Fragile X Syndrome: Steps towards Therapy

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In memory of my dear Nona, who always wanted me to become a doctor, even though a different kind.

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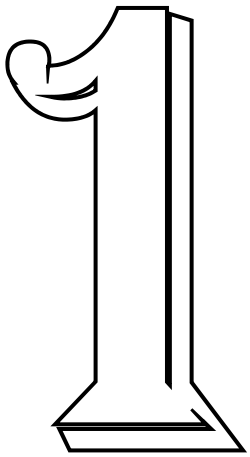
Abbreviation list

ABC	Aberrant Behavior Checklist
ADHD	Attention Deficit Hyperactivity Disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
Arc	Activity-regulated cytoskeleton-associated protein
ASD	Autism Spectrum Disorders
CA1	Cornu Ammonis 1 region
CaMKII	Calcium/calmodulin-dependent protein kinase II
cKO	Conditional knockout
cON	Conditional restoration (ON)
CTEP	2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy) phenyl)-1H-imidazol-4yl)ethynyl)pyridine
DHPG	(S)-3,5-Dihydroxyphenylglycine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FMR1	Fragile X mental retardation 1(gene)
FMRP	Fragile X mental retardation protein
FRAXA	Fragile X Association
FXPOI	Fragile X-associated premature ovarian insuficiency
FXR1/2	Fragile X related gene
FXS	Fragile X syndrome
FXTAS	Fragile X-associated tremor/ataxia syndrome
GABA	Gamma(γ)-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GluR	Glutamate receptor
GSK3	Glycogen synthase kinase 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KO	Knockout
LC-MS	Liquid chromatography and mass spectometry
LTP/LTD	Long-term potentiation/ Long-term depression
mACh	Muscarinic Acetylcholine receptor
mGluR	Metabotropic glutamate receptor
MMP	Matrix metallo-proteinase
MPEP	2-Methyl-6-(phenylethynyl)pyridine
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NMDA	N-methyl-D-aspartate
NOS	Nitric Oxide Synthase

OCD	Obsessive-compulsive disoder
PAK	P21-activated protein kinase
PBS	Phosphate buffered saline
PCR	Polymarase chain reaction
PFA	Paraformaldehide
PI3K	Phosphoinositide 3-kinase
PPI	Prepulse inhibition
PSD	Postsynaptic density
SAPAP	Synapse-associated protein 90/postsynaptic density-95-associated protein
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SN	Synaptoneurosome
SPSN	Sociability and preference for social novelty
STEP	Striatal-enriched tyrosine phosphatase
STR	Stranger
UTR	Untranslated region
VAS	Visual Analog Scale
WT	Wild-type
YAC	Yeast artificial chromosome



Fragile X syndrome: from gene to therapy

Andreea S Pop, Baltazar Gomez-Mancilla, Giovanni Neri, Rob Willemsen and Fabrizio Gasparini

(adapted after the review **Fragile X syndrome: A preclinical review on metabotropic glutamate receptor 5 (mGluR5) antagonists and drug development,** Psychopharmacology**,** 2013, submitted**)**

Rescue of dendritic spine phenotype in *Fmr1* KO mice with the mGluR5 antagonist AFQ056/ Mavoglurant

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Psychopharmacology, 2013

Abstract

Fragile X syndrome (FXS) is the leading monogenic cause of intellectual disability and autism. The disease is a result of lack of expression of the fragile X mental retardation protein (FMRP). Brain tissue of patients with FXS and mice with Fmrp deficiency has shown an abnormal dendritic spine phenotype. We investigated the dendritic spine length and density of hippocampal CA1 pyramidal neurons in 2-week-old, 10-week-old and 25-week-old *Fmr1* KO. Next, we studied the effect of long-term treatment with an mGluR5 antagonist, AFQ056/Mavoglurant, on the spine phenotype in adult *Fmr1* KO mice. We observed alterations in the spine phenotype during development, with a decreased spine length in 2-week-old *Fmr1* KO mice compared with age-match wild-type littermates, but with increased spine length in *Fmr1* KO mice compared with WT controls at 10 weeks and 25 weeks. No difference was found in spine density at any age. We report a rescue of the abnormal spine length in adult *Fmr1* KO mice after long-term treatment with AFQ056/Mavoglurant. This finding suggests that long-term treatment at later stage is sufficient to reverse the structural spine abnormalities and represents a starting point for future studies aimed at improving treatments for FXS.

Keywords: Fragile X syndrome; FMRP; Dendritic spines; Hippocampal CA1; AFQ056/ Mavoglurant; *FMR1*

2.1 Introduction

Fragile X syndrome (FXS) is the leading monogenic cause of intellectual disability (ID) and autism (Hagerman et al. 2010), with a prevalence of 1:2500 to 1:6000 (Hagerman 2008;Coffee et al. 2009). This syndrome is caused by the lack of fragile X mental retardation protein (FMRP), as a result of an expansion of the trinucleotide (CGG) repeat in the 5'UTR region of the fragile X mental retardation 1 gene (*FMR1*) (Verkerk et al. 1991). The length of the repeat is normally between 5 and 55, whereas in patients with FXS this repeat length is higher than 200. This CGG expansion results in silencing of the gene and lack of FMRP expression (Devys et al. 1993).

FMRP is an RNA-binding protein (Brown et al. 2001;Darnell et al. 2001) that plays a crucial role in regulation of protein synthesis following stimulation of group 1 metabotropic glutamate receptors (mGluRs) in dendritic spines (Antar et al. 2004; Aschrafi et al. 2005). Spines are short protrusions of the dendrites of excitatory neurons where connections with other neurons are formed, and altered spine morphology has been reported in many ID syndromes, including Down syndrome and Rett syndrome (Purpura 1974; Kaufmann and Moser 2000;Fiala et al. 2002). Analysis of post-mortem brain tissue of patients with FXS has demonstrated that these patients have abnormally long spines with a tortuous aspect, mixed with normal short spines (Rudelli et al. 1985;Hinton et al. 1991). Other studies reported an increased density of spines compared to controls (Irwin et al. 2001).

Dendritic spines are dynamic structures that change in shape and strength over time and following synaptic activity (Zuo et al. 2005). Several studies conducted in the *Fmr1* knockout (KO) mouse have found different spine phenotypes depending on the brain area or the age of the mice (Comery et al. 1997;Nimchinsky et al. 2001; Irwin et al. 2002;Galvez and Greenough 2005;McKinney et al. 2005;Grossman et al. 2006;Levenga et al. 2011a). An overview of some of these studies is well presented by Portera-Cailliau in his latest review (Portera-Cailliau 2011).

The proposed theory to explain this abnormal spine morphology in FXS, named the mGluR theory, states that lack of FMRP leads to increased protein synthesis following stimulation of the mGluRs, which subsequently causes increased internalization of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Bear et al. 2004). This internalization reduces the size of the head

of the spines, giving them a longer and thinner shape, leading to impaired synaptic signaling and ultimately to the loss of the synapse. In order to prevent or reverse this, the majority of therapeutic interventions being developed today for FXS focus on drugs whose action reduces the activity of group 1 mGluRs and its downstream signal transduction pathways (Berry-Kravis et al. 2011;Healy et al. 2011).

In this study, we decided to look at the aspect of dendritic spines at three stages of development of *Fmr1* KO mice: pups, young adults and adults. We chose to focus on the CA1 region of the hippocampus, as this area is known to play an important role in learning and memory and recently we have demonstrated subregion-specific dendritic spine abnormalities in the hippocampus of 25-week-old *Fmr1* KO mice (Levenga et al. 2011a). Strikingly, the CA1 region of the hippocampus showed an altered spine phenotype, which was absent in the CA3 region. In addition, an increased expression of the mGluR5 in the CA1 compared to the CA3 area of the hippocampus was demonstrated which may explain this finding.

As an experimental approach towards rescuing the abnormal spine phenotype in 25-week-old *Fmr1* KO mice we chose an mGluR antagonists, AFQ056/ Mavoglurant, that has recently be applied in cultured *Fmr1* KO neurons and clinical trials (Jacquemont et al. 2011;Levenga et al. 2011b).

2.2 Material and methods

2.2.1 Animals

Fmr1 KO mice were generated in our lab as previously described, and named *Fmr1* KO(2) (Mientjes et al. 2006). These mice lack the *Neo* cassette, and the level of *Fmr1* mRNA expression is null. The line was completely backcrossed (>10X) in the C57BL/6J background. In our study we only used male *Fmr1* KO mice and wild-type (WT) littermates of 2 weeks (7 KO and 8 WT), 10 weeks (8 KO and 8 WT) and 25 weeks (10 KO and 10 WT). Mice were housed under standard conditions. All experiments were carried out with the permission of the local ethics committee.

2.2.2 Treatment

Six *Fmr1* KO and six WT littermates of 19 weeks received AFQ056/Mavoglurant (Novartis) mixed in food pellets (150 mg/kg) for 6 weeks. Mice eat approximately 3-4

grams of food per day, resulting in a dose of ~18 mg/kg/day. An additional 3 WT mice received sub-chronically (5 days) AFQ056/Mavoglurant via the food pellets (150 mg of AFQ056/kg of food pellets). Mice were sacrificed after 5 days, brains and blood were collected around midnight (23-24 o'clock) after mice entered de night cycle and had time to consume the food. Samples were analyzed for AFQ056/Mavoglurant levels by liquid chromatography separation followed by mass spectrometry (LC-MS). Measured mean concentrations were 95 nM (± 90) (31 nM; 56 nM; 199 nM) in blood, and 244 nmoles/kg (± 200) (89 nmoles; 162 nmoles; 479 nmoles) in brain, giving a high brain/plasma ratio (2.7). The levels measured in the brain (244 nmoles/kg) are in line with the PK/PD relationship demonstrated in other models.

2.2.3 Perfusion and tissue preparation

Brains were isolated as described previously (Shen et al. 2008). In short, mice were anesthetized with ketamine HCl (300 mg/kg) and xylazine (20 mg/kg) injected intraperitoneally, then perfused transcardially with 0.1M PBS (10 ml for adults and 4 ml for pups) followed by 1.5% paraformaldehyde (PFA) in 0.1M PBS (15 ml for adults and 5 ml for pups). Subsequently, brains were dissected and postfixed overnight in 1.5% PFA. The following day, brains were embedded in 1% agar (50% 0.1M PBS and 50% demi H_2O) and coronally sectioned (150 µm thick) using a vibratome at room temperature. Hippocampi were isolated from each slice and collected in PBS. The slices were diOlistically labeled the same day (see below).

2.2.4 DiOlistic labeling

Tungsten particles (1.3 µm diameter, Biorad) coated with 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Sigma) were used. The coating procedure was described previously (Wu et al. 2004). Labeling and post-fixation of the hippocampal pyramidal neurons was performed as described previously (Levenga et al. 2011a). In short, hippocampal slices were shot with a Helios Gene Gun system (Biorad), with tungsten coated bullets. After 48 hours at 4°C the slices were fixed in 4% PFA for 1h, washed with PBS with Hoechst and mounted on a glass microscope slide with Mowiol (Mowiol 4-88, Hoechst).

2.2.5 Confocal imaging

To visualize the labeled pyramidal neurons we used a Leica confocal microscope (Leica TCS SP5 Confocal) and a 543 nm laser line. Hoechst nuclear staining was used to identify the CA1 region of the hippocampus. A 63x oil immersion objective was used for acquiring images of neurons. The image size was 248 µm x 248 µm. Neurons were scanned with an interval of 0.5 µm along the Z-axis. A total of 5 to 12 neurons were obtained per mouse. Two branching basal dendrites (secondary or tertiary) were chosen from each neuron when quantifying the spines (Figure 2.1), as it was not possible to determine the distance from the cell soma due to lacking of the DiI-filled neuronal body on the slide. The investigator was blinded with regard to the genotype and treatment of the mice.

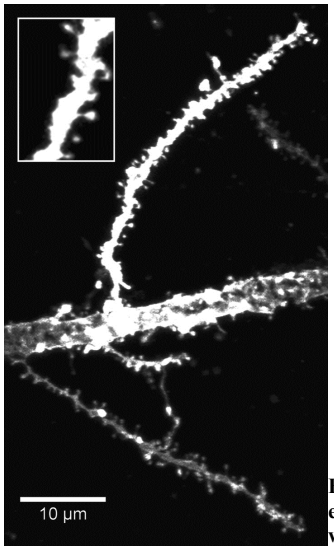

Figure 2.1 Confocal image of a DiOlistic labelled pyramidal excitatory neuron and inset showing a detail of dendritic shaft with spines (*Fmr1* KO, 25-week-old).

2.2.6 Spine measurements and data analysis

Image J software (RSB, NIH) was used to view the confocal images and for spine quantification, as this software provides higher resolution images, compared to MetaMorph software previously used by our group. The maximum projection of the Z-stack was obtained in order to gain a better view. In order to increase the magnification for a better view of the spines without loss of image quality, the resolution of the stack-image was increased by a factor of 5 in the X and Y directions with the plug-in Transform J Scale. The length of a spine was obtained by drawing a

line from its emerging point on the dendrite to the tip of its head. For the statistical analysis the number of spines in each group was taken as the number of subjects per group "N" for spine length, while spine density was calculated per animal, with number of animals as "N". The statistical analysis was done with SPSS (IBM). Mann-Whitney (M-W) statistical test was performed on the spine length between the two experimental groups at each time point, as the data was not normally distributed and Mann-Whitney assesses whether in one group values are larger than in the other (the nonparametric equivalent of the Student t-test). For the spine density at each time point the Student-t test (P) was used, as density is normally distributed. The effect of AFQ056/Mavolgurant treatment on the spine length and density in the 25-week-old *Fmr1* KO and WT, and the effect of age on spine length and density were tested with Kruskal-Wallis (K-W) for spine length and with Two-way ANOVA for spine density with LSD as post-hoc test. The results are presented as the significance value for each statistical test, Mean Rank for the spine length, and Mean (per 10μm) ± SD for spine density.

2.3 Results

The aim of the current study was to look at the dendritic spine phenotype at different time points during development in *Fmr1* KO mice. Therefore we chose 2-week-old, 10-week-old and 25-week-old mice. We also wanted to rescue the previously found immature spine length phenotype at 25 weeks of age with the mGluR5 antagonist, AFQ056/Mavoglurant.

2.3.1 Pups (2-week-old mice)

Spine quantification revealed statistically significant shorter spines (Figure 2.2) in the 2-week-old *Fmr1* KO mice compared with their age-matched WT littermates (M-W= 0.000, N_{KO}= 4543 and N_{WT}= 3438, Mean $Rank_{KO}$= 3871.99 and Mean $Rank_{WT}$= 4148.26). The spine density (Figure 2.6) was not significantly different in the *Fmr1* KO group compared with WT littermates (P= 0.168, N_{KO}= 7 and N_{WT}= 8, $Mean_{KO}$= 10.5 ±1.46 and $Mean_{WT}$= 8.97 ±2.56).

2.3.2 Young adult mice (10-week-old)

Next we wanted to test whether the difference in spine length and spine density between *Fmr1* KO and WT littermates is stable in time. Therefore we quantified spine length (Figure 2.3) and spine density (Figure 2.6) in young adult *Fmr1* KO and WT littermates, at 10 weeks of age. We found significantly longer spines in *Fmr1* KO mice compared with their controls (M-W= 0.035, N_{KO}= 4723 and N_{WT}= 4900, Mean $Rank_{KO}$= 4872.89 and Mean $Rank_{WT}$= 4753.31), but no significant difference in spine density between the two groups (P= 0.392, N_{KO}= 8 and N_{WT}= 8, $Mean_{KO}$= 13.13 ±2.43 and $Mean_{WT}$= 14.03 ±1.56).

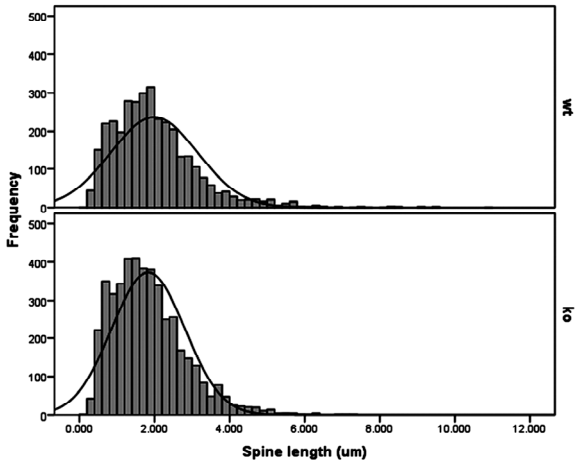

Figure 2.2 Frequency histogram of spine length in 2-week-old *Fmr1* KO mice and WT littermates with the normal curve. A number of 4543 spines and 3438 spines were measured in 7 *Fmr1* KO mice and in 8 WT littermates, respectively. Spine quantification revealed significant shorter spines in the 2-week-old *Fmr1* KO mice compared with their age-matched WT littermates (M-W= 0.000, Mean $Rank_{KO}$= 3871.99 and Mean $Rank_{WT}$= 4148.26). The shape and skewness of the curve indicate the statistically significant difference in spine length between the two groups.

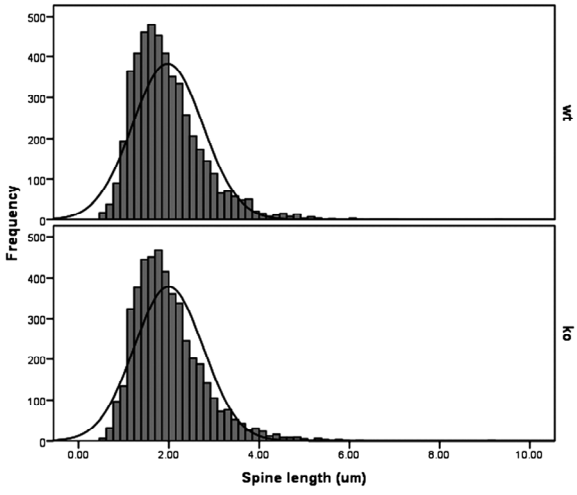

Figure 2.3 Frequency histogram of spine length in 10-week-old *Fmr1* KO mice and WT littermates with the normal curve. For 10 weeks of age, we measured 4723 spines in 8 *Fmr1* KO mice and 4900 spines in 8 WT littermates. We found significantly longer spines in *Fmr1* KO mice compared with their controls (M-W= 0.035, Mean $Rank_{KO}$= 4872.89 and Mean $Rank_{WT}$= 4753.31). The difference between the spine lengths measured in the two groups is small (although significant) indicated also by the similarity of the distribution curve in the two graphs.

