [52]. Although this test has been available for many years, to our knowledge it has not been used in autism research much and only two groups have investigated the behavior of fragile X mice in this test [33, 41]. We are the first to fully characterize the social dominance behavior of our mice under different housing conditions and after applying different rescue strategies and we found a robust social dominant phenotype. Although both other groups showed submissive behavior of fragile X mice, our experimental set-up differed on many important aspects, including 1) we standardized the housing of our test animals which is known to influence social dominance behavior [48, 53], 2) we applied an extensive training protocol in order to reduce the nonsocial anxiety in our mice, 3) our WT and *Fmr1* KO animals participated in an equal number of matches on each day so that a similar test experience was obtained, 4) by performing the same matches on subsequent days we showed a strong stability of our outcomes in time, 5) while last but not least our mice were not able to turn around or crawl over each other in our tube apparatus which led to a clear outcomes [33, 41]. In conclusion, we believe that our *Fmr1* KO mice show a clear social dominant phenotype in the ATT when appropriately controlled for social experience, test experience and nonsocial anxiety.

Nonetheless, studies on social behavior of fragile X patients have also reported a great heterogeneity in social behaviors. Some FXS individuals show clear motivation to engage in social interactions while others show more profound and consistent social withdrawal [54-56]. From these results it is clear that the social behavioral phenotypes in FXS are highly complex. Anxiety is believed to be one of the underlying motivations in engaging social interactions, and although human FXS patients suffer from high levels of social anxiety as well as from additional forms of anxiety (*eg* panic attacks) research on anxiety in *Fmr1* KO mice has shown

different results [57, 58]. A large number of reports showed no differences or lower levels of nonsocial anxiety-like behaviors in fragile X mice while only a few studies were conducted on social anxiety and they show mixed results [33, 41, 46, 59, 60]. However, in our experiments fragile X mice spent more time sniffing a stranger mouse and they showed dominant behavior in the ATT, which points to a reduced social anxiety. The underlying neuronal substrates of anxiety-related behavior include stress induced changes in corticosterone levels, as well as the dopaminergic and serotonergic systems although more research is needed to fully characterize the influence of these substrates on social anxiety [61-64]. Finally, yet importantly, one of the major systems involved in anxiety related behavior is the GABA mediated neurotransmission, which has also been shown to be aberrant in fragile X syndrome [65-70].

Because of our well-controlled behavioral protocol and the robust outcomes with a very low variability between experiments, we decided to focus on the ATT to investigate the involvement of the forebrain and the GABA mediated signaling in social behavior of fragile X mice. The forebrain, which comprises the olfactory bulb, cortex, amygdala and hippocampus has been implicated in social behavior in mice and in humans, and it is speculated that the strength of synaptic glutamate transmission correlates to social ranking in mice [71-73]. In our experiments with the *Cre*-drivers, we showed that a lack of Fmrp in the forebrain leads to socially submissive behavior [74]. This could thus be explained by the aberrant glutamate signaling in the forebrain due to the lack of Fmrp. However, our complete *Fmr1* KO mice showed dominant behavior in the ATT, which makes us believe that although the forebrain might be the most important brain area involved in social behavior, other systems are also heavily involved in regulating dominance behavior. For our additional experiments we used *Gad2-Cre* drivers to delete Fmrp in a small subset of neurons in the brain, namely in the GABA producing, and thus inhibitory, interneurons [75]. These *Gad2-Cre* specific *Fmr1* KO mice clearly showed social dominant behavior despite the presence of Fmrp in the majority of the neurons in the brain; however these animals showed submissive behavior when tested against *Fmr1* KO animals. These findings led to the conclusion that the inhibitory neurotransmission is also involved in regulating social dominance behavior. Additional experiments are planned to elucidate this role further by the use of additional *Cre*-driver lines that target different interneuronal subtypes such as parvalbumin- or somatostatin-expressing interneurons which are both believed to be involved in neurological disorders [76-79].

To further investigate the underlying neural mechanisms involved in social behavior, other pharmacological compounds that target for example monoamine oxidase A, in order to block the metabolism of dopamine, norepinephrine and serotonin, can be used [80]. In addition to experiments in order to clarify the underlying mechanisms, therapeutic compounds that have shown to ameliorate fragile X phenotypes can also be tested in the ATT as well. A large amount of information has already been gathered on the effects of mGluR5 antagonist in humans and mice, thus it would be good to test other new and promising compounds in the ATT. For example drugs that target the GABA system such as ganaxolone, or compounds that target key signaling proteins that have proven to be aberrantly expressed in fragile X mice such as MEK or ERK [70, 81]. This information will increase our knowledge about this type of social behavior as well as the potential therapeutic value of different compounds on social behavior, which

might ultimately result in the development of new therapeutic strategies. The hypothesis that both excitatory (glutamate) and inhibitory (GABA) neurotransmission systems are involved in the pathophysiology of fragile X syndrome, leads to the expectation that only a combination of pharmacological compounds which targets both systems can lead to amelioration of most symptoms of fragile X syndrome however, combination therapies have also not been tested in mice or humans as yet. Last but not least, due to the enormous influence of the genetic background on social behavior, it would be worthwhile to test *Fmr1* KO mice in a series of different genetic backgrounds in order to fully characterize the ATT for the use in fragile X research [82, 83]. In conclusion, although the ATT was shown to be a robust and reliable pre-clinical outcome measure in fragile X research, the underlying mechanisms that lead to this type of social behavior remain elusive. More research is needed to order to investigate the underlying mechanisms of this type of behavior and to validate this test for the broader use in for example autism research.

Since aberrant social behavior is a prominent clinical feature of fragile X patients, it is used as an outcome measure in clinical trials as well. Although there are many rating scales designed to assess autistic-like social behavior, including the Social Responsive Scale and the Autism Diagnostic Observation Scale, these measures are only based on observations of the caregiver or clinician. With the notice that in fragile X clinical trials usually a high placebo effect (30-40%) is observed, these subjective measures are not ideal to detect small improvements in behavior. However, with advancing techniques the social behavior of fragile X patients can easily be recorded by a camera and specialized software. For example, fragile X patients can be observed while they are interacting with an unfamiliar or familiar person under specific circumstances. Although these measures are not yet used in clinical settings, these could ultimately lead to an objective and robust measure of social behavior in fragile X patients, which would also facilitate the translation of the findings from animals to humans.

In addition to the aberrant behavioral characteristics of fragile X mice, several research groups have also shown significant alterations in the expression levels of specific signaling proteins. For example, in *Fmr1* KO mice mTOR phosphorylation and activity is up-regulated as well the phosphorylated ERK levels [30, 84, 85]. In addition, up-regulated levels of STEP or PSD-95 were found in fragile X mice as well [41, 86-88]. However, the reproducibility of these outcomes has always been a challenge due to differences in isolation and quantification methods, the brain areas investigated and the type of samples used (whole brain, brain slices etc.) [81, 89-91]. Thus, in addition to the behavioral outcome measures we tried to find a biochemical outcome measure in fragile X mice. The frontal part of the cortex was chosen because this part is believed to be involved in social behavior, in addition we enriched for synaptic proteins by the isolation of synaptoneurosomes. Although we measured the expression levels of several proteins, the only biomarker significantly altered in fragile X mice was the proportion of active ERK. This could be explained by the fact that we have measured the phosphorylated, and thus active, ERK in relation to the total amount of ERK present, which enables to detect small differences between groups. This outcome measure provides an objective read-out for the altered synaptic plasticity in fragile X mice and potentially also in human patients, although more research is needed to fully validate the p-ERK/ERK ratio as robust outcome measure [84, 92].