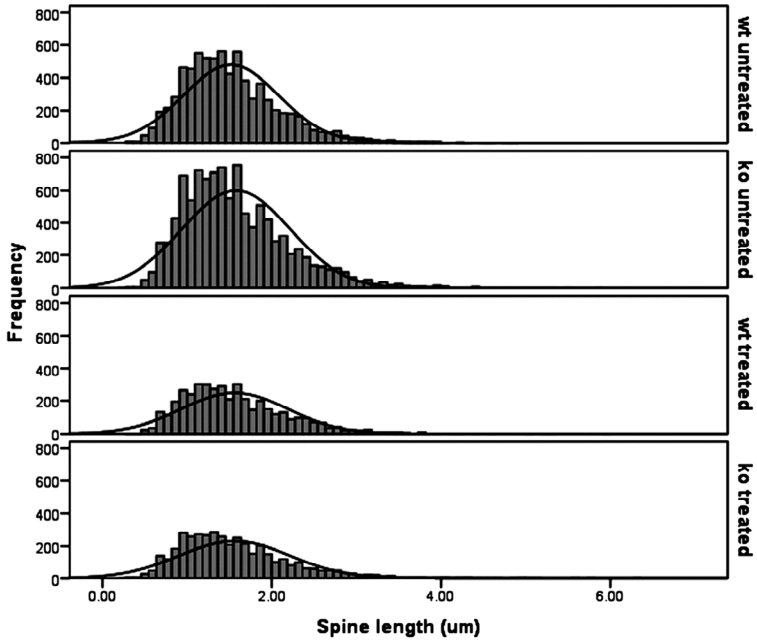

Figure 2.4 Frequency histogram of spine length in 25-week-old *Fmr1* KO mice and WT littermates untreated and treated with AFQ056/Mavoglurant, with the normal curve. Spine length was measured on 10496 spines from 10 untreated *Fmr1* KO mice, on 7502 spine from 10 untreated WT littermates, on 4204 spine from 6 *Fmr1* KO mice treated with AFQ056/Mavoglurant and on 4269 spines from 6 treated WT littermates, all of 25 weeks of age. Spine quantification showed statistically longer spines in the untreated *Fmr1* KO group compared with the untreated WT littermates group (M-W= 0.000, Mean $Rank_{KO}$= 9150.06 and Mean $Rank_{WT}$= 8788.85). No significant difference was found between the treated *Fmr1* KO group and the treated WT group (M-W= 0.398, Mean $Rank_{KOtreated}$= 4214.37 and Mean $Rank_{WTtreated}$= 4259.29). Analysis of the spine length of the treated and untreated mice revealed an overall statistically significant effect of the treatment (K-W= 0.000), with significantly shorter spines found in the treated *Fmr1* KO group compared with the untreated *Fmr1* KO group (M-W= 0.015, Mean $Rank_{KOtreated}$= 7215.70 and Mean $Rank_{KOuntreated}$= 7404.49), but not when compared with the untreated WT group (M-W= 0.236, Mean $Rank_{KOtreated}$= 5902.98 and Mean $Rank_{WTuntreated}$= 5825.77), indicative of rescue of the spine length phenotype to untreated WT level. When comparing the treated and the untreated WT groups there was statistically significant difference found (M-W= 0.027, Mean $Rank_{WTtreated}$= 5978.04 and Mean $Rank_{WTuntreated}$=5833.63). Normal curve was fitted for a better indicative of the skewness and the difference between spine lengths in the experimental groups.

2.3.3 Adult mice (~25-week-old)

Apparently, the abnormal spine length in 2-week-old *Fmr1* KO mice changed by 10 weeks of age from having shorter spines than controls to having an increased spine length within 8 week time. Thus, our next question was what happens later on, during adulthood, in spine development in *Fmr1* KO mice. Spine quantification in 25-week-old mice showed statistically longer spines (Figure 2.4) in the *Fmr1* KO group compared with the WT littermates group (M-W= 0.000, N_{KO}= 10496 and N_{WT}= 7502,

Mean $Rank_{KO}$= 9150.06 and Mean $Rank_{WT}$= 8788.85). No significant difference could be detected between the experimental groups regarding the spine density at this age either (Figure 2.6) (P= 0.993, N_{KO}= 10 and N_{WT}= 10, $Mean_{KO}$= 16.91 ±1.63 and $Mean_{WT}$= 16.70 ±2.1).

Looking at the spine length and density progression in time, our data show a significant difference in spine length (Figure 2.5) over all time points measured in WT mice and in *Fmr1* KO mice (K-W= 0.000 overall and for each of the two genotypes), with significant differences between each two time points (M-W= 0.000). The spine density (Figure 2.6) differed significantly between all ages in both groups (Two-way ANOVA= 0.000 with LSD< 0.05 between each two time points in both genotypes).

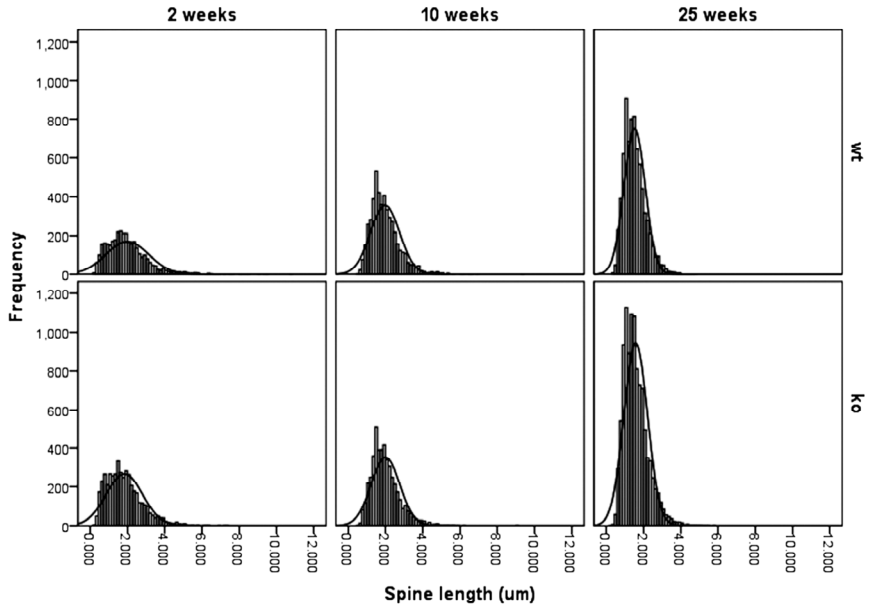

Figure 2.5 Frequency histogram of spine length in time in *Fmr1* KO mice and WT littermates, comparison between 2-week-old, 10-week-old and 25-week-old (untreated). A significant difference was found in spine length over all time points measured in *Fmr1* KO mice and in WT mice (K-W= 0.000 overall and for each of the two genotypes), with significant differences between each two time points for each genotype (M-W= 0.000).

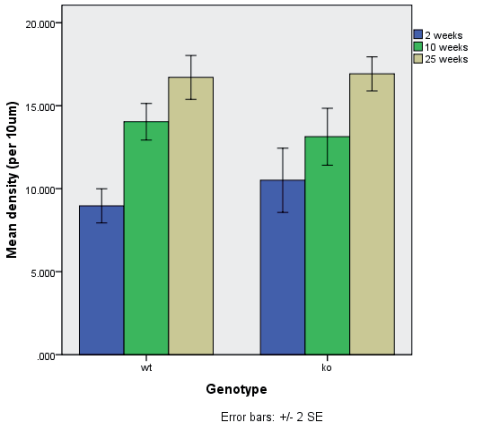

Figure 2.6 Spine density in 2-week-old, 10-week-old and 25-week-old (untreated) *Fmr1* KO mice and WT littermates. No significant difference was found between *Fmr1* KO mice and WT littermates at any chosen time point (2 weeks: P= 0.168, N_{KO}= 7 and N_{WT}= 8, $Mean_{KO}$= 10.5 ±1.46 and $Mean_{WT}$= 8.97 ±2.56; 10 weeks: P= 0.392, N_{KO}= 8 and N_{WT}= 8, $Mean_{KO}$= 13.13 ±2.43 and $Mean_{WT}$= 14.03 ±1.56; 25 weeks: P= 0.993, N_{KO}= 10 and N_{WT}= 10, $Mean_{KO}$= 16.91 ±1.63 and $Mean_{WT}$= 16.70 ±2.1). Spine density differed significantly in time between all ages in both groups (Two-way ANOVA= 0.000 with LSD P-value< 0.05 between each two time points in both genotypes).

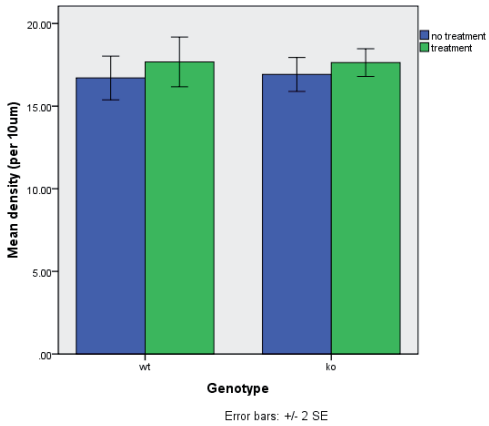

Figure 2.7 Spine density in 25-week-old untreated and AFQ056/Mavoglurant treated *Fmr1* KO and WT littermates. No statistically significant effect of treatment (Two-way ANOVA= 0.197) or for interaction genotype*treatment (Two-way ANOVA= 0.846) on spine density was found. Comparing each two groups no statistically significant difference was obtained (all P-value> 0.1; $N_{KOtreated}$= 6 and $N_{WTtreated}$= 6, $Mean_{KO}$= 17.12 ±1.03 and $Mean_{WT}$= 17.67 ±1.8).

2.3.4 Long-term treatment with AFQ056/Mavoglurant

Clinical studies testing the effect of short-term treatment with AFQ056/Mavoglurant, a specific mGluR5 antagonist, on patients with FXS showed improvement at the behavioral level in a subgroup of these patients. This compound also rescued behavioral deficits in *Fmr1* KO mice and the abnormal spine phenotype in *Fmr1* KO hippocampal cultured neurons. All these previous studies tested the effect of short-term treatment; therefore we were interested to study the effects of long-term treatment in adult *Fmr1* KO mice and WT littermates. AFQ056/Mavoglurant was given to 19-week-old *Fmr1* KO mice and WT littermates for six weeks milled in food pellets. The mice treated for six weeks were sacrificed at 25 weeks of age, together with the age-matched untreated mice. Analysis of the spine length of the treated and untreated mice (Figure 2.4) revealed an overall statistically significant effect of the treatment (K-W= 0.000), with significantly shorter spines in the treated *Fmr1* KO

group compared with the untreated *Fmr1* KO group (M-W= 0.015, $N_{KOtreated}$= 4204 and $N_{KOuntreated}$= 10496, Mean $Rank_{KOtreated}$= 7215.70 and Mean $Rank_{KOuntreated}$= 7404.49). A comparison of the treated *Fmr1* KO group and the untreated WT group showed no significant difference when comparing the values (M-W= 0.236, $N_{KOtreated}$= 4204 and $N_{WTuntreated}$= 7502, Mean $Rank_{KOtreated}$= 5902.98 and Mean $Rank_{WTuntreated}$= 5825.77), indicative of rescue of the spine length phenotype to untreated WT level. No significant difference was found between the treated *Fmr1* KO group and the treated WT group (M-W= 0.398, $N_{KOtreated}$= 4204 and $N_{WTtreated}$= 4269, Mean $Rank_{KOtreated}$= 4214.37 and Mean $Rank_{WTtreated}$= 4259.29), but when comparing the treated and the untreated WT groups there was a statistically significant difference found (M-W= 0.027, $N_{WTtreated}$= 4269 and $N_{WTuntreated}$= 7502, Mean $Rank_{WTtreated}$= 5978.04 and Mean $Rank_{WTuntreated}$=5833.63).

When looking at the spine density (Figure 2.7), we found no statistically significant effect of treatment (Two-way ANOVA= 0.197) or interaction genotype* treatment (Two-way ANOVA= 0.846). Also comparing each two groups no statistically significant difference was found (all LSD P-values> 0.1; $N_{KOtreated}$= 6 and $N_{WTtreated}$= 6, $Mean_{KO}$= 17.12 ±1.03 and $Mean_{WT}$= 17.67 ±1.8).

2.4 Discussion

The hippocampus plays a key role in learning and memory, aspects known to be impaired in patients with FXS. Especially CA1 area is of outmost importance in the above named processes. Abnormal spine phenotype has been found previously in 25-week-old *Fmr1* KO mice in CA1, but not in CA3 hippocampal area, and mGluR5 expression level is higher in the CA1 compared to the CA3 area of the hippocampus (Levenga et al. 2011a). Therefore, we chose to focus on this particular brain area for this study. In the current report, we describe for the first time the dendritic spine phenotype of pyramidal excitatory neurons in the CA1 hippocampal area of *Fmr1* KO mice at three postnatal developmental stages: 2 weeks, 10 weeks and 25 weeks. Additionally, we show an *in vivo* rescuing effect of the specific mGluR5 antagonist AFQ056/Mavoglurant on the dendritic spine length phenotype in the CA1 hippocampal area in 25-week-old *Fmr1* KO mice.

A decreased spine length was found in *Fmr1* KO mice at the age of 2 weeks, which differs from the study of Nimchinsky et al. (2001) who found longer spines in KO mice at this age, but they focused on the cortex. This spine phenotype changed by the age of 10 weeks, when an increased spine length was found in the *Fmr1* KO group compared with the WT littermates group. In the same study by Nimchinsky et al. (2001), the dendritic spine phenotype found in the cortex of 2-week-old mice disappeared at 4 weeks, but was again recorded at 10 weeks by other studies (Galvez and Greenough 2005;McKinney et al. 2005). Our results show that at the same time point, 10 weeks, in the CA1 hippocampal area the same phenotype of longer spines is also seen. This immature spine phenotype is found during adulthood too (25 weeks of age), with longer spines in the *Fmr1* KO mice compared with wild-type littermates. A recent study by Levenga et al. (2011a) also reported an immature spine phenotype in adult *Fmr1* KO mice, but with an increased spine density in the *Fmr1* KO group. No difference in spine density between the *Fmr1* KO and the WT groups was found at any time point in our study. The discrepancy between current findings and the ones from the above-mentioned paper are due to the use of different software for visualizing the confocal images and for spine measurements (ImageJ versus MetaMorph), which allowed for a higher resolution of the images. Increasing image magnification allowed for a more precise distinction between partially overlapping spines or hidden spines, thus due to the new software more spines could be detected either in the background, or from groups of spines, and so a higher density could be counted. Magnification does not influence spine length as it influences density. When looking at other studies reporting results on spine density in *Fmr1* KO mice some found normal density, others increased density, but at different time point and different brain areas, and in different genetic background (Nimchinsky et al. 2001;Galvez and Greenough, 2005;McKinney et al. 2005;Portera-Cailliau 2011, for a review).

Examination of the progression of spine length and density in the *Fmr1* KO mice and their WT littermates at different developmental stages showed a different developmental course. In WT littermates, as expected, spine length decreases significantly throughout life, which correlates with age-dependent changes in the strength and maturity of each spine/synapse. However, in CA1 area of the *Fmr1* KO hippocampus the spine maturation seems to be defective during adulthood compared with the WT littermates. The immature spine phenotype may characterize critical

periods of development for the CA1 region of the hippocampus in *Fmr1* KO mice and underlying learning capacity deficits in adulthood. Perhaps dendritic spine pruning, a process involved in spine elimination, is defective and longer spines are not eliminated resulting in overabundance of immature spines in *Fmr1* KO mice.

Previous studies that focused on the hippocampus of *Fmr1* KO mice have shown controversial results: some have found decreased spine density in the *Fmr1* KO mice compared to WT littermates (Braun and Segal 2000), others a normal density (Grossman et al. 2006) with normal length (Braun and Segal 2000) or with longer spines in the *Fmr1* KO mice (Bilousova et al. 2008). Major drawback in comparing these different studies is caused by the use of different staining (Golgi, diOlistic labeling, transfection) and quantification methods of spines and choice of tissue source; cultured hippocampal neurons versus *in vivo* or *ex vivo* brain tissue, but also different mouse strains. Also the classification of dendritic protrusions into different categories, such as mature and immature, differs between studies, some look at the presence versus absence of a head, other at the ratio between the width and the length of the protrusion, others at the profile. As these criteria can be quite arbitrary we decided not to classify the protrusions, but only to use the length as more objective measure. Another aspect needed to be mentioned is that the mouse model (*Fmr1* KO(2)), with total lack of *Fmr1* mRNA expression, used in our study has not been tested by other groups. Nevertheless, we provide conclusive evidence that during adulthood spine length is altered in the CA1 hippocampal region of *Fmr1* KO mice using an *ex vivo* approach.

For specific reasons (administration via food pellets is not possible for 2-week-old mice), we have chosen to test the effect of the mGluR5 antagonist AFQ056/Mavoglurant on spine length only in adult mice, since, as previously described by our group (Levenga et al. 2011a), at this age *Fmr1* KO mice present with significantly more immature spines than WT littermates in CA1 area of the hippocampus, and the spine phenotype in adult mice most closely resembles the spine phenotype in patients with FXS. To our best knowledge, the current study is the first to test the effect of long-term treatment with AFQ056/Mavoglurant on the spine length in *Fmr1* KO mice. We show evidence that this specific mGluR5 antagonist can rescue the abnormal spine length observed in adult *Fmr1* KO mice. We chose to start long-term treatment (for six weeks) at 19 weeks, as we wanted to examine the effect of

AFQ056/Mavoglurant on the spine phenotype at 25 weeks of age and compare the results with the abnormal spine phenotype in CA1 previously found in untreated 25-weeks-old *Fmr1* KO mice, to see if the treatment can rescue the immature-like spine phenotype. Long-term treatment with AFQ056/Mavoglurant led to a reduction in spine length in the *Fmr1* KO mice comparable with normal values, while in the treated WT mice an increase in the spine length was found when compared with the untreated WT group, but not with treated KO group. This difference in spine length between the two WT groups, although small, is statistically significant, but it could be due to the high number of spines measured. On the other hand, it could very well be possible that interfering with the normal function of mGluR5 would have consequences on spine morphology in unaffected mice. No effect of the treatment on spine density was found in the *Fmr1* KO and WT groups. Thus at all time-points and regardless of treatment no difference in spine density was observed in the experimental groups. Previous studies have shown that compounds from the mGluR5 antagonist category, such as MPEP, fenobam, STX107, AFQ056/Mavoglurant and RO4917523, have a positive effect on the behavioral and morphological spine phenotype of *Fmr1* KO mice and fruit-fly models, and on patients with FXS (McBride et al. 2005;Su et al. 2010). However, the rescue effect of long-term treatment with AFQ056/Mavoglurant found in our study might take place only at a structural level, since we have not tested for functional rescue. An investigation of the functionality of the synapses prior to and following treatment requires electrophysiological and behavioral studies (latter ones currently in progress) that are beyond the scope of this paper.

The reduction in spine length after long-term treatment is a very promising finding indicating the positive effect of AFQ056/Mavoglurant on the immature spine phenotype in *Fmr1* KO mice even at adult age, when neural plasticity is known to be slower than earlier in development. These results also suggest that selective inhibition of the mGluR5 subtype might constitute a valuable therapeutic intervention. As the structural spine deficits can be reversed at adult age, more studies focused on starting treatment at earlier time points in development are needed. Our results also open avenues towards rescue of the behavioral deficits following chronic treatment, and towards getting insights into what behavioral deficits are caused by the abnormal mGluR5 pathway.

As any study, ours also has its limitation, such as: diOlistic labeling of hippocampal slices by means of gene gun does not always give an image of a whole neuron filled with the dye. Therefore, it is not possible to determine the distance of the dendritic segment from the cell soma, however, this problem occurs in both WT and *Fmr1* KO mice.

Conclusion

Our study shows that in the hippocampal CA1 area of *Fmr1* KO mice the spine development seems to follow a different course than in WT littermates. At 2 weeks of age *Fmr1* KO mice have a decrease spine length compared with WT littermates. However, towards adulthood (10 weeks of age) this phenotype changes with *Fmr1* KO mice having a more immature-like phenotype, longer spines than the control group. At adulthood (25 weeks of age), the *Fmr1* KO mice show an overabundance of longer spines compared with WT littermates, however, no difference in spine density at any age could be observed, indicative that the spine maturation and/or pruning is impaired. Finally, this immature spine phenotype can be rescued by long-term treatment with AFQ056/Mavoglurant.

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Chronic administration of AFQ056/Mavoglurant restores social behavior in *Fmr1* KO mice

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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

3.1 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 (Hagerman 2002). 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 (Oberlé et al. 1991;Pieretti et al. 1991;Verkerk et al. 1991). FMRP is highly expressed in neurons of the brain and is an RNA-binding protein involved in controlling mRNA translation at the synapse (Willemsen et al. 2011).

Activation of group 1 metabotropic glutamate receptors (mGluRs), especially mGluR5, leads to local translation of specific mRNAs at the synapse, including *FMR1* itself (Bear et al. 2004;Nakamoto et al. 2007). 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 (Lu et al. 2004;Narayanan et al. 2008;Dictenberg et al. 2008;Sharma et al. 2010). 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 (Kaufmann and Moser 2000;Irwin et al. 2001;Levenga et al. 2011a).

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 (Bakker et al. 1994). Moreover, the *Fmr1* KO mouse exhibits abnormal spine morphology and density on dendrites and enhanced group 1 mGluR LTD (Huber et al. 2002). FXS presents with a distinct behavioral phenotype which overlaps significantly with that of autism (Hagerman et al. 1986;Hagerman 2002;Hagerman et al. 1986;Steinhausen et al. 2002; Hall et al. 2010;Tranfaglia 2011) 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 (Steinhausen et al. 2002;Hall et al. 2010;Tranfaglia 2011). In addition, several of the social behavioral deficits are exhibited in *Fmr1* KO mice, including social dominance, interaction and recognition (Mineur et al. 2005;Spencer et al. 2005;McNaughton et al. 2008;Liu and Smith 2009;Spencer et al. 2011;Pietropaolo et al. 2011). 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 (Levenga et al. 2010). Genetic down-regulation of mGluR5 expression in *Fmr1* KO mice rescued several of the FXS phenotypes (Dolen et al. 2007;Thomas et al. 2011a). 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 (Levenga et al. 2010). 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 (Yan et al. 2005;Nakamoto et al. 2007;De Vrij et al. 2008;Thomas et al. 2011b). Unfortunately MPEP is not mGluR5 specific and has been shown to inhibit NMDA receptors as well (O'Leary et al. 2000;Lea et al. 2005) 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 (Levenga et al. 2011b;Vinueza Veloz et al. 2012). Moreover, in FXS patients with full methylation of the *FMR1* promoter, AFQ056/Mavoglurant treatment did improve behavioral attributes (Jacquemont et al. 2011).

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.

3.2 Materials and Methods

3.2.1 Animals

Generation of male *Fmr1* KO mice and WT littermates was established as described previously (Mientjes et al. 2006). The mice were >10X 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.

3.2.2 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).

3.2.3 Sociability/preference for social novelty task (SPSN)

The SPSN task was a modified version of a protocol described by Nadler and colleagues (Nadler et al. 2004;Naert et al. 2011). 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^{TM} 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^{TM} 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.

3.2.4 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)) (Levenga et al. 2011b). 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 5 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 assay after 3 weeks of administration.

3.2.5 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.).

3.3 Results

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

Sociability was assessed with social approach assay in SPSN task. During this assay 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).

3.3.1.1 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 (Nadler et al. 2004;Moy et al. 2009). Both untreated and AFQ056/Mavoglurant treated WT and *Fmr1* KO mice demonstrated preference for sniffing at STR1 mouse over the novel object (empty) (Figure 3.1A).

Untreated WT and *Fmr1* KO mice (Figure 3.1A, 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* stranger side approached significance ($F(1,20)= 3.235$, $P= 0.087$). Chronic treatment with AFQ056/Mavoglurant indicated for both KO and WT mice (Figure 3.1A, 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*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 (Figure 3.1A, white bars) (repeated measures ANOVA, main effect of treatment: $F(1,20)= 1.882$; $P= 0.185$; treatment*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*treatment interaction (F(1,40)= 3.693, P= 0.062).

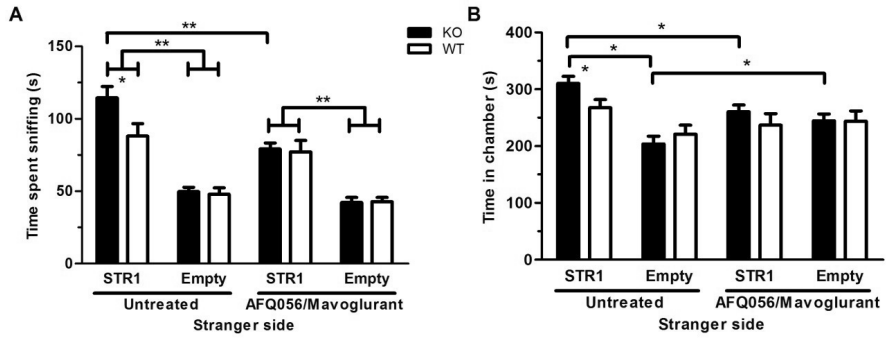

Figure 3.1 Sociability behavior in untreated and chronic AFQ056/Mavoglurant treated WT and *Fmr1* KO mice. A) Time spent sniffing (direct interaction approach) during sociability assay by untreated (left panel) and chronic AFQ056/Mavoglurant treated (right panel) WT and *Fmr1* KO mice. Overall, both 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.

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 3.1A, 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 3.1A, right panel black bars) reduced sniffing time of KO mice at STR1 to levels of WT animals (Figure 3.1A, white bars) (F(1,20)= 1.833, P=0.191).

3.3.1.2 Time in chamber during sociability assay

Total time in chamber represented exploration of the whole chamber (containing STR1 or empty) during sociability assay (Figure 3.1B).

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*chamber side interaction ($F(1,20)= 2.279$, $P= 0.147$) (Figure 3.1B, 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 3.1B, 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 3.1B, 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 3.1A, 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 3.1B) 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* 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 3.1B, black bars) and increased exploration in empty chamber ($P= 0.035$), leading to absence of exploration preference between STR1 and empty chamber (Figure 3.1B, right panel black bars).

The absence of exploration preference in WT animals (both treated and untreated) for STR1 chamber compared with empty chamber (Figure 3.1B, white bars) made it difficult to compare KO and WT groups. Comparing time in chamber with sniffing time measurements (results section 3.3.1.1) suggested that sniffing time is a more sensitive measurement to study direct social interaction during sociability assay, as described previously (Nadler et al. 2004;Moy et al. 2009).

3.3.1.3 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 3.2).

Comparing all groups (untreated and AFQ056/Mavoglurant treated WT and *Fmr1* KO mice; Figure 3.2) determined a significant time*stranger side*genotype* 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 ($F(1,40)= 14.989$, $P< 0.001$) and approached significance for genotype ($F(1,40)= 3.806$, $P= 0.058$).

Untreated *Fmr1* KO animals (Figure 3.2B) 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 3.2A) showed a preference for STR1 from 4 minutes onwards. This was confirmed by repeated measures ANOVA which indicated a significant time*stranger side*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*treatment: $F(1,20)= 6.985$, $P= 0.016$) (Figure 3.2D). 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 3.1B). Repeated measures ANOVA indicated no significant difference

between chronic treated WT and KO (Figure 3.2C, 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.

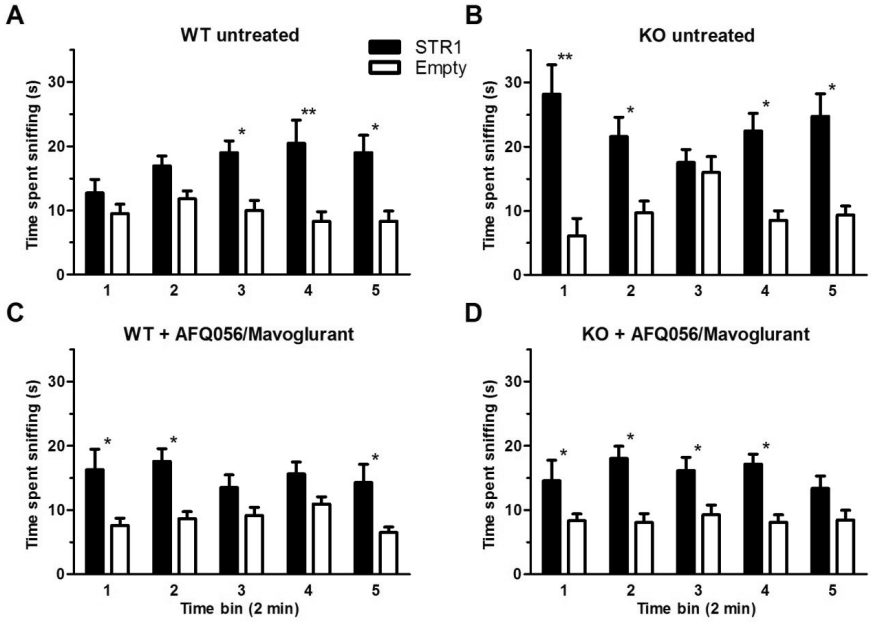

Figure 3.2 Sniffing time over 2 min time bins during sociability assay in untreated and AFQ056/Mavoglurant treated *Fmr1* KO and WT mice. A) Untreated WT mice increased sniffing duration at STR1 over time with a preference for STR1 from 4 min onwards. B) Untreated KO mice demonstrated significant enhanced 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.

3.3.1.4 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 3.3.1.1).

3.3.2 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.

3.3.2.1 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 3.3A).

Comparing untreated WT (Figure 3.3A, left panel, white bars) and KO mice (Figure 3.3A, 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*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 3.3A, right panel, white bars) and *Fmr1* KO mice (Figure 3.3A, 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*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 3.3A, 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 and stranger side (treatment: F(1,20)= 8.628, P= 0.008; stranger side: F(1,20)= 16.479, P< 0.001) (Figure 3.3A 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.

3.3.2.2 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 3.3B, C, D, 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 3.3E) showed high interaction with STR2 over most time points (during first 4 minutes and between 6-8 minutes), while untreated KO mice (Figure 3.3C) 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 3.3B, 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).

3.4 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 and 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.

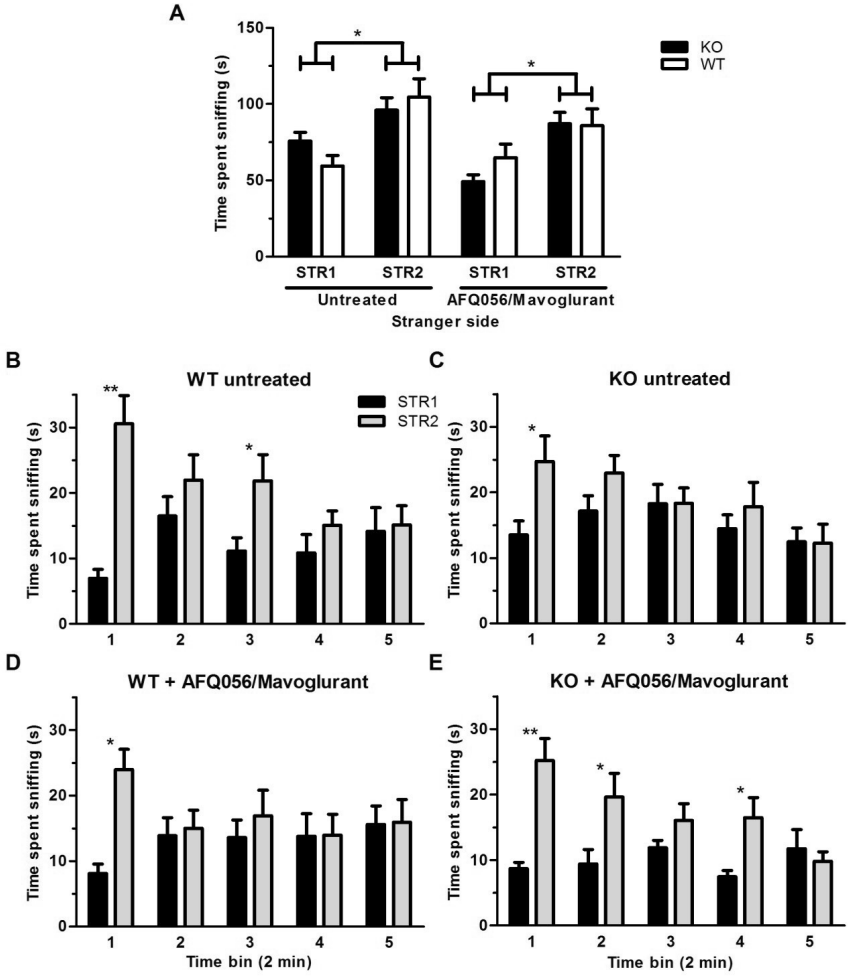

Figure 3.3 Preference for social novelty in untreated and AFQ056/Mavoglurant treated WT and *Fmr1* KO mice. A) Untreated WT (left panel, white bars, N= 11) and *Fmr1* KO (left panel, black bars, N= 11) mice spent more time in proximity to STR2 (sniffing time) compared with STR1 (significant main effect 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.

SPSN is a behavioral task used to study several neuropsychiatric disorders including fragile X syndrome, autism and schizophrenia (McFarlane et al. 2008; McNaughton et al. 2008;Moy et al. 2009;Mines et al. 2010;Silverman et al. 2010;Peca et al. 2011;Spencer et al. 2011). Its two assays measure different components of social behavior (Nadler et al. 2004;Moy et al. 2007). 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 (Nadler et al. 2004;Naert et al. 2011). *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 chambers. 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 (Mineur et al. 2002;Moy et al. 2009). 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 (Spencer et al. 2005;Spencer et al. 2011;Thomas et al. 2011a). This enhanced social approach, described in our task, was not previously reported by other studies (McNaughton et al. 2008;Liu and Smith 2009;Moy et al. 2009;Pietropaolo et al. 2011), possibly due to different background of KO mice or differences in the SPSN protocol. Similar to our findings, abnormal increased sociability has also been described in a mouse model of Rett syndrome (Chao et al. 2010;Schaevitz et al. 2010).

The sociability part of the SPSN task has been described as being the most sensitive to study autism-like symptoms in mice (Yang et al. 2011). Abnormal social behavior is frequently observed in subjects with FXS and is also a major component of autism (Hagerman 2002). 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 (Peca et al. 2011). 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 (Fisch 1993;Tranfaglia 2011).

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 (Liu and Smith 2009; Mines et al. 2010;Pietropaolo et al. 2011). 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 (McFarlane et al. 2008;Moy et al. 2009;Peca et al. 2011).

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 (Bear et al. 2004). Both, FMRP and mGluR5 are expressed in many brain regions and circuits that might be involved in the regulation of social behaviors (Insel and Fernald 2004). 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 (Liu et al. 2010; Mines et al. 2010). A recent study of Silverman et al. (2012) 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 (De Vrij et al. 2008;Burket et al. 2011). 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.

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. 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 (Levenga et al. 2011b). Moreover, AFQ056/Mavoglurant administration in male FXS patients could also improve behavior in a specific subgroup of patients with FXS (Jacquemont et al. 2011).

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 (Steinhausen et al. 2002;Hall et al. 2010;Tranfaglia 2011). 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 (Levenga et al. 2010). 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.

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Synaptic receptor levels in *Fmr1* KO mouse frontal cortex are unchanged compared with controls

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Abstract

Fragile X syndrome (FXS) is caused by silencing of the fragile X mental retardation 1 gene (*FMR1*), and consequently lack of its product, fragile X mental retardation 1 protein (FMRP). This protein plays a regulatory role in translation of target mRNAs at the level of the neuronal synapse, and its absence leads to increased synthesis of other synaptic proteins, which in turn determines a higher internalization rate of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), as proposed by "the mGluR5 theory". In the knockout mouse model for FXS (*Fmr1* KO), the lack of expression of Fmrp is proposed to affect the level of different synaptic proteins and receptors, among which AMPAR, *N*-methyl-D-aspartate receptor (NMDAR), striatal-enriched protein tyrosine phosphatase (STEP) and gamma-aminobutyric acid receptor (GABAR). In order to test this hypothesis we isolated synaptoneurosomes (SNs) from frontal cortex of adult *Fmr1* KO mice and wild-type (WT) littermates, and performed immunoblotting on these samples with the antibodies specific for the above mentioned proteins/receptors of interest. We have not found any significant difference between KO and WT SNs samples for any of the studied protein levels. This could be due to the high variation found within and between samples, but it could also mean that absence of Fmrp does not greatly impact the tested protein levels at the synapse as hypothesized.

Keywords: Fragile X syndrome; FMRP; *FMR1*; AMPA; NMDA; GABA; STEP; Western blot; Synaptoneurosomes; *Fmr1* KO; Frontal cortex;

4.1 Introduction

Fragile X syndrome (FXS) is the most common monogenic inherited form of intellectual disability and autism (Hagerman et al. 2010), being caused by silencing of the fragile X mental retardation 1 gene (*FMR1*), located on the X chromosome, due to more than 200 repeats of the trinucleoids CGG in the 5' untranslated region (UTR) of the gene (Bell et al. 1991;Oberlé et al. 1991;Yu et al. 1991). Silencing of the gene leads to lack of expression of its product, the fragile X mental retardation protein (FMRP). FMRP, an RNA-binding protein, is expressed in many tissues, especially in brain with high levels found in hippocampus and cerebellum, and moderate expression in cerebral cortex (Abitbol et al. 1993;Devys et al. 1993;Hinds et al. 1993;Khandjian et al. 1995). It is localized mainly in the cell cytoplasm (Devys et al. 1993), but it shuttles between cytoplasm and nucleus, and throughout the cell even to the dendritic spines, while binding target mRNAs (Devys et al. 1993;Feng et al. 1997;Ferrari et al. 2007). It has been shown that FMRP travels into the dendrites via large RNA granules containing target mRNAs, motor proteins, other RNA binding proteins and ribosomal subunits (De Diego Otero et al. 2002). It is associating with the mRNAs of proteins like PSD95 (Zalfa et al. 2007), SAPAP1-3 (Brown et al. 2001; Schutt et al. 2009), α-CaMKII, Arc/Ar3.1 (Zalfa et al. 2003), Shank1 (Schutt et al. 2009) and many others. In the postsynaptic density (PSD) these mRNAs are translated into proteins which regulate the morphology of the spine and the functionality of the synapse. In most cases, FMRP acts as a translational repressor of mRNAs encoding proteins that are regulating the internalization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), essential in the proper function of the synapse, including for processes like learning and memory (Lee et al. 2003;Takahashi et al. 2003). The insertion and removal of AMPARs to and from the synaptic plasma membrane depends on the activation of other membrane receptors like *N*-methyl-D-aspartate receptor (NMDAR) and group 1 metabotropic glutamate receptors (mGluRs), during processes called long-term potentiation (LTP) and long-term depression (LTD). During NMDA-dependent LTP AMPARs are inserted into the membrane, whereas during NMDA-dependent LTD and mGluR5-dependent LTD AMPARs are internalized (Huber et al. 2001;Snyder et al. 2001;Xiao et al. 2001;Collingridge et al.