CONCLUDING REMARKS

Although results from a human cell culture model or a behavioral test can never be directly translated to humans, these models are necessary in the understanding of the molecular pathways involved in the disease pathology and for developing and testing potential therapeutic agents. To date, a large amount of therapeutic agents is available for FXS, but despite the positive results in animal models, they have not been very successful in clinical trials. One of the major problems are the clinical endpoints used in fragile X research, it appears that they either do not represent the right characteristics to detect an effect of a treatment or that they are too subjective and thus easily placebo-biased. By the development of a robust behavioral assay and a biochemical marker for fragile X mice, this thesis contributed to the development of objective and translational outcome measures in pre-clinical fragile X research.

REFERENCES

1. Hagerman, R.J., et al., *Advances in the treatment of fragile x syndrome*. Pediatrics, 2009. **123**(1): p. 378-90.
2. Berry-Kravis, E., et al., *Clinic-based retrospective analysis of psychopharmacology for behavior in fragile x syndrome*. Int J Pediatr, 2012. **2012**: p. 843016.
3. Hinton, V.J., et al., *Analysis of neocortex in three males with the fragile X syndrome*. Am J Med Genet, 1991. **41**(3): p. 289-294.
4. Rudelli, R.D., et al., *Adult fragile X syndrome. Clinico-neuropathologic findings*. Acta Neuropathol, 1985. **67**: p. 289-295.
5. Irwin, S.A., et al., *Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: A quantitative examination*. Am J Med Genet, 2001. **98**(2): p. 161-167.
6. Imamura, K. and H. Inoue, *Research on neurodegenerative diseases using induced pluripotent stem cells*. Psychogeriatrics, 2012. **12**(2): p. 115-9.
7. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors*. Cell, 2007. **131**(5): p. 861-72.
8. Urbach, A., et al., *Differential modeling of fragile X syndrome by human embryonic stem cells and induced pluripotent stem cells*. Cell Stem Cell, 2010. **6**(5): p. 407-11.
9. Sheridan, S.D., et al., *Epigenetic characterization of the FMR1 gene and aberrant neurodevelopment in human induced pluripotent stem cell models of fragile x syndrome*. PLoS ONE, 2011. **6**(10): p. e26203.
10. Bar-Nur, O., I. Caspi, and N. Benvenisty, *Molecular analysis of FMR1 reactivation in fragile-X induced pluripotent stem cells and their neuronal derivatives*. J Mol Cell Biol, 2012. **4**: p. 180-183.
11. Gafni, O., et al., *Derivation of novel human ground state naive pluripotent stem cells*. Nature, 2013. **504**(7479): p. 282-6.
12. Li, X.J., et al., *Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells*. Development, 2009. **136**(23): p. 4055-63.
13. Cooper, O., et al., *Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid*. Mol Cell Neurosci, 2010. **45**(3): p. 258-66.
14. Erceg, S., et al., *Efficient differentiation of human embryonic stem cells into functional cerebellar-like cells*. Stem Cells Dev, 2010. **19**(11): p. 1745-56.
15. Lee, H., et al., *Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons*. Stem Cells, 2007. **25**(8): p. 1931-9.
16. Kerr, C.L., et al., *Efficient differentiation of human embryonic stem cells into oligodendrocyte progenitors for application in a rat contusion model of spinal cord injury*. Int J Neurosci, 2010. **120**(4): p. 305-13.
17. Hu, B.Y., et al., *Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency*. Proc Natl Acad Sci U S A, 2010. **107**(9): p. 4335-40.
18. Vierbuchen, T., et al., *Direct conversion of fibroblasts to functional neurons by defined factors*. Nature, 2010. **463**(7284): p. 1035-41.

19. Telias, M., M. Segal, and D. Ben-Yosef, *Neural differentiation of Fragile X human Embryonic Stem Cells reveals abnormal patterns of development despite successful neurogenesis*. Dev Biol, 2013. **374**(1): p. 32-45.
20. Eiges, R., et al., *Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived from Preimplantation Genetically Diagnosed Embryos*. Cell Stem Cell, 2007. **1**(5): p. 568-577.
21. Gerhardt, J., et al., *The DNA replication program is altered at the FMR1 locus in fragile X embryonic stem cells*. Mol Cell, 2014. **53**(1): p. 19-31.
22. Willemsen, R., et al., *Timing of the absence of FMR1 expression in full mutation chorionic villi*. Hum Genet, 2002. **110**(6): p. 601-5.
23. Colak, D., et al., *Promoter-bound trinucleotide repeat mRNA drives epigenetic silencing in fragile X syndrome*. Science, 2014. **343**(6174): p. 1002-5.
24. Bear, M.F., K.M. Huber, and S.T. Warren, *The mGluR theory of fragile X mental retardation*. Trends Neurosci, 2004. **27**(7): p. 370-7.
25. Dolen, G., et al., *Correction of Fragile X Syndrome in Mice*. Neuron, 2007. **56**(6): p. 955-962.
26. Thomas, A.M., et al., *Genetic reduction of group 1 metabotropic glutamate receptors alters select behaviors in a mouse model for fragile X syndrome*. Behav Brain Res, 2011. **223**: p. 310-321.
27. De Vrij, F.M.S., et al., *Rescue of behavioral phenotype and neuronal protrusion morphology in FMR1 KO mice*. Neurobiol Dis, 2008. **31**: p. 127-132.
28. Yan, Q.J., et al., *Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP*. Neuropharmacology, 2005. **49**: p. 1053-1066.
29. Thomas, A.M., et al., *Group I metabotropic glutamate receptor antagonists alter select behaviors in a mouse model for fragile X syndrome*. Psychopharmacology (Berl), 2011. **19**: p. 47-58.
30. Michalon, A., et al., *Chronic Pharmacological mGlu5 Inhibition Corrects Fragile X in Adult Mice*. Neuron, 2012. **74**(1): p. 49-56.
31. Levenga, J., et al., *Potential therapeutic interventions for fragile X syndrome*. Trends Mol Med, 2010. **16**: p. 516-527.
32. Gantois, I., et al., *Chronic administration of AFQ056/Mavoglurant restores social behaviour in Fmr1 knockout mice*. Behav Brain Res, 2013. **239** p. 72-79.
33. Spencer, C.M., et al., *Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome*. Genes Brain Behav, 2005. **4**(7): p. 420-30.
34. Mineur, Y.S., et al., *Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse*. Hippocampus, 2002. **12**(1): p. 39-46.
35. Crawley, J.N., *Mouse behavioral assays relevant to the symptoms of autism*. Brain Pathol, 2007. **17**(4): p. 448-59.
36. Moy, S.S., et al., *Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice*. Genes Brain Behav, 2004. **3**(5): p. 287-302.
37. Nadler, J.J., et al., *Automated apparatus for quantitation of social approach behaviors in mice*. Genes Brain Behav, 2004. **3**(5): p. 303-14.
38. Yang, M., J.L. Silverman, and J.N. Crawley, *Automated three-chambered social approach task for mice*. Curr Protoc Neurosci, 2011. **Chapter 8**: p. Unit 8 26.
39. Brodkin, E.S., et al., *Social approach-avoidance behavior of inbred mouse strains towards DBA/2 mice*. Brain Research, 2004. **1002**(1-2): p. 151-157.
40. Naert, A., Z. Callaerts-Vegh, and R. D'Hooge, *Nocturnal hyperactivity, increased social novelty preference and delayed extinction of fear responses in post-weaning socially isolated mice*. Brain Research Bulletin, 2011. **85**(6): p. 354-362.
41. Goebel-Goody, S.M., et al., *Genetic manipulation of STEP reverses behavioral abnormalities in a fragile X syndrome mouse model*. Genes Brain Behav, 2012. **11**: p. 586-600.
42. Liu, Z.H., D.M. Chuang, and C.B. Smith, *Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome*. Int J Neuropsychopharmacol, 2010: p. 1-13.
43. Liu, Z.H. and C.B. Smith, *Dissociation of social and nonsocial anxiety in a mouse model of fragile X syndrome*. Neurosci Lett, 2009. **454**(1): p. 62-6.
44. Mines, M.A., et al., *GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of fragile X syndrome and autism*. PLoS ONE, 2010. **5**(3): p. e9706.
45. Moy, S.S., et al., *Social Approach in Genetically-Engineered Mouse Lines Relevant to Autism*. Genes Brain Behav, 2009. **8**: p. 129-142.
46. McNaughton, C.H., et al., *Evidence for social anxiety and impaired social cognition in a mouse model of fragile X syndrome*. Behav Neurosci, 2008. **122**(2): p. 293-300.
47. Moy, S.S., et al., *Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains*. Behav Brain Res, 2007. **176**(1): p. 4-20.