2004). Zhang et al. (2008) demonstrated that AMPA receptor endocytosis is dependent also on striatal-enriched protein tyrosine phosphatase (STEP), which is located in postsynaptic terminals (Oyama et al. 1995) and associates with NMDA complex inhibiting its function (Pelkey et al. 2002).

Huber et al. (2002) showed that the absence of FMRP leads to increased protein synthesis and altered synaptic plasticity, including enhanced LTD. According to the "mGluR theory" developed by Bear et al. (2004) the increased activity of mGluRs in FXS leads to increased protein synthesis in the postsynaptic density, and consequently to permanent internalization of AMPA receptors and subsequent degradation, as the break posed by FMRP is lacking. Zhao et al. (2005) reported that NMDA-dependent LTP in prefrontal cortex also requires FMRP. Several other studies on different brain areas of the knockout mouse model for FXS, the *Fmr1* KO mouse, have looked at levels of AMPAR subunits, NMDAR subunits and mGluR5, but also at other postsynaptic proteins, such as STEP, PSD-95, SAPAP3, but not all studies show the same outcome. Giuffrida reported normal levels of AMPA, NMDA and mGluR5 receptors in total protein homogenates and synaptic membrane preparations from forebrain of *Fmr1* KO mice (Giuffrida et al. 2005). Normal levels of AMPA receptor subunits GluA1 and GluA2/3 have been found in homogenate preparation from cortex of 1-week-old *Fmr1* KO mice, but reduced in SN fraction, whereas at 2 weeks of age only GluA1 subunit was reduced in SNs, while GluA2/3 and GluN2B levels were reduced in homogenate (Till et al. 2012).

In addition to the mGluR theory, a GABA hypothesis has been proposed (D'Hulst and Kooy 2007), stating that GABA signaling is also affected in FXS, being known that many patients manifest epileptic seizures, sleep disorder which are connected to this inhibitory pathway. It has been found that $GABA_A$ receptor subunit mRNAs are targets of FMRP (Miyashiro et al. 2003), and that in the subiculum of *Fmr1* KO mice $GABA_A$ current is reduced and expression of its $\alpha 5$ and δ subunits is altered (Curia et al. 2009). Also in the striatum of these mice GABA transmission is abnormal (Centonze et al. 2007). In the cortex of *Fmr1* KO mice reduced levels of mRNA for 8 out of 18 $GABA_A$ receptor subunits have been found (D'Hulst et al. 2006), while in the hippocampus and brainstem the $GABA_A$ β subunits levels were found to be decreased compared with control values (El Idrissi et al. 2005).

Given the previous studies on AMPA and $GABA_A$ levels in the *Fmr1* KO mice in some brain areas, we were interested to investigate the state of these receptors, but also of STEP, in SNs from frontal cortex of adult *Fmr1* KO mice, by immunoblotting. We chose frontal cortex as investigation area, because previous studies showed different results (Giuffrida et al. 2005;Till et al. 2012), and because we were interested in corroborating the obtained results with the ones from a social behavior test on the *Fmr1* KO mice. As this behavior is presumably connected to the fronto-cortical areas, we were wondering whether at molecular level alterations can also be detected. We could not find any significant difference between the receptor levels in *Fmr1* KO mice and their controls in frontal cortex SNs.

4.2 Materials and Methods

4.2.1 Animals

Fmr1 KO mice were generated in our lab as previously described, and named *Fmr1* KO(2) (Mientjes et al. 2006). These mice lack the *Neo* cassette, and the level of *Fmr1* mRNA expression is null. The line was completely backcrossed (>10X) in the C57BL/6J background. In our study we only used male mice, *Fmr1* KO (7) and wild-type (WT) littermates (4) of 13±1 weeks of age. Mice were housed under standard conditions. All experiments were carried out with the permission of the local ethics committee.

4.2.2 Antibodies

The first antibodies used for immunoblotting were: GluR1 (Millipore; rabbit; 1:1000), STEP/PTPN5 (Abcam; mouse; 1:1000), $GABA_A$ α1 (Millipore; rabbit; 1:1000), Synapsin (Synaptic Systems; mouse; 1:2000), actin (Sigma; mouse; 1:1000) and GAPDH (Millipore; mouse; 1:1000). As secondary antibodies rabbit anti-mouse (Dako; 1:10000) and goat anti-rabbit (Dako; 1:10000) were used.

4.2.3 Synaptoneurosome preparation

Synaptoneurosomes (SNs) were isolated from frontal cortex of ~13-week-old male *Fmr1* KO (7) and WT (4) mice (samples were used individually and pooled) following

an adapted protocol previously described (Till et al. 2012). In short, frontal cortex from each animal was isolated by cutting the anterior cortex until hippocampus in ice-cold PBS, and gently homogenized in a Teflon-glass homogenizer in HEPES buffer (10 mM HEPES, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, protease inhibitor cocktail (Roche)). The homogenate was centrifuged for 1 min at 4^{o}C at 2000x g. The supernatant was filtered through two layers of polypropylene mesh (pore size 100 μm: Millipore) and subsequently filtered through a Durapore filter (pore size 0.5 μm: Millipore). The filtrate was centrifuged for 10 min at 4^{o}C at 1000x g and the SNs pellets were lysed in 100 μl 1% SDS buffer. Protein concentration was measured with a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Samples were boiled in Laemmli buffer.

4.2.4 Electronmicroscopy

SNs from frontal cortex were re-suspended in 10 μl of Milli-Q and spotter onto Formvar coated grids (200 mesh). Adsorbed SNs were directly negatively stained using uranyl acetate. Grids were examined by a Philips CM100 electron microscope at 80 KV.

4.2.5 Immunoblotting

Proteins (7.5 μg/15 μl) were resolved by SDS-PAGE, and transferred onto nitrocellulose membrane. The membranes were blocked for 1 h in 5% milk (Elk) in PBS-Tween-20 (0.05%), washed two times in PBS-Tween-20, and incubated with first antibodies in 0.5% BSA in PBS-Tween-20 overnight. Next day the blots were washed and incubated with the secondary antibody in PBS-Tween-20 for 1 h at room temperature. Further, the membranes were washed and scanned with Odyssey infrared imaging system (LI-COR Biosciences).

4.2.6 Data analysis

Images obtained following scanning of the blots were analysed with Odyssey 3.0 software. The signal intensity values were averaged for 3 lines, and top/bottom background value was subtracted. Fluorescence levels for the proteins of interest were normalized to the loading control levels (actin/GAPDH). Data from individual samples

was analysed with two sample equal variance t-test, and from pooled samples with a two sample paired t-test with aliquots as data points. Level of significance was set at 0.05. Data are expressed as mean ± SD and percentage.

4.3 Results

Our current study investigated the level of GluR1 subunit of AMPAR, STEP/PTPN5 and $GABA_A$ α1 subunit in SNs from frontal cortex of 13-week-old *Fmr1* KO mice compared with their age-matched WT littermates.

For a more reliable study we first established the best protein concentration to be used for immunoblotting for all proteins of interest as being 7.5 μg/15 μl loading volume (Figure 4.1). Once the protein concentration was determined the next step was to verify the enrichment of our SN fraction compared to the initial homogenate, before filtration steps. Enrichment was tested by comparing synapsin levels in WT frontal cortex SN fraction with the one in unfiltered homogenate, as synapsin is a well-known synaptic protein, thus it should appear in a higher concentration in SNs. A 1.76 fold increase in synapsin level was found in the SN fraction indicating enrichment of the fraction (Figure 4.2A). Corroborating this result with the electron-microscopic aspect of the SN fraction (Figure 4.2B) we can assess that the end-fraction isolated following the above mentioned protocol is indeed synaptoneurosomal, thus it is an enriched substrate for studying the synaptic proteins of interest.

In order to test the reliability of the immunoblotting method for our samples and proteins of interest we resolved and blotted on the same membrane six aliquots of the same sample (*Fmr1* KO) (Figure 4.3). Quantification revealed a 34 to 50% difference between the lowest and the highest values measured for each protein when comparing the normalized data. The highest and the lowest values did not correspond to the same aliquot for all proteins. This big variation between virtually identical samples led us to the hypothesis that actin might not be the most reliable loading control.

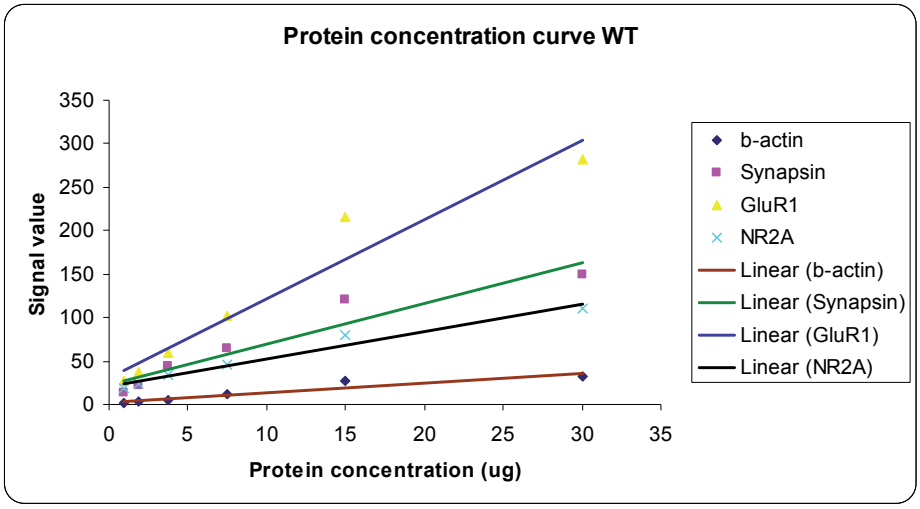
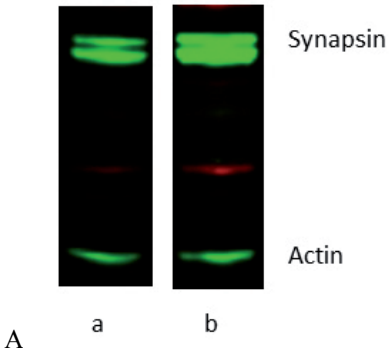


Figure 4.1 Protein concentration curve. Several protein concentrations have been loaded on gel and blotted (30 µg, 15 µg, 7.5 µg, 3.75 µg, 1.875 µg, 0.9375 µg, each in 15 µl). Different antibodies were used for incubation and testing the best protein concentration to be further used in Western blot experiments. 7.5 µg/ 15µl was chosen, as it is the highest value still in the linear region of the concentration curve.



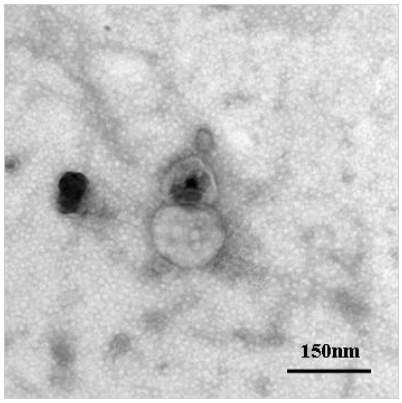


Figure 4.2 Proof of enrichment in SNs fraction from mouse frontal cortex. A) Western blot comparing synapsin levels in homogenate (a) and SN fraction (b) from frontal cortex of an adult WT mouse (13-week-old). A 1.76-fold increase in synapsin level was found in the SN fraction. Values were normalized to actin. B) Electron-microscopic image of the SN fraction using negative staining with uranyl acetate.

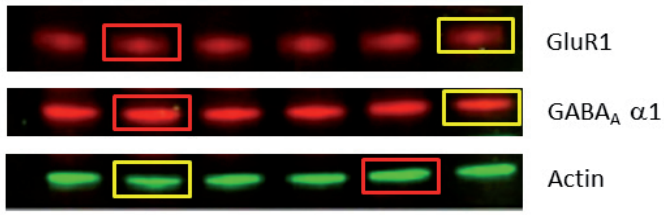


Figure 4.3 Testing reliability with actin. Six aliquots of the same sample (frontal cortex SNs from one 13-week-old *Fmr1* KO mouse) were blotted and values measured for each protein were compared. The yellow rectangle indicates the lowest value found for each sample after normalizing to actin, while the red rectangle indicates the highest value. For actin the raw data is indicated. Between the two extreme values for GluR1 a 32.5% difference was found, while for GABA$_A$ α1 a 49.8% difference. In the case of actin a 31.1% difference was found. The extreme values did not correspond to the same aliquot for all proteins tested.

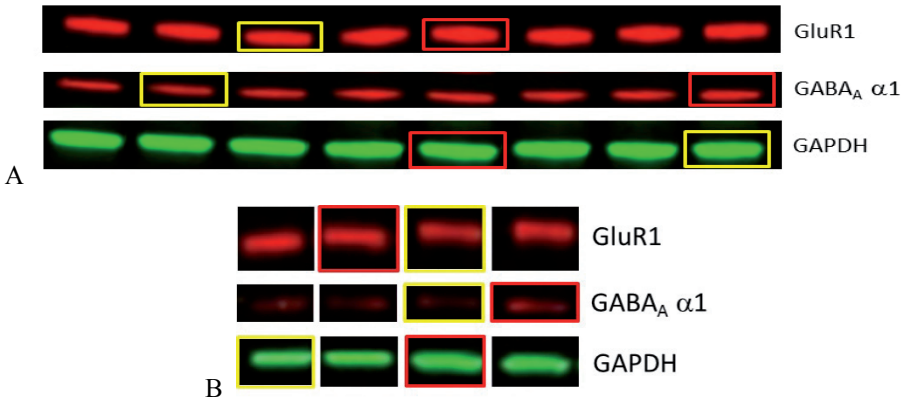


Figure 4.4 Testing reliability with GAPDH. A) The same sample (pooled frontal cortex SNs from 4 WT mice 13-week-old) was loaded 8 times on one gel. Although in this case too the extreme values belonged to different slots for different proteins, the difference between the lowest (yellow rectangle) and the highest values (red rectangle), normalized to GAPDH, was smaller than when actin was used as loading control (GluR1: 10.18%; GABA$_A$ α1: 23.50%; GAPDH: 13.10%), indicating that GAPDH might be a more reliable loading control. B) The same sample (pooled frontal cortex SNs from 4 WT mice 13-week-old) was loaded on 4 different gels, immunoblotted in parallel. In this case the difference between aliquots was higher than when loaded on the same gel, for all proteins tested (GluR1: 61.43%; GABA$_A$ α1: 56.02%; GAPDH: 51.60%). In this experiment also the lowest (yellow border) and the highest (red border) normalized values belonged to different gels for different proteins. For GAPDH raw values are compared.

Therefore, for our next experiment we used GAPDH as a loading control. Frontal cortex SN fractions from 4 WT mice were pooled and loaded eight times on a gel, and immunoblotted (Figure 4.4A). The eight values obtained for each sample were compared between each other for each protein of interest. The same sample was also loaded on four gels, immunoblotted at the same time to study the variation between gels, as within a gel (Figure 4.4B). The variation found for a sample loaded more times on one gel was smaller than the variation when the same sample was loaded on

different gels. Also, GAPDH seems to be a more reliable loading control than actin, as its variation is less within one gel.

Seeing that comparing samples loaded on the same gel gives a more reliable outcome than data coming from different gels, we loaded frontal cortex SN fractions from *Fmr1* KO mice and WT littermates on a gel and performed a Western blot to study the levels of GluR1, STEP/PTPN5 and $GABA_A$ α1 (Figure 4.5A). GAPDH was used as loading control for normalizing the data. A high variation was observed within a group for all proteins, with the difference between the lowest and the highest value larger for the WT group (ranging from 26% to 59%) compared with the KO group (between 17% and 47%). A higher mean was found in the *Fmr1* KO group compared with the WT group for each of the studied proteins (for GluR1: 1.71±0.29 for KO and 1.63±0.45 for WT; for STEP/PTPN5: 0.25±0.03 for KO and 0.23±0.09 for WT; for $GABA_A$ α1: 0.41±0.11 for KO and 0.37±0.13 for WT), but without a statistical significance (all P-values > 0.05; Figure 4.5B).

In order to eliminate the variation between mice as a variable we pooled frontal cortex SN fractions from *Fmr1* KO mice and WT littermates and loaded them as two samples 8 times on one gel and performed immunoblotting for the same proteins of interest (Figure 4.6). When comparing the values measured for the aliquots of the same pooled sample a lower variation was found compared with the variation between individual SN samples from the *Fmr1* KO and the WT mice. The difference between the lowest and the highest values measured for each protein of interest in the 8 aliquots of each sample ranged from 24% to 37% for the KO sample, and from 10% to 24% for the WT sample. The protein levels in the two experimental groups were very similar for each studied protein (mean of the 8 aliquots ±SD: $GABA_A$ α1: 0.27±0.03 for KO and 0.27±0.02 for WT; STEP/PTPN5: 0.16±0.02 for KO and 0.17±0.01 for WT; GluR1: 0.50±0.04 for KO and 0.51±0.02 for WT), thus no statistically significant difference between protein levels in *Fmr1* KO and WT frontal cortex SNs could be detected (all P-values > 0.05).

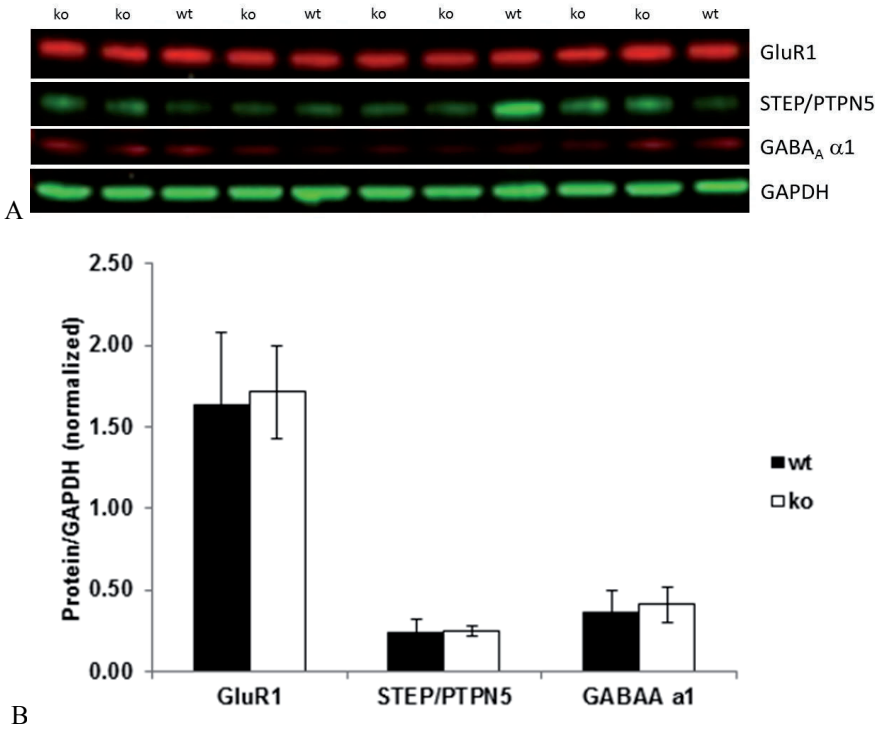
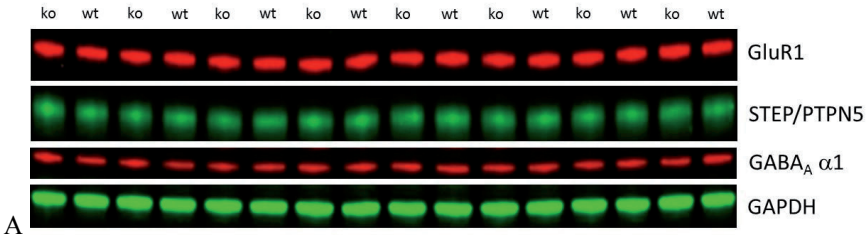


Figure 4.5 Protein levels in frontal cortex SNs from *Fmr1* KO mice and WT littermates. A) Western blot of *Fmr1* KO (N= 7) and WT (N= 4) frontal cortex SN samples for GluR1, STEP/PTPN5 and GABA$_A$ α1 protein levels. GAPDH was used as loading control. The variation within a group between the lowest value and the highest is smaller for KO group than for WT for all proteins (GAPDH: 16.87% for KO and 26.44% for WT; GABA$_A$ α1: 46.69% for KO and 58.86% for WT; STEP/PTPN5: 33.60% for KO and 51.73% for WT; GluR1: 33.48% for KO and 47.36% for WT). B) Average GluR1, STEP/PTPN5 and GABA$_A$ α1 protein levels normalized to GAPDH in *Fmr1* KO mice (N= 7) and WT littermates (N=4). The errors bars represent SD. No statistically significant difference between groups was found for any protein tested (mean ±SD and P-value - for GABA$_A$ α1: 0.41±0.11 for KO and 0.37±0.13 for WT, P= 0.90; for STEP/PTPN5: 0.25±0.03 for KO and 0.23±0.09 for WT, P= 0.80; for GluR1: 1.71±0.29 for KO and 1.63±0.45 for WT, P= 0.63).

4.4 Discussion

To our knowledge, the current study is the first to investigate the level of synaptic receptors in frontal cortex SN fraction from the *Fmr1* KO(2) mouse model for FXS. According to the mGluR5 theory and the GABA hypothesis the functionality of synapses is impaired in patients with FXS, therefore we wanted to study whether an abnormal synaptic protein level could be indeed found in frontal cortex of *Fmr1* KO mice. The SNs were isolated from 13-week-old male *Fmr1* KO and WT littermates and immunoblotted for GluR1 subunit of AMPAR, STEP/PTPN5 and α1 subunit of

$GABA_A$ receptor. We resolved on SDS-PAGE and blotted individual aliquots of the SN samples obtained from each mouse to look at the variation between mice in the *Fmr1* KO and the WT groups, and we also pooled aliquots per experimental group to reduce the variation between mice. Our results indicated that in both cases no significant difference between KO and WT groups could be found for any of the proteins of interest.



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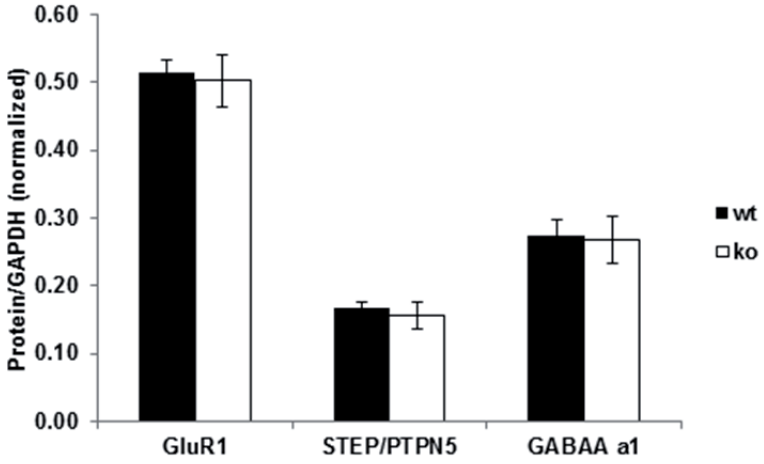

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Figure 4.6 Protein levels in pooled frontal cortex SNs from *Fmr1* KO mice and WT littermates. A) Western blot on pooled *Fmr1* KO (from 7 mice) and WT (from 4 mice) frontal cortex SN samples for GluR1, STEP/PTPN5 and $GABA_A$ α1 protein levels. The two pooled samples were loaded 8 times for variation. GAPDH was used as loading control. The variation between the identical KO samples was found to be higher than between the WT samples for all proteins tested. For the KO aliquots, the difference between the lowest and the highest values was 24.33% for GAPDH, 30.62% for $GABA_A$ α1, 36.78% for STEP/PTPN5, and 23.70% for GluR1. In the case of WT aliquots the difference ranged from 10.18% for GluR1 to 13.10% for GAPDH, to 14.45% for STEP/PTPN5, and to 23.50% for $GABA_A$ α1. These differences are smaller than the ones found between the individual SN samples from the 7 *Fmr1* KO and 4 WT mice. B) GluR1, STEP/PTPN5 and $GABA_A$ α1 protein levels normalized to GAPDH in pooled *Fmr1* KO sample from 7 mice and pooled WT sample from 4 mice. The columns represent average value of the 8 aliquots with SD as error bar. No statistically significant difference between groups was found for any protein tested (mean ±SD and P-value - for $GABA_A$ α1: 0.27±0.03 for KO and 0.27±0.02 for WT, P= 0.73; for STEP/PTPN5: 0.16±0.02 for KO and 0.17±0.01 for WT, P= 0.22; for GluR1: 0.50±0.04 for KO and 0.51±0.02 for WT, P= 0.39).

Fragile X syndrome is characterized by intellectual disability, autistic-like behavior, and abnormal dendritic spine morphology, among others. These features of the syndrome are presumably connected, as dysregulation at the level of neuronal synapses affects cognitive processes; therefore it has been hypothesized that the behavioral, intellectual and dendritic phenotypes could have their roots in synaptic protein abnormalities. AMPARs are the principal glutamatergic receptors in the excitatory synapses. They are tetramers comprised of GluR2 and GluR1 or GluR3 subunits, with GluR1 subunit largely expressed in many brain areas (Hollmann and Heinemann 1994). Till et al. (2012) have found normal levels of AMPA receptor subunits GluR1 and GluR2/3 in homogenate preparation from neocortex of 1-week-old *Fmr1* KO mice, but reduced in SN fraction, whereas at 2 weeks of age only GluR1 subunit was reduced in SNs, while GluR2/3 in homogenate. In our current experiment in SN fraction from frontal cortex of adult *Fmr1* mice, 13-week-old, no statistically significant difference between KO and WT groups could be shown. This outcome is different than what was found by Till et al., but the brain area and age of mice were different in the two experiments, which could account for the lack of differences in protein levels between KO and WT mice in our experiment compared with theirs. Our results are similar to the ones of Giuffrida et al. (2005) who also found normal levels of AMPA receptors in total protein homogenates and synaptic membrane preparations from forebrain of *Fmr1* KO mice.

A study by Zhang et al. (2008) showed that AMPA receptor endocytosis is dependent on striatal-enriched protein tyrosine phosphatase (STEP), located in postsynaptic terminals (Oyama et al. 1995). Goebel-Goody et al. (2012) found a higher level of STEP in hippocampal SNs in *Fmr1* KO mice compared with the WTs, as FMRP is presumed to be a negative regulator of STEP, while we did not find any significant difference in STEP levels between *Fmr1* KO and WT mice in frontal cortex SN fraction.

Several research groups showed alteration in GABAergic pathways in the *Fmr1* KO mice, with reduced $GABA_A$ current in subiculum, reduced expression of $\alpha 5$ and δ subunits in the same area (Curia et al. 2009), decreased level of β subunit in hippocampus of these mice (El Idrissi et al. 2005), but also reduced levels of mRNA have been found in the frontal cortex of *Fmr1* KO mice for 8 out of 18 $GABA_A$

receptor subunits, among which α1, 3 and 4, β 1 and 2, and γ1 and 2 (D'Hulst et al. 2006). Our study failed to find significant difference in protein level expression for the α1 subunit of $GABA_A$ receptor in SN fraction from frontal cortex of *Fmr1* KO and WT mice. Thus, although mRNA level of $GABA_A$ α1 subunit has been shown to be decreased in the frontal cortex of *Fmr1* KO mouse model, the protein expression level seems to be unaltered at the synapses.

The fact that we did not find any alteration in synaptic protein levels tested in the frontal cortex of *Fmr1* KO mice could be also due to the method and protocols we used in this experiment. We followed a synaptoneurosome isolation protocol used previously in the studies of Goebel-Goody et al. (2012) and Till et al. (2012). We tested whether the fraction obtained was enriched in synaptic elements, and established that it was, by both immunoblotting for synapsin and electron-microscopy. We also determined the optimal protein concentration of the SN fraction to be used for immunoblotting. The two studies mentioned above, showing difference in GluR1 and STEP levels between *Fmr1* KO and WT mice, also used Western blot as experimental method, with chemiluminescence as immunodetection method in the study of Goebel-Goody et al. (2012), and Odyssey in the study of Till et al. (2012). We also used the Odyssey system for immunodetection. Although our experiments followed the protocols presented in the two studies, we have not found significant differences between the two experimental groups. When analyzing the data we observed up to 60% variation between values measured for samples coming from different mice with the same genotype, but what was more worrying was the variation measured within one sample, loaded multiple times, between 10 and 50%, depending on the protein tested, whether the same sample was loaded on the same gel or on different gels ran in parallel, and also depending on the loading control. In order to eliminate variability caused by the researcher in loading the samples or performing the protocol we repeated several times the immunoblotting of the same sample multiple times on one gel by different researchers, as this should give the most reliable results. In all trials variability was observed, although loaded aliquots were virtually identical. Therefore we propose that Western blot might not be the most reliable method for determining the significance of small differences (< 30%) in protein levels between groups or that

there is indeed no difference in the studied protein levels in the frontal cortex of *Fmr1* KO and WT groups.

Conclusion

In the present study we investigated whether the synaptic receptor levels are altered in the frontal cortex of *Fmr1* KO mice compared with WT littermates. As this study is the first, to our knowledge, to study the GluR1, STEP/PTPN5 and $GABA_A$ α1 levels in SN from frontal cortex of adult *Fmr1* KO mice, it is difficult to compare our findings to previously existing ones. Thus, it can be that there is indeed no alteration in synaptic protein levels in the frontal cortex of *Fmr1* KO mice, which does not exclude a functional alteration, or that the difference cannot be detected by Western blot following the protocol we used in this experiment. We tested individual and pooled SN samples from the same mice, but we found that the variation between mice, between blots and within a blot between the same sample loaded multiple times is too large to give a statistically significant difference between the experimental groups. Further research is needed by other methods.

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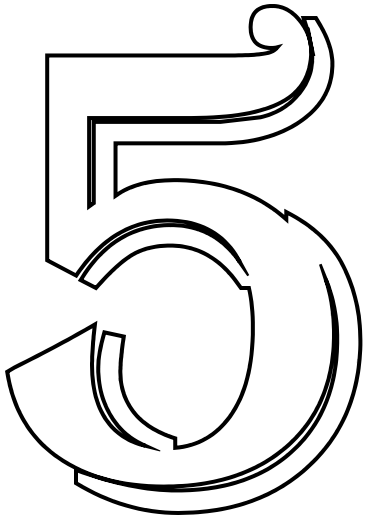


Chronic treatment with minocycline and baclofen fails to show a rescue of sociability deficits in *Fmr1* KO mice

Andreea S Pop, Shimriet Zeidler, Celine EF de Esch, Ronald AM Buijsen, Ingeborg M Nieuwenhuizen, Ilse Gantois, Ben A Oostra and Rob Willemsen

Abstract
One of the leading single-gene causes of intellectual disability and autism spectrum disorders is fragile X syndrome (FXS). Due to silencing of the fragile X mental retardation 1 gene (*FMR1*) and absence of its protein, fragile X mental retardation protein (FMRP), individuals with FXS present with intellectual disability, autistic like behavior and macroorchidism, among other features. In addition, social behavior, known to be disrupted in autistic people, has been found to be impaired in patients with FXS. Impaired social behavior has been also observed in the knockout mouse model for FXS, the *Fmr1* KO mouse. These mice showed increased interaction with a novel mouse than their wild-type littermates do when tested by the automated three chamber test for sociability, although both genotypes prefer socializing with the novel mouse versus a novel object. Rescue of this phenotype was obtained by long-term treatment with an mGluR5 antagonist, AFQ056/Mavoglurant. Therefore, we were interested whether intervention in other cellular pathways known to be affected in FXS would also lead to a rescue of the same sociability phenotype. We administered minocycline, a matrix metalloproteinase-9 inhibitor, and baclofen, a $GABA_B$ agonist, to *Fmr1* KO mice and WT littermates for two weeks, prior to testing them. The results obtained showed no rescuing effect of the treatments on exaggerated sociability in *Fmr1* KO mice.

Keywords: Fragile X syndrome; Social behavior; *Fmr1* KO; FMRP; *FMR1*; Autistic-like behavior; Minocycline; Baclofen; Automated three chamber test;

5.1 Introduction

The leading inherited monogenic cause of intellectual disability and autistic spectrum disorders is the fragile X syndrome (FXS) (Hagerman et al. 2010). The first characteristic described in 1943 by J. Purdon Martin and Julia Bell for what later was named FXS, was the intellectual disability. Later studies added macroorchidism, epileptic seizures, repetitive behavior, hyperactivity, anxiety, irritability, social deficits, language deficits, working and short-term memory problems, deficits in executive function, mathematical and visuo-spatial abilities (Garber et al. 2008, a review). These features seem to be caused by the lack of fragile X mental retardation protein (FMRP), the protein product of the fragile X mental retardation 1 gene (*FMR1*). This gene is located on the X chromosome at position q27.3 (Verkerk et al. 1991), and due to more than 200 CGG repeats on its 5' untranslated region (UTR) the promoter region becomes methylated and silenced (Bell et al. 1991;Oberlé et al. 1991;Yu et al. 1991). As FMRP is an RNA-binding protein, it binds *in vitro* approximately 4% of different types of brain mRNAs, including its own, and controls mRNA translation at the synapse (Willemsen et al. 2011). In this subcellular compartment it acts as a negative regulator of synaptic protein synthesis by associating with the mRNAs of proteins like PSD95, SAPAP1-3, α-CaMKII, Arc/Ar3.1, Shank1 (Brown et al. 2001;Zalfa et al. 2003;Zalfa et al. 2007;Schutt et al. 2009) and many more. Thus, absence of FMRP disrupts local protein synthesis at the synapse, and consequently affects synaptic plasticity.

This effect has been observed in the knockout mouse model for FXS, the *Fmr1* KO mouse, which mimics most of the features of the human syndrome. Studies on this mouse model have shown that lack of Fmrp leads to an increased internalization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), important for synaptic plasticity and functioning. This theory is called the "mGluR theory" (Bear et al. 2004), and it states that activity of group 1 metabotropic glutamate receptors (mGluRs) is increased in FXS which leads to increased protein synthesis in the postsynaptic density, and consequently to permanent internalization of AMPA receptors. Another theory proposed that the inhibitory pathway, coordinated by the γ-aminobutyric acid (GABA) receptors, is also disturbed in FXS (D'Hulst and

Kooy 2007), as many patients manifest epileptic seizures and sleep disorders which are connected to this inhibitory pathway.

A major challenge is to pinpoint which of the characteristics of the FXS are caused by which dysregulation in the normal functioning of specific brain areas, therefore many studies have been conducted, mostly on the *Fmr1* KO mouse model, as this model offers a good experimental substrate. It has been observed that these mice show repetitive behavior (measured mainly by marble burying or grooming), abnormal social behavior (measured by the automated three chamber test, or direct/indirect social interaction tests), audiogenic seizures, anxiety deficiency (in open field or open-arms plus maze, dark-light box), although the results vary depending on the mouse background, age, test, protocol etc. (Peier et al. 2000;Mineur et al. 2002;Nielsen et al. 2002;Restivo et al. 2005;Spencer et al. 2005;Bilousova et al. 2008;Moy et al. 2009; Pietropaolo et al. 2011;Spencer et al. 2011;Michalon et al. 2012). Studies focused on the social behavior of *Fmr1* KO mice demonstrated increased sociability in KO mice (Spencer et al. 2005;Gantois et al. 2013), no difference (McNaughton et al. 2008), or even decreased social interest (Mineur et al. 2002); these results being also influenced by the mouse strain and experimental protocols used.

The goals of the current research are finding the cause of the abnormal phenotypes and developing an effective treatment. Several pharmacological substances have been tested on *Fmr1* KO mice to correct at least in part the behavioral abnormalities. Most common tested drugs include mGluR5 antagonists, such as MPEP, fenobam, AFQ056/Mavoglurant, STX107, RO4917523, CTEP and an mGluR1 antagonist, JNJ16259685. Administration of these drugs showed a reverse of some behavioral phenotypes like abnormal prepulse inhibition (PPI) (De Vrij et al. 2008), decreased anxiety, susceptibility to audiogenic seizures, repetitive-like behavior (Yan et al. 2005;Burket et al. 2011;Thomas et al. 2011b), improvement of motor learning deficiency observed on the Erasmus Ladder (Vinueza Veloz et al. 2012), learning and memory deficits (Michalon et al. 2012) and impaired social behavior (Gantois et al. 2013). From the $GABA_A$ agonists used in *Fmr1* KO mice both diazepam and ganaxolone rescued audiogenic seizures (Heulens et al. 2012), while the $GABA_B$ agonist racemic baclofen can decrease audiogenic seizure susceptibility (Pacey et al. 2009;Henderson et al. 2012), especially through its R-enantiomer, arbaclofen. This substance was found to rescue audiogenic seizures at lower doses than the racemic

baclofen itself, and to reduce repetitive behavior (Henderson et al. 2012) and open-field hyperactivity (Paylor 2008).