48. Palanza, P., L. Gioiosa, and S. Parmigiani, *Social stress in mice: Gender differences and effects of estrous cycle and social dominance*. Physiology & Behavior, 2001. **73**(3): p. 411-420.

49. Silverman, J.L., et al., *Repetitive self-grooming behavior in the BTBR mouse model of autism is blocked by the mGluR5 antagonist MPEP*. Neuropsychopharmacology, 2010. **35**(4): p. 976-89.

50. Kwon, C.H., et al., *Pten regulates neuronal arborization and social interaction in mice*. Neuron, 2006. **50**(3): p. 377-88.

51. Sala, M., et al., *Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: a neurobehavioral model of autism*. Biol Psychiatry, 2011. **69**(9): p. 875-82.

52. Lindzey, G., H. Winston, and M. Manosevitz, *Social dominance in inbred mouse strains*. Nature, 1961. **191**: p. 474-6.

53. Bartolomucci, A., et al., *Social status in mice: behavioral, endocrine and immune changes are context dependent*. Physiology & Behavior, 2001. **73**(3): p. 401-410.

54. Roberts, J.E., et al., *Social Approach and Autistic Behavior in Children with Fragile X Syndrome*. J Autism Dev Disord, 2006. **37**: p. 1748-1760.

55. Tranfaglia, M.R., *The Psychiatric Presentation of Fragile X: Evolution of the Diagnosis and Treatment of the Psychiatric Comorbidities of Fragile X Syndrome*. Dev Neurosci, 2011. **33**: p. 337-348.

56. Gross, C., E.M. Berry-Kravis, and G.J. Bassell, *Therapeutic strategies in fragile X syndrome: dysregulated mGluR signaling and beyond*. Neuropsychopharmacology, 2012. **37**(1): p. 178-95.

57. Merenstein, S.A., et al., *Molecular-clinical correlations in males with an expanded FMR1 mutation*. Am J Med Genet, 1996. **64**: p. 388-94.

58. Hagerman, R.J., *The physical and behavioural phenotype*, in *Fragile-X syndrome: diagnosis, treatment and research*, R.J. Hagerman and P. Hagerman, Editors. 2002: The Johns Hopkins University Press, Baltimore. p. 3-109.

59. Mineur, Y.S., L.X. Huynh, and W.E. Crusio, *Social behavior deficits in the Fmr1 mutant mouse*. Behav Brain Res, 2005. **168**: p. 172-175.

60. Vinueza Veloz, M.F., et al., *The effect of an mGluR5 inhibitor on procedural memory and avoidance discrimination impairments in Fmr1 KO mice*. Genes Brain Behav, 2012. **11**: p. 325-331.

61. Liebowitz, M.R., *Update on the diagnosis and treatment of social anxiety disorder*. Journal of Clinical Psychiatry, 1999. **60**: p. 22-26.

62. Nutt, D.J., et al., *Mechanisms of action of selective serotonin reuptake inhibitors in the treatment of psychiatric disorders*. European Neuropsychopharmacology, 1999. **9**: p. S81-S86.

63. Holsboer, F., *The rationale for corticotropin-releasing hormone receptor (CRH-R) antagonists to treat depression and anxiety*. Journal of Psychiatric Research, 1999. **33**(3): p. 181-214.

64. Cases, O., et al., *Aggressive-Behavior and Altered Amounts of Brain-Serotonin and Norepinephrine in Mice Lacking Maoa*. Science, 1995. **268**(5218): p. 1763-1766.

65. Centonze, D., et al., *Abnormal Striatal GABA Transmission in the Mouse Model for the Fragile X Syndrome*. Biol Psychiatry, 2007. **63**: p. 963-973.

66. Curia, G., et al., *Downregulation of Tonic GABAergic Inhibition in a Mouse Model of Fragile X Syndrome*. Cereb Cortex, 2009. **19**: p. 1515-1520.

67. D'Hulst, C., et al., *Decreased expression of the GABA(A) receptor in fragile X syndrome*. Brain Res, 2006. **1121**: p. 238-245.

68. D'Hulst, C., et al., *Expression of the GABAergic system in animal models for fragile X syndrome and fragile X associated tremor/ataxia syndrome (FXTAS)*. Brain Res, 2009. **1253**: p. 176-183.

69. Selby, L., C. Zhang, and Q.Q. Sun, *Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein*. Neurosci Lett, 2007. **412**: p. 227-232.

70. Heulens, I., et al., *Pharmacological treatment of fragile X syndrome with GABAergic drugs in a knockout mouse model*. Behav Brain Res, 2012. **229**: p. 244-249.

71. Timmer, M., et al., *Evidence for a role of oxytocin receptors in the long-term establishment of dominance hierarchies*. Neuropsychopharmacology, 2011. **36**(11): p. 2349-56.

72. Wang, F., et al., *Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex*. Science, 2011. **334**(6056): p. 693-7.

73. Kessels, H.W. and R. Malinow, *Synaptic AMPA receptor plasticity and behavior*. Neuron, 2009. **61**(3): p. 340-50.

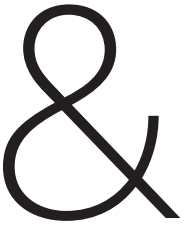
74. Guo, H., et al., *Specificity and efficiency of Cre-mediated recombination in Emx1-Cre knock-in mice*. Biochem Biophys Res Commun, 2000. **273**(2): p. 661-5.

75. Taniguchi, H., et al., *A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex*. Neuron, 2011. **71**(6): p. 995-1013.

76. Gonzalez-Burgos, G. and D.A. Lewis, *NMDA receptor hypofunction, parvalbumin-positive neurons, and cortical gamma oscillations in schizophrenia*. Schizophr Bull, 2012. **38**(5): p. 950-7.