Besides the two main pathways involved in FXS, other targets have been investigated. Among these is the matrix metalloproteinase-9 that can influence spine morphology (Ethell and Ethell 2007), thus synaptic plasticity too, and higher levels than normal have been found in *Fmr1* KO mice (Bilousova et al. 2008). In both patients and animal models for FXS an aberrant spine phenotype has been found (Rudelli et al. 1985;Hinton et al. 1991;Irwin et al. 2000;Irwin et al. 2001;Nimchinsky et al. 2001;Irwin et al. 2002;Galvez and Greenough 2005;McKinney et al. 2005; Grossman et al. 2006;Bilousova et al. 2008;Cruz-Martin et al. 2010;Levenga et al. 2011). Bilousova et al. (2008) showed that minocycline, an antibiotic of the tetracycline family, and an MMP-9 inhibitor, is able to rescue the dendritic spine phenotype in the hippocampus of *Fmr1* KO mice, to induce maturation of dendritic spines in hippocampal neuron *in vitro*, and to improve mice performance in a spontaneous alternation task, while another study showed reversal of ultrasonic vocalization production deficits in *Fmr1* KO mice (Rotschafer et al. 2011). Both minocycline and arbaclofen are currently tested in clinical trials. In humans, the results so far have indicated improvements in language, attention, anxiety, social communication (Utari et al. 2010), irritability, Visual Analog Scale for behavior (VAS) (Paribello et al. 2010) following minocycline administration, while a randomized, controlled, phase II trial with arbaclofen (STX209) showed improvements on VAS-rated problem behaviors, on Aberrant Behavior Checklist (ABC)- Social Avoidance, on Vineland-Socialization measure of adaptive function and on multiple global assessments in more severely affected patients with FXS (Berry-Kravis et al. 2012).

Given that both minocycline and arbaclofen showed improvements in some aspects of the FXS phenotype both in human and in mice, we were interested to what extent these two substances would rescue the exaggerated sociability phenotype in *Fmr1* KO mice. As racemic baclofen is commercially available and at a lower price than arbaclofen, its R-enantiomer, and has also been proven to have therapeutic effect in *Fmr1* KO mice, we chose racemic baclofen for our study. Therefore, we administered for two weeks minocycline and racemic baclofen (separately) in drinking water to *Fmr1* KO and WT mice before testing them for sociability in the automated

three chamber test. Social interaction between the test mouse and another novel mouse, and between the test mouse and an empty cage were measured as sniffing time. The results obtained indicated no significant rescuing effect of the treatment for both drugs on the sociability of *Fmr1* KO mice. This outcome could be due to the dosage used, but it can also mean that sociability in *Fmr1* KO mice is not influenced by either $GABA_B$ or MMP-9 pathways.

5.2 Materials and Methods

5.2.1 Animals

In our study only male mice were used, *Fmr1* KO and wild-type (WT) littermates of 12- 13 weeks of age. *Fmr1* KO mice were generated in our lab as previously described and named *Fmr1* KO(2) (Mientjes et al. 2006). The line was completely backcrossed (>10X) in the C57BL/6J background. Mice were housed under standard conditions, 12 hours dark-light cycle, 2-4 per cage, with food and water *ad libitum*. Mice were transferred to the behavioral animal facility at the age of 8-9 weeks and tested around 12-13 weeks of age. Two weeks prior to testing each mouse was handled by the experimenter 3 times a week, for eliminating handling stress during the behavioral experiments. Experiments were performed during light phase (between 9AM and 2PM) and animals were always transported to the experimental room at least 30 min before the start of the experiment to acclimate. The stranger mice were age-matched males of a C57BL/6J background (Elevage Janvier, Le-Genest-Saint-Isle, France). All experiments were carried out with the permission of the local ethics committee. The experimenter was blind for genotype and treatment.

The experiment consisted of three main parts: control, minocycline and baclofen experiments. Each of the three experiments was performed separately. For the control experiment 17 *Fmr1* KO and 19 WT littermates were used. For the minocycline experiment two groups of *Fmr1* KO mice and two of WT mice were used, one of each receiving only vehicle, in this case sucrose water to compensate for the bitter taste of the drug (*Fmr1* KO: N= 11, WT: N= 10), the other one minocycline in sucrose water (*Fmr1* KO: N= 11, WT: N= 10). For the baclofen experiment also four groups of mice were used: one group of *Fmr1* KO (N= 13) and one of WT (N=

13) received vehicle, aspartame is this case, while one group of *Fmr1* (N= 12) and one of WT (N= 11) received baclofen in aspartame water.

5.2.2 Sociability test

We used a modified version of the automated three chamber test described in Gantois et al. (2013) and Nadler et al. (2004). In short, the setup was made of transparent Plexiglass and consisted of three chambers: a central chamber, a left and a right chamber, separated by sliding doors. Left and right chamber contained cylindrical wire cups that could accommodate stranger mice (Figure 5.1). Two cameras mounted above the setup transferred images to a computer with ANY-maze™ Video Tracking System software (Stoelting Co., IL, USA). Testing comprised of two phases: acclimation phase and sociability phase. During the acclimation assay, the test mouse was placed in the central compartment for 5 min without access to the left and right chambers. During the sociability assay, a stranger mouse (STR) was placed into the wire cup randomly in either the left or right chamber. The other chamber contained an empty wire cup. The sliding doors were opened and the test mouse could freely explore all three chambers. After 5 min, the test mouse was guided into the central compartment and sliding doors were closed. Preferential exploration of STR over the empty cup were recorded and analyzed with ANY-maze™ Video Tracking System software (Stoelting Co., IL, USA). Time spent in each chamber, number of entries to each room and distance traveled were automatically recorded. Manually scored behavior (time spent sniffing the STR or the empty wire cup) was defined as the time the test animal was in direct nose contact with the wire cups, and was scored in a blinded manner. The stranger mice were never in contact with the test mice prior to testing day. Each stranger mouse was used maximum 2 times a day.

5.2.3 Drug treatment

Commercially available Minocycline (Pharmachemie BV, Haarlem, the Netherlands) was administered in 5% sucrose water at a concentration of 0.2 mg/ml. An adult mouse of about 30 g drinks around 5 ml/day, which results in a daily dose of approximately 30 mg/kg/day. This dose has been previously shown to cross the blood-brain barrier (Lee et al. 2006) and to rescue abnormal dendritic spine morphology, reduce anxiety, improve some behavioral performances in *Fmr1* KO mice (Bilousova

et al. 2008) and correct ultrasonic vocalization production deficit (Rotschafer et al. 2011). Drinking water was kept in dark bottles and refreshed 3 times a week. Drinking water uptake was similar between cages and treatments.

Racemic baclofen (Spruyt-Hillen, Ijsselstein, the Netherlands) was added to 0.1% aspartame water in a concentration of 1 mg/ml, leading to approximately 150 mg/kg/day. It has previously been shown that baclofen crosses the blood-brain barrier (van Bree et al. 1991), and that a dose of 0.5 mg/ml of arbaclofen in drinking water gives better plasma and brain concentration values than administering 6 mg/kg/day intraperitoneal (Henderson et al. 2012). As arbaclofen reduces audiogenic seizures at half a dose of racemic baclofen (Henderson et al. 2012), we decided to use a concentration of 1 mg/ml racemic baclofen in drinking water in our chronic treatment study. Drinking water was kept in dark bottles and refreshed daily.

5.2.4 Statistical analysis

Data was analyzed with SPSS (IBM, NY, USA) for Windows statistical software. Overall effects were evaluated with Repeated Measures Analysis of variance (RM ANOVA) with genotype (KO and WT) and/or treatment (no treatment, sucrose, minocycline, aspartame and baclofen) as between-subjects factors and stranger side (empty and STR) as within-subjects factor. Tukey's test (α-level set at 0.05) was used for *post hoc* evaluation. Within the same treatment data was analyzed with Two-Way ANOVA. Data are represented as mean ± standard error of the mean (S.E.M.).

Figure 5.1 The automated three chamber test set-up. A Plexiglass box consisting of 3 chambers: a central chamber, a left and a right chamber, separated by sliding doors. Left and right chambers contained cylindrical wire cages that can house a stranger mouse.

5.3 Results

The scope of the current study was to investigate whether long-term treatment with minocycline and baclofen could rescue impaired social behavior of *Fmr1* KO mice. Therefore, we conducted three experiments: a control experiment to establish the sociability phenotype of *Fmr1* KO mice, a minocycline experiment comparing treated mice with mice receiving vehicle (sucrose), and a baclofen experiment comparing treated mice with mice receiving another vehicle (aspartame). Five *Fmr1* KO groups and five WT littermates groups were used in the five experimental conditions: control: *Fmr1* $KO_{control\ (c)}$: N= 17, WT_c: N= 19; sucrose: *Fmr1* $KO_{sucrose\ (s)}$: N= 11, WT_s: N= 10; minocycline: *Fmr1* $KO_{minocycline\ (m)}$: N= 11, WT_m: N= 10; aspartame *Fmr1* $KO_{aspartame\ (a)}$: N= 13, WT_a: N= 13; and baclofen: *Fmr1* $KO_{baclofen\ (b)}$: N= 12; WT_b: N= 11.

5.3.1 Sniffing time

Time spent by the test mouse in nose contact with the empty wire cage or the stranger (STR) mouse was manually recorded by the experimenter and expressed as sniffing time.

Control experiment. For the control experiment both groups sniffed more the new mouse than the empty cage ($P< 0.001$), and *Fmr1* KO_c mice spent significantly more time interacting with the STR mouse compared with WT_c (*Fmr1* KO_c= 58.324s ± 3716, WT_c= 42.137s ± 3.515, P= 0.002). A significant effect of stranger side (T-W ANOVA, F(1,68)= 81.698, $P< 0.001$) and of genotype*stranger side interaction were found (T-W ANOVA, F(1,68)= 8.581, P= 0.005) (Figure 5.2A).

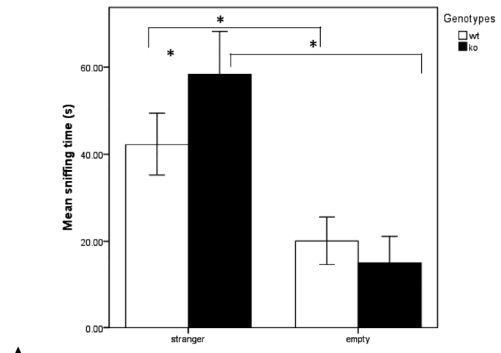
Sucrose water and aspartame water groups were taken as controls for the treated groups (minocycline and baclofen respectively), therefore the effect of the sweetener on sniffing time was tested. An effect of genotype only was found for control versus sucrose groups comparison (RM ANOVA, F(1,53)= 7.333, P= 0.009). In the case of aspartame versus control groups significant effect of genotype only was found (RM ANOVA, F(1,58)= 12.891, P= 0.001).

Minocycline treatment. Sucrose was used as sweetener for the minocycline drinking water, as the drug has a bitter taste. If we compare minocycline treatment to its

control, sucrose, no significant effects of minocycline treatment or genotype were found. In the minocycline, like in the sucrose condition both genotypes spent more time sniffing STR mouse than empty cage (for *Fmr1* KO_m P= 0.025, for WT_m P< 0.001, for *Fmr1* KO_s P< 0.001, for WT_s P< 0.001), without a significant difference between *Fmr1* KO and WT groups for sniffing time STR mouse (*Fmr1* KO_m= 41.200s ± 4.703, WT_m= 46.600s ± 4.932; *Fmr1* KO_s= 52.418s ± 4.489, WT_s= 51.520s ± 4.280). An effect of the stranger side on sniffing time was found in both minocycline and sucrose conditions (T-W ANOVA, F(1,38)= 71.979, P< 0.001 for sucrose; F(1,38)= 25.392, P< 0.001 for minocycline) (Figure 5.2B).

Baclofen treatment. Aspartame was used as a sweetener for the baclofen water as the latter has a bitter taste, thus baclofen treated groups were compared with their controls, aspartame groups. The effect of genotype was significant (RM ANOVA, F(1,45)= 5.521, P= 0.023), but no effect of the baclofen treatment on sniffing time was found (Figure 5.2C). All four groups (*Fmr1* KO_b, WT_b, *Fmr1* KO_a and $WT_{a)}$ spent more time sniffing STR mouse than empty cage (P< 0.001), without a significant difference between genotypes in sniffing time STR mouse for the baclofen treated groups (*Fmr1* KO_b= 56.375s ± 3.506, WT_b= 51.700s ± 3.662, P> 0.1), whereas for the aspartame groups a higher sniffing time STR mouse was found in *Fmr1* KO_a mice compared with WT_a mice (*Fmr1* KO_a= 57.862s ± 2.640, WT_a= 42.208s ± 2.640, P< 0.001). A significant effect of genotype, stranger side and interaction genotype*stranger on sniffing time to each stimulus was found for the aspartame groups (T-W ANOVA, F(1,48)= 5.139, P= 0.028 for genotype; F(1,48)= 136.307, P< 0.001 for stranger side; F(1,48)= 13.414, P= 0.001 for genotype*stranger), but only an effect of the stranger side for the baclofen treated groups (F(1,42)= 123.414, P< 0.001).

Repeated Measures ANOVA on all groups indicated an overall effect of genotype on sniffing time (F(1,117)= 13.118, P< 0.001), but no effect of treatment, or genotype*treatment interaction was found. When comparing effect of treatment on sniffing time per genotype no significant effect of treatment was seen in any of the two genotypes. Comparing all experimental conditions per genotype for sniffing time no significant difference was obtained for either side in any of the two genotypes (Figure 5.5A).



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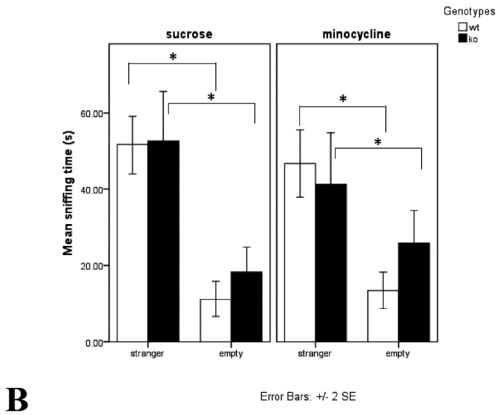

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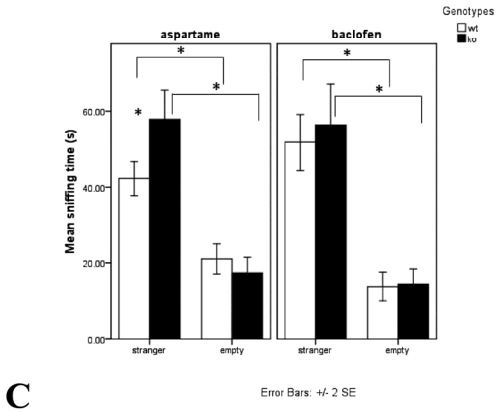

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Figure 5.2 Sniffing time. A) *Control experiment*. Both *Fmr1* KO and WT groups spent significantly more time sniffing the novel mouse than the empty cage (P< 0.001), and *Fmr1* KO mice showed an increased sniffing time of the new mouse compared with WT mice (*Fmr1* KO_c= 58.324s ± 3716, WT_c= 42.137s ± 3.515, P= 0.002). B) *Minocycline experiment*. All four groups showed preference for sniffing the new mouse than the empty cage (P< 0.001 for both sucrose groups and for minocycline treated WT group, and P= 0.025 for minocycline treated *Fmr1* KO). No difference between *Fmr1* KO and WT in time sniffing either the new mouse or the empty cage has been found in either condition (*Fmr1* KO_s= 52.418s ± 4.489, WT_s= 51.520s ± 4.280, P> 0.05; *Fmr1* KO_m= 41.200s ± 4.703, WT_m= 46.600s ± 4.932, P> 0.05). C) *Baclofen experiment*. All four groups spent more time sniffing the novel mouse than the empty chamber (P< 0.001), but only in the aspartame condition *Fmr1* KO mice showed an increased sociability compared with WT mice, as sniffing time of the new mouse (*Fmr1* KO_a= 57.862s ± 2.640, WT_a= 42.208s ± 2.640, P< 0.001).

5.3.2 Time in chamber

Sniffing time is considered to be a more reliable measure for sociability, but time spent in chamber with a new mouse or with empty cage is also considered an indicator for sociability. Therefore we also quantified and analyzed time spent by mice in the two side chambers.

Control experiment. When we compared time in chamber for the control condition, we found that the time spent by both genotypes in the chamber with STR mouse was significantly higher than the time spent in the chamber with the empty cage (for *Fmr1* KO_c $P< 0.001$; for WT_c $P= 0.001$), and no significant difference was found between the two genotypes for time in chamber with STR mouse (*Fmr1* KO_c= 170.212s ±12.366, WT_c= 148.389s ± 11.697). Only an effect of stranger side on time in chamber was found (T-W ANOVA, $F(1,68)= 35.346$, $P< 0.001$) (Figure 5.3A).

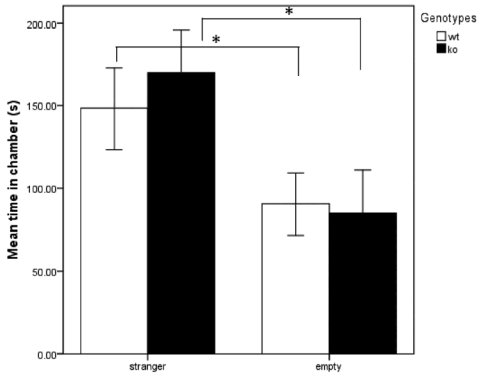
A comparison between control and sucrose groups showed no significant effects of genotype or treatment. For the control and aspartame conditions, the comparison showed only a significant effect of genotype on time in chamber (RM ANOVA, $F(1,58)= 6.430$, $P= 0.014$).

Minoycline treatment. In order to see if minocycline had an effect on time in chamber we compared the minocycline groups with their controls, the sucrose groups. RM ANOVA revealed a significant effect of genotype only on time in chamber between the two conditions ($F(1,38)= 5.271$, $P= 0.027$), without an effect of minocycline treatment. Within the minocycline treated groups only WT_m mice spent significantly more time in the chamber with STR mouse ($P= 0.003$), while *Fmr1* KO_m mice showed no preference for the company of another mouse or the empty cage. This led to a larger amount of time spent by *Fmr1* KO_m mice in the empty chamber compared with WT_m mice (*Fmr1* KO_m= 133.273s ± 14.488, WT_m= 83.900s ± 15.196, $P= 0.024$), whereas for the time in STR chamber no difference between the two genotypes was found. For the minocycline treatment we found only a significant effect of genotype* stranger interaction on time in chamber (T-W ANOVA, $F(1,38)= 6.921$, $P= 0.012$), while stranger side effect approached significance ($F(1,38)= 3.634$, $P= 0.064$) (Figure 5.4B). If we looked at the sucrose groups, both *Fmr1* KO_s and WT_s spent significantly more time in the chamber with STR mouse than in the chamber with the empty cage

(P= 0.002 for *Fmr1* KO_s, and P< 0.001 for WT_s), the difference between the two genotypes for time in chamber of STR mouse not being significant (*Fmr1* KO_s= 154.191s ± 13.132, WT_s= 174.890s ± 13.773). A significant effect of stranger side on time in chamber was found for the sucrose groups (T-W ANOVA, F(1,38)= 39.701, P< 0.001) (Figure 5.3B).