77. Lewis, D.A., *The chandelier neuron in schizophrenia*. Dev Neurobiol, 2011. **71**(1): p. 118-27.
78. Tallent, M.K., *Somatostatin in the dentate gyrus*. Prog Brain Res, 2007. **163**: p. 265-84.
79. Binaschi, A., G. Bregola, and M. Simonato, *On the role of somatostatin in seizure control: clues from the hippocampus*. Rev Neurosci, 2003. **14**(3): p. 285-301.
80. Holmes, A., *Targeted gene mutation approaches to the study of anxiety-like behavior in mice*. Neurosci Biobehav Rev, 2001. **25**(3): p. 261-73.
81. Osterweil, E.K., et al., *Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile x syndrome*. Neuron, 2013. **77**(2): p. 243-50.
82. Spencer, C.M., et al., *Modifying behavioral phenotypes in Fmr1KO mice: genetic background differences reveal autistic-like responses*. Autism Res, 2011. **4**(1): p. 40-56.
83. Errijgers, V. and R.F. Kooy, *Genetic modifiers in mice: the example of the fragile X mouse model*. Cytogenet Genome Res, 2004. **105**(2-4): p. 448-54.
84. Wang, X., et al., *Activation of the extracellular signal-regulated kinase pathway contributes to the behavioral deficit of fragile x-syndrome*. J Neurochem, 2012. **121**(4): p. 672-9.
85. Sharma, A., et al., *Dysregulation of mTOR signaling in fragile X syndrome*. J Neurosci, 2010. **30**(2): p. 694-702.
86. Zalfa, F., et al., *A new function for the fragile X mental retardation protein in regulation of PSD-95 mRNA stability*. Nat Neurosci, 2007. **10**: p. 578-587.
87. Schutt, J., et al., *Fragile X mental retardation protein regulates the levels of scaffold proteins and glutamate receptors in postsynaptic densities*. J Biol Chem, 2009. **284**(38): p. 25479-87.
88. Wang, H., S.S. Kim, and M. Zhuo, *Roles of FMRP in dopaminergic stimulation induced synapse-associated protein synthesis and subsequent AMPA receptor internalization*. J Biol Chem, 2010. **285**: p. 21888-21901.
89. Till, S.M., et al., *Altered maturation of the primary somatosensory cortex in a mouse model of fragile X syndrome*. Hum Mol Genet, 2012. **21**(10): p. 2143-2156.
90. Giuffrida, R., et al., *A reduced number of metabotropic glutamate subtype 5 receptors are associated with constitutive homer proteins in a mouse model of fragile X syndrome*. J Neurosci, 2005. **25**(39): p. 8908-16.
91. Osterweil, E.K., et al., *Hypersensitivity to mGluR5 and ERK1/2 Leads to Excessive Protein Synthesis in the Hippocampus of a Mouse Model of Fragile X Syndrome*. J Neurosci, 2010. **30**(46): p. 15616-27.
92. Berry-Kravis, E., et al., *Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome*. J Dev Behav Pediatr, 2008. **29**(4): p. 293-302.

ADDENDUM



SUMMARY

SAMENVATTING

CURRICULUM VITAE

PHD PORTFOLIO

LIST OF PUBLICATIONS

DANKWOORD

SUMMARY

Worldwide, 2–3% of the population is affected by mild to severe intellectual disability (ID), while autism spectrum disorder (ASD) is a very common disorder with increasing prevalence rates over the past decades, up to 6 per 1000 newborns. Fragile X syndrome (FXS) is a disorder characterized by both ID and ASD and has an estimated prevalence of 1:4000 males and 1:6000 females. 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. Lack of FMRP results in an increased mRNA translation at the synapse upon glutamate stimulation, which leads to an altered synaptic plasticity. One of the signaling pathways believed to cause the pathophysiology of FXS is the mGluR signaling pathway; however the inhibitory γ-aminobutyric acid (GABA) system has also shown to be involved. The generation of mouse models for FXS served as a pre-clinical starting point and paved the way for drug discovery research. To date, 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.

In **chapter one** we introduced the challenging routes to the identification of reliable outcome measures in preclinical studies using both cellular models and *Fmr1* knockout (KO) mice. Several translational endpoints have been described for *Fmr1* KO mice, including an immature spine phenotype with more longer and thinner spines. Among the most robust behavioral phenotypes are increased locomotor activity and the susceptibility to audiogenic seizures consistent with observations in human fragile X patients. However, the wide variety of genetic backgrounds and protocols used, have led to different results for the same behavioral phenotype. These difficulties do hamper the translation of the outcomes of potential therapeutic interventions to human patients. By now, disease-specific human induced pluripotent stem cells and differentiated cell types like neurons can also be used in FXS research, which potentially facilitates the translation to human patients although these *in vitro* models need to be carefully characterized before they can be widely implemented. Clinical studies using a variety of existing and new drugs are being carried out in FXS patients in order to correct intellectual and behavioral deficits. Translational outcome measures in FXS patients usually consist of non-objective measures such as orthopedagogic score lists filled out by parents or caregivers. Despite the positive effects of the therapeutic interventions in animal models, thus far no overall significant treatments effects have been reported for the majority of patients in clinical trials. In addition, a high-placebo effect is noticed which leads to the conclusion that there is an unmet need for objective and robust outcome measures in both pre-clinical and clinical research.

In **chapter two**, we initiated the investigation of a human cell model for fragile X syndrome to study the epigenetic silencing of the *FMR1* gene during development, which has now become possible by the use of induced pluripotent stem cells. We started with an investigation of the epigenetic marks of the *FMR1* promoter in a rare human cell line carrying a fully expanded CGG repeat which is, in contrast to what normally occurs in FXS, completely unmethylated. This rare fibroblast line was derived from an individual with normal intelligence and these

fibroblasts show normal to slightly increased *FMR1* expression levels, increased H3 acetylation and H3K4 methylation in combination with a reduced H3K9 methylation, all indicative of an actively transcribed *FMR1* gene. After reprogramming of these fibroblasts into pluripotent stem cells, it turned out that the expanded repeat was completely methylated in all clones. These cells showed no *FMR1* expression and the epigenetic histone marks also indicated a repressed *FMR1* promoter. In conclusion, the standard reprogramming method induced changes to the epigenetic state of the original cells and although these pluripotent cells would provide a good basis for an *in vitro* cellular model for FXS, this rare cell line cannot be used to study the epigenetic silencing mechanisms in time. In addition, more research is needed to fully characterize the pluripotent stem cells before they can be used as an *in vitro* model for fragile X research.

In **chapter three,** we then continued with the establishment of a social behavioral assay for fragile X mice. We used a three-chambered set-up to investigate their sociability, *ie* the interaction time with a stranger mouse, and in phase two the preference for social novelty, *ie* the interaction time with a novel stranger mouse compared to the interaction time with the initial stranger mouse. In the sociability phase of the test the *Fmr1* KO mice spent significantly more time sniffing the stranger mouse than wild type (WT) mice. In the following phase, the preference for social novelty phase, there was no difference in behavior between wild type and *Fmr1* KO mice. In addition, we tested whether chronic treatment with the mGluR5 receptor antagonist AFQ056/Mavoglurant would offset the aberrant social behavior of fragile X mice. Three weeks after commencing treatment with food pellets containing AFQ056/Mavoglurant, the *Fmr1* KO mice did not show aberrant social behavior compared to treated WT animals. These results support the involvement of the mGluR5 signaling pathway in this type of social behavior. In addition, these results lead to the conclusion that social behavior is an objective outcome measure in preclinical research with fragile X mice.

In chapter four and five we further explored the social behavior of fragile X mice, this time by the use of the automated tube test (ATT). This automated set-up measures the social dominance behavior of mice by determining the winner of a match between two mice, *ie* the mouse that proceeds forward through the tube until the other mouse is back in his starting position. In **chapter four** we validated this test for the use in fragile X research, by investigating the difference in behavior between WT and *Fmr1* KO mice. We observed a robust phenotype of the fragile X mice in the ATT; the *Fmr1* KO mice won the majority of the matches of their WT opponents. We also found that the housing of the mice had an influence on their tube behavior; when WT or *Fmr1* KO animals were housed in couples with a similar genotype, then the *Fmr1* KO animals did not display a phenotype in the tube. In addition, we used a genetic and pharmacological approach to investigate the involvement of the mGluR5 signaling pathway on social dominance behavior. Our results showed that mGluR5 signaling pathway is involved in social dominance behavior, however the rescue of the behavior of *Fmr1* KO mice was not complete, which led to the conclusion that additional signaling pathways are involved as well. In **chapter five** we continued with the ATT, but this time we investigated the effects of region specific deletion of *Fmr1* expression by the use of the following driver lines: *Emx1-Cre,* which is expressed in the forebrain, and *Gad2-Cre,* expressed by inhibitory neurons. Lack of Fmrp in

the glutamatergic neurons of the forebrain resulted in submissive behavior. In contrast, lack of Fmrp in the GABAergic neurons resulted in socially dominant behavior. Although these results suggest that the balance between excitation and inhibition is involved in regulating social behavior, more research is needed to establish the specific role of the inhibitory system in social behavior.