Baclofen treatment. To study the effect of baclofen treatment on *Fmr1* KO and WT mice, we compared the results from these groups with the ones from their controls, the aspartame groups. No effect of baclofen treatment on time in chamber was found. When comparing the time spent in each side chamber, we found a significantly larger amount of time in STR chamber compared with empty chamber in all four groups (P< 0.001), with no difference between genotypes in time spent in one or in the other chamber for the baclofen treated groups (Figure 5.4C), whereas *Fmr1* KO_a mice spent significantly more time than WT_a mice in the chamber with the new mouse (*Fmr1* KO_a= 167.892s ± 7.225, WT_a= 143.662s ± 7.225, P= 0.022) (Figure 5.3C). Only stranger side had a significant effect on time in chamber in baclofen treated groups (T-W ANOVA, F(1,42)= 86.628, P< 0.001). A significant effect of stranger side and of genotype*stranger interaction on time in chamber was found for the aspartame groups (T-W ANOVA, F(1,48)= 84.415, P< 0.001 for stranger; F(1,48)= 6.169, P= 0.017 for genotype*stranger).

RM ANOVA on all groups indicated an overall effect of genotype on time in chamber (F(1,117)= 10.530, P= 0.002), but no effect of treatment or genotype* treatment interaction was found. When comparing effect of treatment on time in chamber per genotype no significance was seen in the WT or *Fmr1* KO groups. Comparing all experimental conditions per genotype for time in chamber no significant difference was obtained for either side in WT groups (Turkey's *post hoc*: P> 0.1), whereas for *Fmr1* KO groups significant difference was only found between control and minocycline (Turkey's *post hoc*: P= 0.042), minocycline and baclofen (Turkey's *post hoc*: P= 0.017) for the empty side; the difference between minocycline and aspartame approached significance (Turkey's *post hoc*: P= 0.052) (Figure 5.5B).



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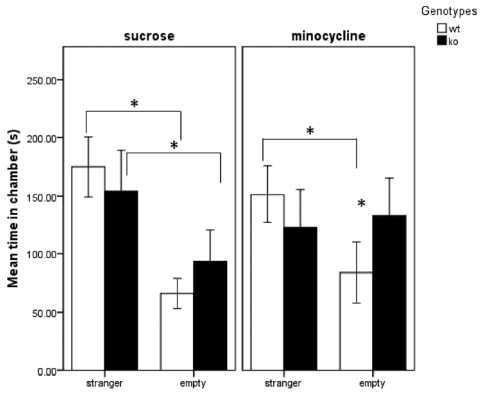

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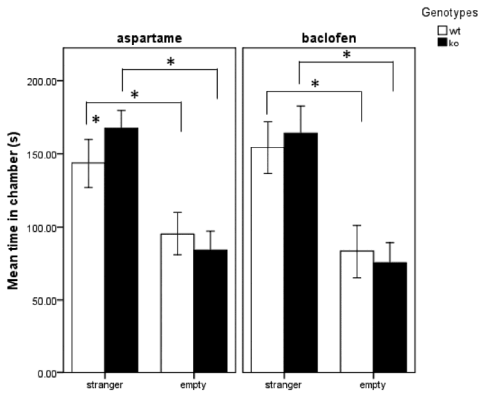

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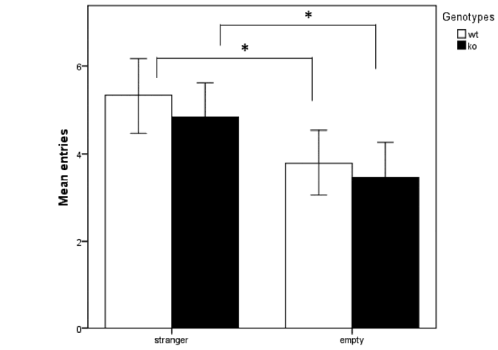
Figure 5.3 Time in chamber. A) *Control experiment*. The time spent by both genotypes in the chamber with STR mouse was significantly higher than the time spent in the chamber with the empty cage (*Fmr1* KO_c P< 0.001; WT_c P= 0.001), and no significant difference was found between the two genotypes for time in either chamber. B) *Minocycline experiment*. For the minocycline treatment we found only the WT group showing preference for spending time in the chamber with STR mouse versus the empty chamber (P= 0.003), but *Fmr1* KO mice spent more time in the empty chamber than WT mice did (*Fmr1* KO_m= 133.273s ± 14.488, WT_m= 83.900s ± 15.196, P= 0.024). Both sucrose *Fmr1* KO and WT groups spent significantly more time in the chamber with STR mouse than in the chamber with the empty cage (*Fmr1* KO P= 0.002, WT P< 0.001), without a significant difference between genotypes. C) *Baclofen experiment*. In the baclofen treated groups we found a significantly larger amount of time spent in STR chamber compared with empty chamber in both groups (P< 0.001), without any difference between genotypes in time spent in one or the other chamber. For the aspartame groups the difference between time spent in STR chamber and time in empty chamber was significant for both genotypes (P< 0.001), and aspartame *Fmr1* KO mice spent significantly more time than WT mice in the chamber with STR mouse (*Fmr1* KO_a= 167.892s ± 7.225, WT_a= 143.662s ± 7.225, P= 0.022).



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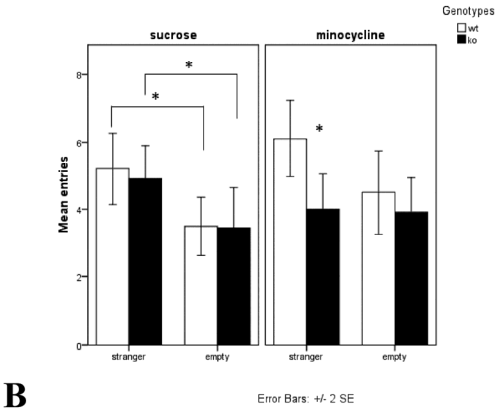

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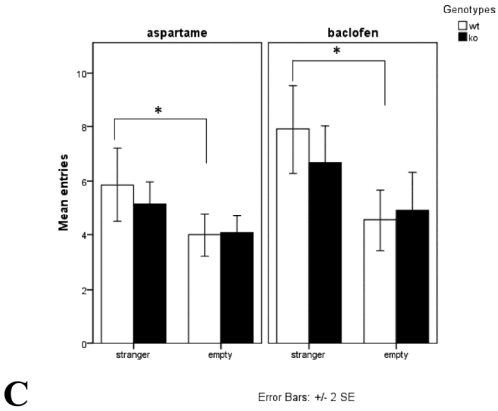


C

Figure 5.4 Number of entries. A) *Control experiment*. Both genotypes entered significantly more into STR chamber compared with the empty chamber (*Fmr1* KO P= 0.022; WT P= 0.007), but without differences between genotypes for each chamber. B) *Minocycline experiment*. None of the two minocycline treated groups showed a preference for entering into one side chamber or the other, but a significant difference was found between *Fmr1* KO and WT in number of entries to STR chamber (*Fmr1* KO_m= 4 ± 0.541 versus WT_m= 6.1 ± 0.567, T-W ANOVA, P= 0.011). As for the sucrose treated groups both genotypes entered more into STR chamber than into the empty chamber (*Fmr1* KO_s P= 0.05; WT_s P= 0.03). No difference between genotypes in entries to one chamber was found. C) *Baclofen experiment*. For the baclofen treated mice only WT group entered significantly more times into STR chamber than into the empty chamber (P= 0.002), without any difference between genotypes. The same was seen in the aspartame condition where only WT mice entered more into STR chamber (P= 0.007), without a significant difference between genotypes for either one of the chambers.

5.3.3 Number of entries

The number of entries to each room is an indicator for general activity.

Control experiment. Both genotypes entered significantly more into the STR chamber compared with the empty chamber (*Fmr1* KO_c: P= 0.022; WT_c: P= 0.007), but without differences between genotypes for each chamber. Statistical analysis of the number of entries to each side chamber in the control condition revealed only a significant effect of stranger side on number of entries (T-W ANOVA, F(1,68)= 13.174, P= 0.001) (Figure 5.4A). In order to establish whether sucrose and aspartame were comparable to control values we performed RM ANOVA on these groups. No significant effect on number of entries for control versus sucrose groups, or for aspartame versus control was found.

Minocycline treatment. In order to investigate the effect of minocycline on number of entries to the side chambers we compared the minocycline groups and the sucrose groups. RM ANOVA revealed no significant effect of minocycline treatment. For the minocycline treated groups only, we found a significant effect of genotype (T-WANOVA, F(1,38)= 5.982, P= 0.020), with none of the two genotypes showing a significant preference for entering into one chamber or the other, although for WT_m group the difference between entries to the two chambers approached significance (P= 0.053). This also contributed to the significant difference found between *Fmr1* KO_m and WT_m in number of entries into STR chamber (*Fmr1* KO_m= 4 ± 0.541 versus WT_m= 6.1 ± 0.567, P= 0.011) (Figure 5.4B). If we looked at the sucrose groups, both genotypes entered more into STR chamber than into the empty chamber (*Fmr1* KO_s: P= 0.05; WT_s: P= 0.03). No difference between genotypes in entries to one chamber was found. An effect of stranger side on number of entries was found for sucrose groups (T-W ANOVA, F(1,38)= 9.193, P= 0.004) (Figure 5.5B).

Baclofen treatment. Next we compared the mean number of entries to each side chamber between the baclofen and aspartame groups, in order to establish the effect of baclofen treatment on the two genotypes. RM ANOVA on the two conditions found significant effect of treatment on number of entries (F(1,45)= 6.053, P= 0.018). Only WT_b mice entered significantly more time in the STR chamber than in the empty chamber (P= 0.002), whereas *Fmr1* KO_b did not show a preference for one side or the other. Even so there was no significant difference found between the genotypes in number of entries to one chamber or the other (Figure 5.5C), but a significant effect of

stranger side on number of entries was found (T-W ANOVA, F(1,42)= 13.513, P= 0.001). In the case of the aspartame groups, we obtained a significant preference for entries into STR chamber only for WT_a (P= 0.007), and not for *Fmr1* KO_a mice. No difference between the two genotypes was found in number of entries to each chamber (Figure 5.4C). A significant effect of stranger side on entries was found for the aspartame groups (T-W ANOVA, F(1,48)= 9.779, P= 0.003).

RM ANOVA on all groups indicated an overall effect of treatment on the number of entries (F(4,117)= 4.913, P= 0.001), and effect of genotype alone approached significance (F(1,117)= 3.584, P= 0.061). When comparing effect of treatment on number of entries per genotype, for both *Fmr1* KO and WT groups a significant between-subjects effect of treatment was found (RM ANOVA, F(4,58)= 2.553, P= 0.048 for *Fmr1* KO; F(4,58)= 2.902, P= 0.029 for WT). Comparing all experimental conditions per genotype for entry number the only significant difference in WT groups was obtained between control and baclofen groups (Turkey's *post hoc*: P= 0.017), and between sucrose and baclofen (Turkey's *post hoc*: P= 0.038) for entries to the STR side, whereas for *Fmr1* KO groups significant difference was found only between minocycline and baclofen (Turkey's *post hoc*: P= 0.005) for the STR side. The difference between control and baclofen for entries to the STR chamber approached significance (Turkey's *post hoc*: P= 0.056) (Figure 5.5C).

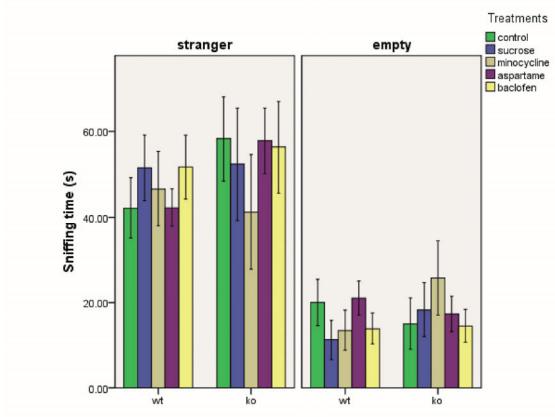
5.3.4 Distance traveled

The distance traveled during the two phases was considered an indicator of exploratory and locomotor behavior, therefore we compared this factor between the two experimental groups and between experimental conditions, to see also if the treatments influenced these behaviors.

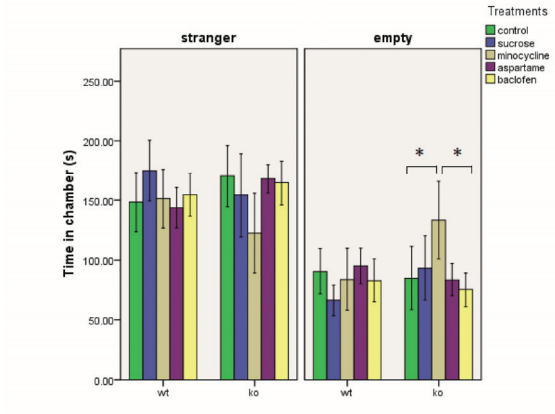
Acclimation phase. During acclimation phase (Figure 5.6A), the distance traveled was significantly influenced by treatment alone (T-W ANOVA, F(4,117)= 8.916, P< 0.001). Within the *Fmr1* KO groups treatment had a significant effect on distanced traveled (T-W ANOVA, F(4,59)= 3.280, P= 0.017), with statistically significant increase in the baclofen treated group compared with minocycline and aspartame conditions (Turkey's *post hoc*: *Fmr1* KO_b= 14.424m ± 1.100 versus *Fmr1* KO_m= 9.407m ± 1.149, P= 0.021; *Fmr1* KO_b= 14.424m ± 1.100 versus *Fmr1* KO_a= 9.920m ±

1.057, P= 0.035). For the WT groups treatment also significantly influenced distance traveled during acclimation phase (T-W ANOVA, F(4,58)= 6.403, P< 0.001), with the baclofen treated group travelling significantly more than all other WT groups (Turkey's *post hoc*: WT_b= 15.889m ± 1.266 versus WT_c= 10.352m ± 0.963, P= 0.008; WT_b= 15.889m ± 1.266 versus WT_s= 7.965m ± 1.328, P= 0.001; WT_b= 15.889m ± 1.266 versus WT_m= 8.877m ± 1.328, P= 0.003; WT_b= 15.889m ± 1.266 versus WT_a= 8.696m ± 1.165, P= 0.001). When comparing *Fmr1* KO and WT groups for each experiment we found a significant difference only between the two groups receiving only sucrose water, with the *Fmr1* KO mice travelling significantly more compared with the WT sucrose group (*Fmr1* KO_s= 11.748m ± 1.258 versus WT_s= 7.965m ± 1.354, P= 0.025). No significant difference between the two genotypes in the other experimental conditions was found.

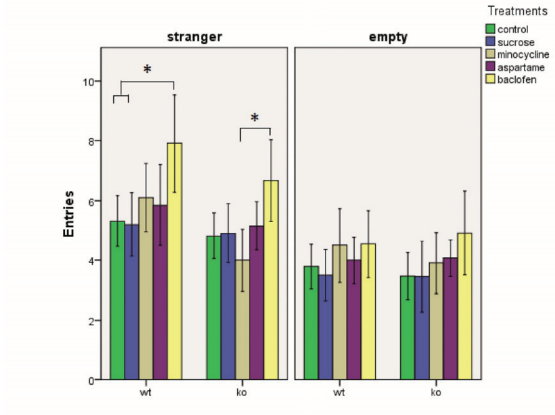
Sociability phase. We also calculated the distance traveled during the sociability phase (Figure 5.6B) and we obtained a significant effect of the treatment (T-W ANOVA, F(4,117)= 10.184, P< 0.001). When comparing between treatments within the same genotype, we found that treatment influenced distance traveled during the sociability phase in both genotypes (T-W ANOVA, F(4,59)= 4.192, P= 0.005 for *Fmr1* KO, and F(4,58)= 7.669, P< 0.001 for WT), again with baclofen treated WT groups differing significantly from the other conditions (Turkey's *post hoc*: WT_b= 26.725m ± 1.314 versus WT_c= 18.618m ± 1.099, P< 0.001; WT_b= 26.725m ± 1.314m versus WT_s= 18.103m ± 1.515, P< 0.001; WT_b= 26.725m ± 1.314 versus WT_m= 18.894m ± 1.515, P= 0.001; WT_b= 26.725m ± 1.314 versus WT_a= 19.507m ± 1.329, P= 0.001), while in the case of *Fmr1* KO groups the baclofen treated group only differed from the sucrose and minocycline groups (Turkey's *post hoc*: *Fmr1* KO_b= 25.824m ± 1.331 versus *Fmr1* KO_m= 18.705m ± 1.391, P= 0.004; *Fmr1* KO_b= 25.824m ± 1.331 versus *Fmr1* KO_s= 20.243m ± 1.391, P= 0.040). Comparing the distance traveled during sociability phase for each condition between the genotypes, we found significant differences between *Fmr1* KO and WT mice only in the control groups (*Fmr1* KO_c= 23.059m ± 1.119 versus WT_c= 18.618m ± 1.092, P= 0.011), while in other conditions the mice belonging to the two genotypes traveled similar distances.



A



B



C

Figure 5.5 Overview of the experiment results. A) *Sniffing time in all groups, on both stranger mouse and empty cage.* A between-subjects effect of genotype (RM ANOVA, $F(1,117)= 13.118$, $P< 0.001$) was found. No significant difference between the five conditions was observed in sniffing time in both genotypes, on either stimulus. B) *Time in each side chamber in all groups.* A between-subjects effect of genotype (RM ANOVA, $F(1,117)= 10.530$, $P= 0.002$) was found. Although no effect of the treatment on time in chamber was observed for the *Fmr1* KO groups, a comparison between treatments within the genotype showed that the minocycline treated *Fmr1* KO spent significantly more time in the empty chamber than the control and baclofen treated *Fmr1* KO mice (Turkey's *post hoc*, $P= 0.042$ and $P= 0.017$, respectively). C) *Number of entries to each side chamber in all experiments.* An overall between-subject effect of treatment on number of entries was found (RM ANOVA, $F(4,117)= 4.916$, $P= 0.001$). For both genotypes a significant between-subjects effect of treatment was found (RM ANOVA, $F(4,58)= 2.553$, $P= 0.048$ for *Fmr1* KO; $F(4,58)= 2.902$, $P= 0.029$ for WT). A significant difference within the WT genotype was obtained between control and baclofen groups (*Turkey's post hoc*, $P= 0.017$), and between sucrose and baclofen (*Turkey's post hoc*, $P= 0.038$) for entries to the STR side, whereas for *Fmr1* KO groups significant difference was found only between minocycline and baclofen (*Turkey's post hoc*, $P= 0.005$) for the STR side.