In the last chapter the findings of the different chapters are being discussed in the light of the existing literature. Within this thesis, we provide some initial results on a potential human *in vitro* cellular model for fragile X research, and although more research is needed to characterize this model further, this might lead to a valuable cellular model in the future. This thesis also introduces two behavioral set-ups in which the fragile X mice display a reproducible phenotype with a correlation to the aberrant social behavior displayed by fragile X patients. With these results this thesis contributes to the development of robust and reliable outcome measures that facilitate the evaluation and translation of the results found in pre-clinical research.

SAMENVATTING

Wereldwijd wordt 2-3% van de bevolking getroffen door een lichte tot ernstige verstandelijke beperking, terwijl autisme spectrum stoornis (ASS) een veel voorkomende aandoening is met een prevalentie die in de afgelopen decennia is toegenomen tot 6 per 1000 pasgeborenen. Fragiele X syndroom (FXS) is een stoornis die wordt gekenmerkt door een verstandelijke beperking en ASS, en heeft een geschatte prevalentie van 1 op 4000 mannen en 1 op 6000 vrouwen. De oorzaak van FXS is een lange aaneenschakeling (>200 units) van CGG trinucleotiden in de DNA sequentie van het *FMR1* gen. Deze lange CGG aaneenschakeling noemen we een volledige mutatie en deze induceert de binding van methyl groepen aan het *FMR1* gen (methylering). Door deze methylatie wordt het *FMR1* gen uitgeschakeld. Door deze uitschakeling wordt het product van het *FMR1* gen, het fragiele X mentale retardatie eiwit (FMRP), niet meer aangemaakt. Het ontbreken van FMRP in zenuwcellen (neuronen) is de oorzaak van de verstandelijke beperking van patiënten met FXS. In de hersenen leidt een gebrek aan FMRP tot een verhoogde productie van allerlei andere eiwitten na stimulatie door de neurotransmitter glutamaat. Dit leidt uiteindelijk tot een verstoorde communicatie tussen neuronen onderling. Een van de signaleringsroutes verantwoordelijk voor de pathofysiologie van FXS is de stimulerende metabotrope glutamaat-receptor (mGluR) route, maar het is ook aangetoond dat het remmende γ-aminoboterzuur (GABA) neurotransmitter systeem betrokken is bij FXS. De ontwikkeling van een muismodel voor FXS, de *Fmr1* knock-out muis (*Fmr1* KO) die ook het eiwit FMRP niet aanmaakt, heeft de weg vrijgemaakt voor onderzoek naar therapieën voor FXS. De geavanceerde kennis over de moleculaire en cellulaire mechanismen betrokken bij FXS hebben geleid tot de ontwikkeling van therapeutische interventies om de intellectuele problemen en de gedragsproblemen bij patiënten met FXS te verbeteren.

Hoofdstuk één is een inleiding waarin de uitdagingen in het vinden van betrouwbare eindpunten voor fragiele X onderzoek in het laboratorium worden beschreven. Een betrouwbaar eindpunt is een testuitslag die een duidelijk verschil kan laten zien tussen controles (bijvoorbeeld wild type muizen) en testsamples (bijvoorbeeld *Fmr1* KO muizen) zowel vóór als na een eventuele behandeling met een geneesmiddel. Het is met name belangrijk dat deze eindpunten in muizen of cel modellen ook kunnen worden vertaald naar patiënten, zodat de effecten van therapieën in het laboratorium goed beoordeeld kunnen worden. Er zijn verschillende eindpunten beschreven in *Fmr1* KO muizen, je kunt bijvoorbeeld de neuronen bestuderen onder de microscoop en de neuronale uitlopers karakteriseren door de lengte en de breedte van de dendritische uitstulpingen (spines) te kwantificeren. In eerder onderzoek is aangetoond dat de *Fmr1* KO muizen meer langere en dunnere spines hebben dan wild type muizen. De spines zijn verantwoordelijk voor de communicatie tussen de neuronen dus uit deze resultaten blijkt dat in fragiele X muizen de communicatie tussen neuronen verstoord is. De meest robuuste afwijkende gedragskenmerken van *Fmr1* KO muizen zijn de hyperactiviteit en de gevoeligheid voor audiogeen geïnduceerde epileptische aanvallen, deze komen ook overeen met waarnemingen bij fragiele X patiënten. Helaas gebruiken onderzoeksgroepen wereldwijd fragiele X muizen met een wisselende genetische achtergrond in combinatie met uiteenlopende testprotocollen, wat leidt tot verschillende uitkomsten voor dezelfde metingen. Dit probleem belemmert de interpretatie van de onderzoeken naar potentiële therapeutische

interventies in de diermodellen. Inmiddels kunnen humaan geïnduceerde stamcellen worden gebruikt in FXS onderzoek, het gebruik van specifieke humane cellen maakt de vertaling van gevonden resultaten in het laboratorium naar patiënten eenvoudiger. Echter, deze *in vitro* modellen moeten eerst zorgvuldig worden gekarakteriseerd voordat ze op grote schaal kunnen worden toegepast.

Momenteel worden er klinische studies met geneesmiddelen uitgevoerd bij FXS patiënten om de intellectuele- en gedragsproblemen te corrigeren. Ondanks de positieve effecten van de therapeutische middelen in de fragiele X muizen zijn er dusver geen behandelingen gerapporteerd die een positief effect hebben in de meerderheid van de patiënten in klinische studies. Uitkomstmaten in onderzoek bij FXS patiënten bestaan meestal uit niet-objectieve maten zoals orthopedagogische scorelijsten die worden ingevuld door ouders of verzorgers, waardoor de uitkomsten onbetrouwbaar kunnen zijn. Dit zou het grote placebo-effect kunnen verklaren dat wordt gezien in de klinische studies. Deze problemen leiden tot de conclusie dat er een onvervulde behoefte is aan objectieve en robuuste uitkomstmaten in zowel preklinisch als klinisch onderzoek naar FXS.

In **hoofdstuk twee** zijn we gestart met onderzoek naar een humaan cel model voor het fragiele X syndroom. Wij wilden de uitschakeling van het *FMR1* gen in de tijd bestuderen door de methylering van het gen te onderzoeken in combinatie met andere specifieke epigenetische kenmerken. Epigenetische kenmerken zijn alle veranderingen van het DNA die geen invloed hebben op de onderliggende DNA sequentie. We zijn gestart met het analyseren van de karakteristieke epigenetische kenmerken van het *FMR1* gen in een zeldzame cellijn. De cellijn is zeldzaam omdat de cellen een volledige mutatie hebben, zoals bij FXS, maar die echter in tegenstelling tot wat normaal gebeurt in FXS, niet gemethyleerd is. Het *FMR1* gen is dus actief in deze cellen waardoor FMRP wordt aangemaakt ondanks de aanwezigheid van een volledig verlengde CGG repeat. De cellen (fibroblasten) zijn afkomstig van een huidbiopt van een persoon met een normale intelligentie, wat dus verklaard kan worden door de normale aanmaak van FMRP.

Het is bekend uit eerder onderzoek dat nog niet-gespecialiseerde oftewel ongedifferentieerde humane embryonale stamcellen, afkomstig van embryo's met een volledige mutatie, nog FMRP produceren. Wij hebben vervolgens een nieuwe techniek gebruikt om de fibroblasten te herprogrammeren tot stamcellen. Deze stamcellen, genaamd geïnduceerde pluripotente stamcellen, zijn opnieuw in staat om te differentiëren naar verschillende soorten cellen. Onze hypothese was dat je met het gebruik van deze cellijn de uitschakeling van het *FMR1* gen kan volgen in de tijd die de geïnduceerde stamcellen nodig hebben om te differentiëren naar neuronen. Het bleek echter dat de CGG aaneenschakeling direct na herprogrammering meteen volledig gemethyleerd was. Deze ge-her programmeerde cellen vertoonden geen *FMR1* expressie meer en ook de overige epigenetische kenmerken waren karakteristiek voor een uitgeschakeld *FMR1* gen. Concluderend kunnen we zeggen dat de standaard herprogrammeringsmethode veranderingen introduceert in de epigenetische kenmerken van de originele cellijn. Hoewel de pluripotente stamcellen een goede basis zijn voor een *in vitro* model voor FXS, kan deze zeldzame cellijn niet worden gebruikt om de uitschakeling van het *FMR1* gen te volgen in de tijd.

In **hoofdstuk drie** beschrijven we een sociale gedragstest voor fragiele X muizen. We hebben een opstelling met 3 kamers gebruikt om hun sociale interactie met andere muizen in twee fases te onderzoeken. Deze drie kamers liggen naast elkaar, de middelste ruimte is een habituatie ruimte en in de twee buitenste kamers staat een klein muizenkooitje. Tijdens het experiment kan de test muis drie ruimtes in en uitlopen, in de eerste fase staat er in één van de buitenste ruimtes een kooitje met een vreemde muis, in de andere buitenste ruimte staat alleen een leeg kooitje. In de tweede fase zit er ook een nieuwe vreemde muis in het eerst lege kooitje, dus nu kan de test muis in twee verschillende kamers aan een andere muis snuffelen. In de eerste fase meten we de tijd die ze snuffelen aan de vreemde muis in vergelijking met het lege kooitje, en daarna in de tweede fase de sociale voorkeur, namelijk de tijd die ze snuffelen aan de nieuwe vreemde muis in vergelijking met de vreemde muis uit de eerste fase. In de eerste fase van de test besteedden de *Fmr1* KO muizen veel meer tijd aan de vreemde muis dan wild type (WT) muizen. In de tweede fase was er geen verschil in gedrag tussen wild type en *Fmr1* KO muizen. Daarnaast hebben we getest of een chronische behandeling met de mGluR5 receptor antagonist AFQ056/Mavoglurant, het afwijkende sociale gedrag van de fragiele X muizen kan corrigeren. Drie weken na het begin van de behandeling met voer dat AFQ056/Mavoglurant bevatte, vertoonden de *Fmr1* KO muizen geen afwijkend sociaal gedrag meer ten opzichte van behandelde WT dieren. Deze resultaten bewijzen de betrokkenheid van de mGluR5 signaleringsroute in dit type sociaal gedrag. Bovendien leiden deze resultaten tot de conclusie dat sociaal gedrag een objectief eindpunt is in preklinisch onderzoek met fragiele X muizen.

In hoofdstuk vier en vijf onderzoeken we het sociale gedrag van de fragiele X muizen verder, dit keer door het gebruik van de geautomatiseerde buis test (ATT). Deze automatische set up in de vorm van een rechthoekige buis, meet het sociale dominantie gedrag van muizen door een winnaar te bepalen na een wedstrijd tussen twee muizen. Bij een wedstrijd worden twee muizen in een aparte ruimte aan het uiteinde van de buis geplaatst, ieder aan een andere kant van de buis. Daarna kunnen de muizen naar binnen lopen totdat ze elkaar tegenkomen in het midden. Hier bevindt zich een deurtje dat pas opengaat als beide muizen in het midden zijn. Vervolgens kunnen de muizen elkaar besnuffelen en wordt de winnaar bepaald. De winnaar is de muis die vooruit gaat door de buis totdat de andere muis terug is in de ruimte waar hij begon. In **hoofdstuk vier** valideren we deze test voor het gebruik in het fragiele X onderzoek, door het verschil in gedrag tussen WT en *Fmr1* KO muizen te onderzoeken. We zien een robuust verschil in gedrag (een fenotype) tussen de fragiele X muizen en WT muizen in de ATT. De *Fmr1* KO muizen winnen de meerderheid van de wedstrijden van hun WT tegenstanders. We vonden ook dat de huisvesting van de muizen invloed heeft op het gedrag in de ATT; wanneer WT of *Fmr1* KO dieren zijn gehuisvest in koppels met een vergelijkbaar genotype, dan laten de *Fmr1* KO dieren geen fenotype in de ATT zien. Daarnaast hebben we een genetische en farmacologische strategie gebruikt om de betrokkenheid van de glutamaat signaleringsroute op sociaal dominant gedrag te onderzoeken. Onze resultaten laten zien dat de mGluR5 signaleringsroute ook betrokken is bij dit type sociaal dominant gedrag. Echter, het gedrag van de *Fmr1* KO muizen wordt niet volledig hersteld door de beïnvloeding van de mGluR5 signaleringsroute. Dit leidt tot de conclusie dat er ook aanvullende signaalroutes zijn betrokken bij deze vorm

van sociaal gedrag. In **hoofdstuk vijf** onderzoeken we de effecten van het uitschakelen van de *Fmr1* expressie in specifieke gedeelten van het brein in de ATT. Dit hebben we gedaan door twee genetische muislijnen te gebruiken die ervoor zorgen dat een bepaald eiwit (Cre recombinase) tot expressie komt in specifieke hersengebieden. De driver lijn *Emx1*-Cre, waar Cre recombinase tot expressie komt in de voorhersenen (cortex, amygdala, hippocampus) en de driver lijn *Gad2*-Cre, waar Cre recombinase tot expressie komt in remmende GABA neuronen. Door deze muislijn te kruisen met een speciale *Fmr1* conditionele KO muislijn, schakel je *Fmr1* uit in die specifieke gebieden waar Cre recombinase tot expressie komt. Uit de experimenten blijkt dat de muizen waar FMRP afwezig is in de activerende glutamaat neuronen in de voorhersenen, de meerderheid van de wedstrijden verliezen van WT muizen in de ATT. Echter, muizen winnen de meerderheid van de wedstrijden van WT muizen indien FMRP afwezig is in de GABAerge neuronen. Deze resultaten suggereren dat de balans tussen de activerende glutamaat neuronen en de remmende GABA neuronen een belangrijke rol speelt in de vorming van sociaal dominant gedrag. Er is echter meer onderzoek nodig om de specifieke rollen van deze systemen in sociaal dominant gedrag volledig te karakteriseren.

In het laatste hoofdstuk worden de bevindingen van de verschillende hoofdstukken besproken in het licht van de bestaande literatuur. In dit proefschrift geven we een beschrijving van een mogelijk humaan cellulair model voor het fragiele X onderzoek. Hoewel meer onderzoek nodig is om dit model verder te karakteriseren, kan dit leiden tot een waardevol humaan *in vitro* model in de toekomst. Dit proefschrift introduceert ook twee gedragstesten waarin de fragiele X muizen een reproduceerbaar fenotype vertonen met een correlatie in het afwijkende sociale gedrag van fragiele X patiënten. Met deze resultaten draagt dit proefschrift bij aan de ontwikkeling van robuuste en reproduceerbare translationele eindpunten welke de evaluatie en de vertaling van de preklinische resultaten vergemakkelijkt.

CURRICULUM VITAE

Celine EF de Esch
Dr Zamenhofstraat 57G
3061 SE Rotterdam
celine_esch@hotmail.com

Work experience

2010- 2014 PhD student in the department of Clinical Genetics, Erasmus University Medical Center, Rotterdam working on fragile X syndrome.

In fragile X syndrome, the *FMR1* gene is silenced and the consequent loss of its protein product FMRP leads to the symptoms of the disorder, including intellectual disability and autism. Goals of my research were to gain insights in the molecular pathways and timing of the epigenetic changes that cause transcriptional silencing of *FMR1* by using induced pluripotent stem cells and to elucidate the role of FMRP in synaptic transmission and plasticity by investigating neuronal morphology, protein expression and animal behavior using different transgenic mouse lines.

2008-2009 Zebrafish project at the Netherlands Organization of Applied Scientific Research (TNO) Quality of Life, Department of Toxicology and Applied Pharmacology, Zeist, the Netherlands.

Goals of this research project were setting-up behavioral tests such as a locomotor assay for zebrafish and validate these using model compounds such as ethanol.

Education

2002-2007 **Master of Science (MSc) in Biomedical Sciences**
Majors in Toxicology and Human Pathobiology at the Radboud University, Nijmegen in the Netherlands.

1996-2002 **Pre-university college degree** at Sintermeerten College, in Heerlen, the Netherlands.

PHD PORTFOLIO

Name PhD student: Celine de Esch
Erasmus MC Department: Clinical Genetics
Research School: MGC en CBG
PhD period: 15/1/2010 – 25/06/2014
Promotor: Prof.dr. R. Willemsen

1. PhD training (*continued*)

	Year	Workload (Hours/ECTS)
General courses		
• Biomedical English Writing and Communication	2012	4 ECTS
• Research Integrity	2012	2 ECTS
• Laboratory animal science	2007	N/A
• Statistics	2010	5.7 ECTS
• Safely working in the laboratory	2010	0.25 ECTS
• Literature Course	2011	2 ECTS
Specific courses (e.g. Research school, Medical Training)	2010	1 ECTS
• MolMed course on Diagnostics V	2010	3 ECTS
• Cell and Developmental Biology course	2010	3 ECTS
• Genetics course	2010	3 ECTS
• Biophysics and Biochemistry course	2011	1 ECTS
• MGC AIO course: Transgenesis, gene targeting and in vivo imaging (3 days)	2012	0.75 ECTS
• The Next Generation Sequencing data analysis course	2012	0.75 ECTS
• Leiden: iPS cell Course		
Seminars and workshops	2010	0.25 ECTS
• Introduction to the confocal microscope and live imaging	2010	1 ECTS
• PhD workshop Cologne (1st)	2010	0.25 ECTS
• Photoshop and InDesign Course (2 sessions)	2011	1 ECTS
• PhD workshop Maastricht (2nd)	2011	0.6 ECTS
• Get out of your lab days	2012	0.25 ECTS
• PhD day 2012	2013	1 ECTS
• PhD workshop Luxembourg (3rd)	2013	0.25 ECTS
• Workshop: Negotiation for Scientists (ElroyCOM)	2013	0.25 ECTS
• The do's and don't's of networking and collaboration	2014	0.4 ECTS
• Loopbaan oriëntatie		

1. PhD training (*continued*)

	Year	Workload (Hours/ECTS)
Presentations		
• Wednesday Morning Meetings	2010-2014	1 ECTS
• Thursday/Friday work discussions	2010-2014	1 ECTS
• PhD Workshop Luxembourg	2013	0.5 ECTS
• FRAXA Meeting Boston 2013	2013	1 ECTS
(Inter)national conferences		
• NIRM Stem cell symposium 2010	2010	1 ECTS
• ENDP Meeting 2011	2011	0.25 ECTS
• FRAXA Investigators meeting 2011	2011	1 ECTS
• A decade of Neuroscience 2012	2012	0.25 ECTS
• FENS meeting 2012	2012	1 ECTS
• FRAXA Investigators Meeting 2013	2013	1 ECTS

2. Teaching activities

	Year	Workload (Hours/ECTS)
• Junior Med School	2010-2014	1 ECTS
• Clinical Genetics VAA	2012-2013	1 ECTS
• Master Student	2010-2011	2 ECTS
• Neuroscience Master Student	2012-2014	2 ECTS
Other		
• Party Committee Cluster 15	2010-2011	1 ECTS

LIST OF PUBLICATIONS

1. **de Esch CE**, Ghazvini M, Schelling-Kazaryan N et al., Epigenetic characterization of the *FMR1* promoter after reprogramming of a human fibroblast line carrying an unmethylated full mutation into pluripotent stem cells. *Manuscript under review by Stem Cell Reports*.
2. **de Esch CE**, van den Berg, Buijsen et al., Altered social interaction in fragile X mice. *Manuscript in preparation*.
3. Okray Z, **de Esch CE**, van Esch H, et al., A de novo clinical point mutation in *FMR1* coding sequence unveils a conserved role for the carboxy-terminus in FMRP localization and function. *Manuscript in preparation*.
4. **de Esch CE**, Zeidler S, and Willemsen R. Translational endpoints in fragile X syndrome. Neurosci Biobehav Rev in press, 2014.
5. Gantois I, Pop AS, **de Esch CE** et al., Chronic administration of AFQ056/Mavoglurant restores social behaviour in Fmr1 knockout mice. Behav Brain Res, 2013. 239: p. 72-9.
6. Pop AS, Levenga J, **de Esch CE** et al., Rescue of dendritic spine phenotype in Fmr1 KO mice with the mGluR5 antagonist AFQ056/Mavoglurant. Psychopharmacology (Berl), 2014. 231(6): p. 1227-35.
7. **de Esch C**, Slieker R, Wolterbeek A et al., Zebrafish as potential model for developmental neurotoxicity testing: a mini review. Neurotoxicol Teratol, 2012. 34(6): p. 545-53.
8. **de Esch C,** van der Linde H, Slieker R et al., Locomotor activity assay in zebrafish larvae: influence of age, strain and ethanol. Neurotoxicol Teratol, 2012. 34(4): p. 425-33.
9. Radonjic M, Cappaert NL, de Vries EF et al., Delay and impairment in brain development and function in rat offspring after maternal exposure to methylmercury. Toxicol Sci, 2013. 133(1): p. 112-24.
10. Nguyen N, **de Esch C**, Cameron B et al., Positioning of leukocyte subsets in the portal and lobular compartments of HCV-infected liver correlates with local chemokine expression. J Gastroenterol Hepatol, 2013.

DANKWOORD

Na een periode van 4,5 jaar ligt hier dan een proefschrift en is het promoveren binnen handbereik! In deze periode heb ik veel geleerd, en niet alleen op wetenschappelijk gebied, maar ook veel plezier gehad. Een promotieonderzoek doe je nooit alleen, dus hier wil ik graag de mensen bedanken die hebben bijgedragen aan de totstandkoming van dit proefschrift.

Allereerst Ben Oostra, Rob Willemsen en Robert Hofstra diegenen die mij de mogelijkheid hebben gegeven om hier aan een promotieonderzoek te beginnen, bedankt voor alle begeleiding en ondersteuning. Rob, je was zo gastvrij om mij als toenmalige TNO-er 'stage' te laten lopen bij de zebravissen. Daar heb ik, mede dankzij de hulp van Herma en Edwin, niet alleen veel geleerd, maar dat heeft uiteindelijk ook geleid tot mijn promotieplek in Rotterdam. Ben, bedankt voor jouw ideeën en de kritische kijk op mijn werk tijdens jouw periode als hoofd van de onderzoeksgroep. Rob, jouw deur stond altijd open ondanks de enorme extra drukte die het met zich meebracht om de leiding over te nemen. Bedankt voor alle ideeën, het vertrouwen en de vrijheid die je me gegeven hebt om dit onderzoek te kunnen uitvoeren! Jeannette, Bep en Marieke, bedankt voor alle ondersteuning hierbij en voor jullie hulp bij het verzenden van pakketjes (het is weer maandag), het registreren van studenten en het uitdelen van de labjournaals!

All remaining members of the reading committee, Frank Kooy, Steven Kushner en Jeroen Pasterkamp, thank you for all the time you invested in my thesis and I am looking forward to discuss my results with you during my defense.

Femke en Josien, jullie hebben me in het begin op weg geholpen met mijn onderzoek en van jullie heb ik alles geleerd over neuronen kweken en spines tellen. Bedankt voor alle gezelligheid, ideeën en hulp hierbij, zelfs vanuit New York. Femke, ook nu je op een andere afdeling werkt, doen we nog samen onderzoek wie had dat gedacht? Renate en Edwin, bij jullie kon ik altijd terecht met alle willekeurige labvragen en ook met alle bisulfiet en qPCR vragen en problemen, bedankt daarvoor. Edwin bedankt voor het 'redden' van het oude qPCR apparaat, het komt nog goed van pas! Renate, je bent onmisbaar geworden in de groep, veel succes en plezier met je verdere carrière. Andreea, jij was er ook vanaf mijn begin bij en Shimriet jij kwam wat later. Bedankt voor alle gezelligheid tijdens onze uitjes en alle hulp op het lab. Ronald, volgens mij krijg jij uiteindelijk ook een heel mooi boekje, succes met al het werk dat op je ligt te wachten! Bedankt voor al je hulp, steun en ideeën vanaf dag één. Samen met Shimriet en Fenne heb je nog even te gaan en ik wens jullie allemaal heel veel succes de komende paar jaar.

Steven Kushner, thank you for your extensive collaboration with the fragile X group. Thank you for all your ideas and thoughts, I am sure we will get a nice tube paper out soon. Wouter, mede dankzij jou hebben we een prachtige gedragstest voor onze fragiele X muizen. Ik bewonder je enorme inzet op ieder moment van de dag voor het onderzoek ondanks het feit dat je zo'n andere achtergrond hebt. Bedankt voor alle hulp en heel veel succes met je toekomstige carrière. Ook allerlei andere mensen uit de groep van neurobiologische psychiatrie hebben bijgedragen aan mijn onderzoek, Sashini, Nilhan, Ivo en Denise: many thanks for your help.

Arnold Reuser bedankt voor jouw bijdrage aan de werk besprekingen, voor de gezelligheid op het lab en het oogje in het zeil houden. Je had het altijd in de gaten als er iets mis was met de stikstof. Andre Hoogeveen, Aida Bertoli Avella and Vincenzo Bonifati thank you for all your

contributions during work discussions and research meetings. Ingeborg, Herma, Marianne en Guido zonder jullie was al het proefdier, lab en kweekwerk niet mogelijk geweest. Bedankt voor jullie onmisbare bijdrages. Mark en Marianne, bedankt voor alle hulp met de westernblots op ieder willekeurig moment. Lies-anne bedankt voor al het snij- en kleurwerk, het is weer gelukt om een set mooie plaatjes in een proefschrift te krijgen! Leontine, dankjewel voor alle hulp met de fibroblasten assays. Jij was er altijd voor antwoord op alle vragen of voor extra controle samples, net als Rachel, bedankt. Pim Pijnappel, Erik, Mehrnaz en Tracy, jullie hebben mij enorm geholpen met het kweken en karakteriseren van de iPS cellen, bedankt voor al jullie medewerking, hulp en ideeën hierbij. Maar ook Joost Gribnau en Friedemann, bedankt voor jullie waardevolle bijdrages aan al het stamcel werk.

Josja, Adriana, Claudia, Simone, Alan, Kate, Marian, Vanessa, Erik, Yunia, Erwin and Maria thank you for your contributions to the nice atmosphere in the lab, the work discussions and research meetings. Niet in alleen in het lab was het gezellig ook op 'kantoor': Atze, Rajendra, Danny, Shimriet, Erik en Bianca hartstikke bedankt voor alle gezellige gesprekken tussendoor maar ook voor de gezelligheid tijdens alle uitjes en feestjes. Francesca, Isa, Romana, Luna, and Cathryn thank you for all the fun we had, mostly outside of the lab though, and thank you for introducing me to the best cocktail bar of Rotterdam. I will never forget the Take That concert, those memories will last forever. Widagdo, my first student! Not only was it great fun to have you in the lab, I am proud of everything you have accomplished so far. Thank you for all your work and I wish you all the best for your future career. All other students that contributed to my research in one way or another: Adi, Sotos, Michelle, Rob V, Karina, Laura en Shami many thanks for all your help.

Naast de mensen op de negende verdieping wil ik ook nog graag allerlei andere mensen in het faculteitsgebouw bedanken voor hun bijdrage aan de totstandkoming van dit boekje: Melle, Leo en Arthur voor alle bestellingen; Pim, Leo, Sjozef, Mario, Ton en Nils voor alle computer problemen en vragen; Huub, Charlotte en Ineke voor alle hulp met de muizen en AMS; Benno en Marjoleine voor alle administratieve hulp en Gert voor alle hulp met de confocale microscoop. In addition, our collaborators from different institutions: Fabrizio Gasparini, George Portielje, Pieter and Ilse Gantois, many thanks for your contributions to the different chapters in this thesis as well.

Israa en Joantine, bedankt dat jullie het aandurven om mijn paranimfen te zijn. Israa, ik was meteen onder de indruk van jouw nauwkeurige manier werken! Geen opdracht was je teveel, bedankt voor de enorme berg werk die je hebt verzet en heel veel succes met je verdere carrière. Joantine, bedankt voor alle leuke wandelprestaties die we samen hebben behaald en voor alle emotionele ondersteuning de afgelopen jaren. Hopelijk blijven we elkaar zien waar onze carrières ons ook mogen brengen. Net als Chrystel, mijn 'zusje', die ik heb leren kennen toen ik begon met studeren in Nijmegen. We hebben ondertussen al heel wat meegemaakt samen, bedankt voor alle steun en lol die we altijd hebben! Hopelijk blijven we leuke dingen doen samen, ook in de toekomst!

Alle 'oude' vrienden en familie uit Limburg en alle 'nieuwe' vrienden uit Rotterdam, ook jullie hebben bijgedragen aan dit proefschrift! Ook al was ik diegene die weinig tijd had, ver weg woonde en te weinig belde of kaartjes stuurde, jullie zijn er nog steeds altijd voor mij. Niet

alleen met alle hulp bij verhuizingen natuurlijk, maar ook met alle gezelligheid en broodnodige ontspanning! Er is een hoop gebeurd in de afgelopen jaren, inclusief bruiloften en het krijgen van kinderen maar hopelijk blijven we de tijd vinden om elkaar te zien, bedankt voor jullie vriendschap! Natuurlijk ook bedankt voor het luisteren naar al mijn wetenschappelijke verhalen over muizen en cellen, hopelijk kunnen jullie nog een presentatie aan zodat we daarna mijn promotie samen kunnen vieren!

Finally yet importantly, mijn ouders die mij altijd in alles gesteund hebben of ik nou naar Nijmegen wilde of naar Sydney in Australië. Het begon allemaal het (voor)lezen van boeken ik klein was en kijk wat dat uiteindelijk heeft opgeleverd! Bedankt voor het creëren van een stevige basis waardoor ik zover kon komen en bedankt voor jullie onmisbare vertrouwen in mij!

Celine