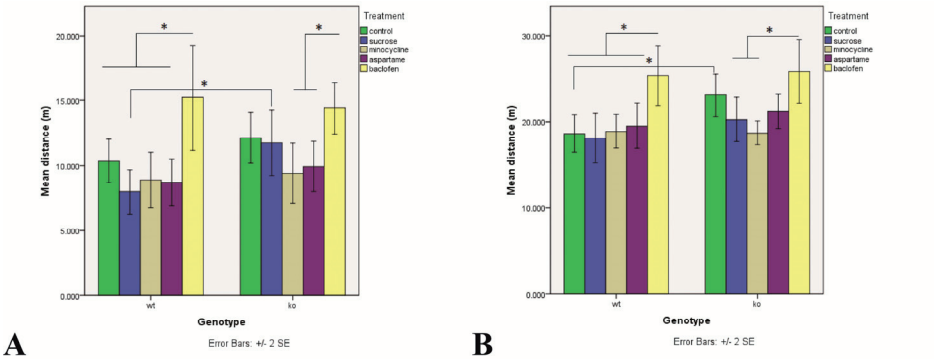


Figure 5.6 Distance traveled. A) *Acclimation phase*. An overall effect of treatment (T-W ANOVA, F(4,117)= 8.916, P< 0.001) on distanced traveled during acclimation phase was found. Between the *Fmr1* KO groups treatment had a significant effect on distanced traveled (T-W ANOVA, F(4,59)= 3.280, P= 0.017), with statistically significant increase in the baclofen treated group compared with minocycline and aspartame conditions (Turkey's *posthoc*: *Fmr1* KO_b= 14.424m ± 1.100 versus *Fmr1* KO_m= 9.407m ± 1.149, P= 0.021; *Fmr1* KO_b= 14.424m ± 1.100 versus *Fmr1* KO_a= 9.920m ± 1.057, P= 0.035). Between the WT groups treatment also significantly influenced distance traveled (T-W ANOVA, F(4,58)= 6.403, P< 0.001), with the baclofen treated group travelling significantly more than all other WT groups (Turkey's *posthoc*: WT_b= 15.889m ± 1.266 versus WT_c= 10.352m ± 0.963, P= 0.008; WT_b= 15.889m ± 1.266 versus WT_s= 7.965m ± 1.328, P= 0.001; WT_b= 15.889m ± 1.266 versus WT_m= 8.877m ± 1.328, P= 0.003; WT_b= 15.889m ± 1.266 versus WT_a= 8.696m ± 1.165, P= 0.001). Only between *Fmr1* KO mice and WT mice receiving sucrose a significant difference in distance traveled was found (*Fmr1* KO_s= 11.748m ± 1.258 versus WT_s= 7.965m ± 1.354, P= 0.025). B) *Sociability phase*. An overall effect of treatment was found significant (T-W ANOVA, F(4,117)= 10.184, P< 0.001). In both genotypes treatment influenced the distance traveled (T-W ANOVA, F(4,59)= 4.192, P= 0.005 for *Fmr1* KO, and F(4,58)= 7.669, P< 0.001 for WT), with baclofen treated WT groups differing significantly from the other conditions (Turkey's *posthoc*: WT_b= 26.725m ± 1.314 versus WT_c= 18.618m ± 1.099, P< 0.001; WT_b= 26.725m ± 1.314m versus WT_s= 18.103m ± 1.515, P< 0.001; WT_b= 26.725m ± 1.314 versus WT_m= 18.894m ± 1.515, P= 0.001; WT_b= 26.725m ± 1.314 versus WT_a= 19.507m ± 1.329, P= 0.001). Between the *Fmr1* KO groups the baclofen treated differed only from the sucrose and minocycline groups (Turkey's *posthoc*: *Fmr1* KO_b= 25.824m ± 1.331 versus *Fmr1* KO_s= 20.243m ± 1.391, P= 0.040; *Fmr1* KO_b= 25.824m ± 1.331 versus *Fmr1* KO_m= 18.705m ± 1.391, P= 0.004). Within treatments we found significant differences between *Fmr1* KO and WT mice only in the control groups (*Fmr1* KO_c= 23.059m ± 1.119 versus WT_c= 18.618m ± 1.092, P= 0.011).

5.4 Discussion

One of the core features of autism and therefore also FXS is abnormal social behavior, but unlike in ASD where individuals show social withdrawal, patients with FXS, especially young children, seek more social contact and show interest in communication (Hall et al. 2010;Tranfaglia 2011). Gantois et al. (2013) found a similar phenotype in *Fmr1* KO mice which showed increased sociability in comparison with WT littermates; this could be rescued by AFQ056/Mavoglurant, an mGluR5 antagonist. The current study investigates whether the same abnormal social behavior of *Fmr1* KO mice can be rescued by other drugs, acting on different molecular pathways: minocycline, an MMP-9 inhibitor, and baclofen, a $GABA_B$

agonist. To our knowledge this study is the first to investigate the effect of long-term treatment with these two drugs on sociability in *Fmr1* KO mice. Unfortunately, we did not find any significant rescuing effect of these substances on *Fmr1* KO mice aberrant sociability phenotype, tested by the automated three chamber test. This result could be due to the dose we used, or it could mean that $GABA_B$ and MMP-9 are not involved in this type of social behavior deficits in the *Fmr1* KO mouse model. The overall interaction of side (STR/empty), genotype and treatment was significant for sniffing time, meaning that the results we obtained for both sides of the setup were dependent on the genotype and the treatment condition. But, as there was no effect of treatment we cannot state that the treatments we used influenced sociability as sniffing time of a new mouse. The situation for time in chamber is somehow similar: no overall effect of interaction between side, genotype, and/or treatment was found, but we did find an effect of treatment on time in side chambers in *Fmr1* KO mice, as the *post hoc* test revealed significant differences between minocycline treated and control-untreated *Fmr1* KO mice, and between minocycline and baclofen treated KO mice. But these comparisons were not forming the scope of our study. The locomotor and exploratory behavior and activity, measured by entry number and distance traveled, appeared to be affected by treatment in both genotypes, and *post hoc* analysis revealed that baclofen treatment significantly affected these parameters.

In our study we looked only at the sociability phase of the automated three chamber test, also known as the Sociability and Preference for Social Novelty (SPSN) test, as lack of preference for a conspecific versus an object is a core feature of autism (Kanner 1968). Moreover, the preference for social novelty in C57Bl/6J mice is not as strong as sociability, as in some studies these mice show preference for a novel mouse versus a familiar one (Moy et al. 2004;Moy et al. 2007;Moy et al. 2009;Pietropaolo et al. 2011), while in another study not (Pearson et al. 2010). We also used only a 5 min protocol for the sociability phase, as the more significant behavior can be observed at the beginning of the interaction phase, when the new stimulus raises more interest, whereas as time goes by the interest lowers and habituation takes place. This could be seen in the study of Gantois et al. (2013), where the biggest difference between untreated *Fmr1* KO and WT mice took place in the first 4-6 min. Therefore, we decided that testing mice for only 5 min would give a stronger phenotype. Indeed, when we analyzed the data obtained for the sociability phase for the control *Fmr1* KO

and WT littermates we found that, as expected, both genotypes sniffed significantly more the stimulus mouse than the empty cage, and, what was more important, *Fmr1* KO mice showed a higher interest in the new mouse than WTs did. This proves that the sociability phenotype is quite strong and reliable, even for only 5 min, as also in the study of Gantois et al. (2013) *Fmr1* KO mice showed a higher sniffing time of the new mouse than WT group did. Time spent in the stranger mouse chamber was higher than in the empty cage chamber in both genotypes, without any significant difference between genotypes. This behavior was previously found also by other three studies (McNaughton et al. 2008;Pietropaolo et al. 2011;Gantois et al. 2013). Although these mice show preference for conspecifics versus objects, we cannot state that their social behavior is unaltered, as both our study and that of Gantois et al. (2013) showed an exaggerated sociability of *Fmr1* KO mice compared with WT, measured as sniffing time, considered an even more appropriate measurement for sociability, as it involves active social approach and interaction.

Distance traveled and number of entries to each chamber are considered indicators of activity, exploratory behavior. For each of the two genotypes the number of entries to the stranger mouse chamber was higher than to the empty cage chamber, which could contribute to the longer time spent in that chamber. As for distance, although during the habituation phase no significant difference between the two groups was found, indicating a basal similar locomotor activity, similar exploratory behavior, during the sociability phase *Fmr1* KO traveled significantly more, being thus more active. This is somehow surprising considering that these mice also spent more time sniffing the new mouse. Thus, the increased social approach in *Fmr1* KO mice was not caused by difference in activity, as they spent equal time in the chamber with the stranger mouse as WTs did, and the number of entries to this chamber was not different than for WT mice. Increased social approach in *Fmr1* KO mice was previously found in other studies (Spencer et al. 2005;Spencer et al. 2011;Thomas et al. 2011a), but it was background strain dependent, and different tests were used. Other studies found no difference in social approach between *Fmr1* KO mice and WT mice (McNaughton et al. 2008;Pietropaolo et al. 2011), or even decreased sociability (Mineur et al. 2005;Liu and Smith 2009). Only the study of Liu and Smith (2009) used the automated three chamber test, but their mice were of a different background than the ones in our study.

As we were able to reproduce the previous results of Gantois et al. (2013) concerning increased sociability in untreated *Fmr1* KO mice compared with WT littermates, next step was to test if drugs like minocycline and baclofen would rescue this phenotype. Minocycline is an MMP-9 inhibitor, which was found to be upregulated in the absence of Fmrp (Bilousova et al. 2008), whereas baclofen is a $GABA_B$ agonist. $GABA_B$ receptors are localized presynaptically and postsynaptically, the presynaptical ones regulating glutamate release (Vigot et al. 2006). $GABA_B$ R1 subunit has been found to be down regulated in frontal cortex of *Fmr1* KO mice (Pacey et al. 2011). Treatment with minocycline has been shown to rescue abnormal spine phenotype, to improve mice performance in a spontaneous alternation task (Bilousova et al. 2008), while another study showed reversal of ultrasonic vocalization production deficit in *Fmr1* KO mice (Rotschafer et al. 2011). In patients with FXS improvements in language, attention, anxiety, social communication (Utari et al. 2010), irritability, VAS for behavior (Paribello et al. 2010) have been observed following minocycline administration. Racemic baclofen is a $GABA_B$ agonist shown to decrease audiogenic seizure susceptibility, especially through its R-enantiomer, arbaclofen (Pacey et al. 2009;Henderson et al. 2012). In patients, arbaclofen (STX209) showed improvements on different aspects (Berry-Kravis et al. 2012).

Therefore, we wanted to see if these drugs have the same therapeutic effect on sociability as AFQ056/Mavoglurant was found to have in the study of Gantois et al. (2013). Both minocycline and baclofen have a bitter taste, therefore we were forced to used sweeten drinking water as dissolvent in order to assure that mice would drink. For the initial minocycline experiment we used water with sucrose. Sucrose is the most commonly used sweetener for drinking water in mouse experiments, but following the minocycline experiment we observed an effect of sucrose itself on mice activity during habituation phase, and a loss of the sociability phenotype previously found. We considered that this outcome might have influenced the results of the minocycline experiment, therefore for the baclofen experiment we decided to use aspartame instead. In the aspartame groups no difference in baseline activity has been seen between the genotypes, and the social phenotype of *Fmr1* KO mice was found again, indicating that aspartame might be the better sweetener.

We have found no effect of minocycline at the dose used. There was no interaction effect of genotype or treatment, meaning that the treated mice did not differ

from the sucrose mice (their controls) in sniffing time, in sociability. Just as in the sucrose mice, both genotypes spent more time sniffing the new mouse than the empty cage. We did see an effect of genotype, and treatment or genotype interaction with the stranger chamber on the time spent in chambers by both genotypes in the sucrose and minocycline conditions, meaning that treatment influenced the time in chamber within and between these groups unevenly. Unlike in the sucrose groups where both genotypes spent more time in the chamber with the stranger mouse, in the minocycline treated groups only WT spent more time in that chamber, while *Fmr1* KO mice did not show a preference, but they did spend more time in the empty chamber compared with WT mice. In this situation also a correlation between sniffing time and time in chamber is difficult to establish, as *Fmr1* KO mice spent equal amount of time in both side chambers, even more than WT in the empty chamber, but they did sniff more onto the novel mouse than the empty cage.

The minocycline treated *Fmr1* KO mice entered the stranger chamber significantly less times than WTs did, although not differently than they entered the empty chamber, and the distance traveled was not different between the genotypes in either acclimation or sociability phase, indicating a similar activity level in all mice. Overall we can say that minocycline treatment affected time in chamber when compared with sucrose condition, but not in a beneficial way. As sucrose alone affected distance traveled and sniffing time compared with control-untreated groups, we cannot be sure if it also influenced the results from minocycline treated groups, although the treated groups showed no alteration in distance traveled. If looking at the mean values of all *Fmr1* KO groups we see that minocycline treated KOs have the lowest value for time in stranger chamber and the highest for time in empty chamber (significantly different than control-untreated *Fmr1* KO). This lack of preference for proximity to another mouse versus an object resembles a social deficit specific for autism, but the fact that the minocycline treated *Fmr1* KO mice still sniffed the new mouse more than the empty cage indicate that they do show normal social interaction. Therefore it is difficult to establish exactly the effect of minocycline on social behavior in *Fmr1* KO mice. Testing more doses could be helpful in finding whether minocycline has a positive effect on sociability in *Fmr1* KO mice or not, but also testing the same dose in combination with aspartame water, to exclude the effect of sucrose.

Baclofen treated groups were compared with groups receiving aspartame water only. No effect of the treatment was found between these groups in both sniffing time and time in chamber. Although in the aspartame groups *Fmr1* KO mice showed the same phenotype as in control-untreated groups, increased sniffing time on the new mouse compared with the matched WT group, and in the baclofen groups the phenotype was not found, this was due to an increased sniffing time in the WT treated group compared with the aspartame WT group. A similar situation is also observed for the time in chamber. Time spent by baclofen treated WT mice in the stranger chamber was higher than the time spent by the aspartame WT mice, even though not to a significant level. GABA agonists are supposed to act through increasing inhibition, thus also inducing a sedative state. To our surprise the baclofen treated mice (both WT and *Fmr1* KO) showed increased locomotor activity during the acclimation phase compared with aspartame mice, and for the baclofen treated WT group even compared with all other WT groups. During sociability phase baclofen WT group also traveled more than the aspartame WT group (and all the other WT groups). *Fmr1* KO mice treated with baclofen did not show significantly increased activity compared with aspartame *Fmr1* KO group.

As for number of entries, in both baclofen treated groups and aspartame treated groups only WT mice showed an increased entry number into the stranger chamber versus the empty one. The increase in sniffing time, time in chamber and even number of entries between baclofen WT and aspartame WT groups, although not significant, could be due to the significantly increased locomotor activity of the treated group. From all this data it seems that baclofen has more an excitatory effect on both genotypes, but especially on WT mice. This paradoxical phenomenon for GABA has been reported before for phenobarbital as well (Ben-Ari et al. 2007). Although the first impression, when looking at the sniffing time in aspartame and baclofen groups, would be that baclofen had a rescuing effect on the increased sociability in *Fmr1* KO mice, this statement would be false, as the absolute value of sniffing time increased in the WT group, and the WT group was mostly affected by baclofen treatment. Like in the case of minocycline, testing different doses of baclofen would be advisable in order to find whether racemic baclofen is able to rescue the increased sociability in *Fmr1* KO mice or this behavior is not $GABA_B$-dependent.

One of the limitations of our study consist in testing only one dose per drug, which always raises the question if it is the optimal one, if a lower or a higher dose would give the hypothesized result. Another limitation would be using sucrose as sweetener for the minocycline experiment, but as it is the most commonly used it was our first choice too. After using aspartame for the baclofen treatment we concluded that this second sweetening substance would be more reliable. Due to the higher number of mice used, and to having five conditions in total, it was not feasible to test all groups in parallel. Therefore we divided them into three main experiments: control, minocycline (with sucrose) and baclofen (with aspartame). It could be argued that not testing them in parallel would lead to different results, but both minocycline and baclofen treated groups were tested in parallel with their controls: sucrose and aspartame, respectively, and the protocol and environmental conditions were similar for all experiments. Moreover, the aspartame treated groups reproduced the results from the control experiment. Indicating that if the test is conducted in the same way every time, even apart tested groups under similar condition lead to similar outcomes.

As this study is the first, to our knowledge, to test minocycline and racemic baclofen on sociability in the automated three chamber test, we cannot compare the current findings to others'. So far, only AFQ056/Mavoglurant, an mGluR5 antagonist, has been shown to rescue exaggerated sociability in *Fmr1* KO mice in the automated three chamber test (Gantois et al. 2013), indicating that the pathway regulating sociability is (more) glutamate-dependent.

Conclusion

Fmr1 KO mice have been found to have an exaggerated sociability compared with WT littermates, as measured by the automated three chamber test. Long-term treatment with minocycline and racemic baclofen was not able to rescue this phenotype, while the presence of sucrose in drinking water led to loss of this phenotype. To our surprise the only significant effect of treatment was that of baclofen on activity and exploration, increasing them in both genotypes, but especially in WT mice. Further research could be focused on testing different doses of these drugs, using aspartame as sweetener, and combining these substances, and also with AFQ056/Mavoglurant, which already has been proven to rescue the sociability phenotype in *Fmr1* KO mice.

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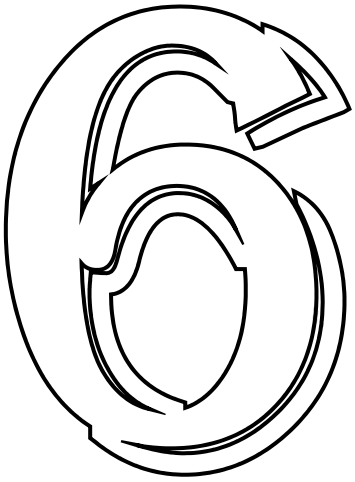

General discussion

"It's official: Fragile X is now the hottest research topic in all of neuroscience!" is the title of an article written in November 2012 by Michael Tranfaglia, Medical Director and Co-Founder of the Fragile X Association Research Foundation (FRAXA), and father of a son with fragile X syndrome (FXS), and posted on the web-page of the foundation (http://www.fraxa.org/newsArticle.aspx?newsroom_id=62). But what makes FXS such a hot topic?

FXS is the leading inherited single-gene cause of intellectual disability and autism spectrum disorders (Hagerman et al. 2010). This offers a good and promising starting point for the research focused on cause and therapy. This disorder was first described as a form of intellectual disability in 1943 by James Purdon Martin and Julia Bell (Martin and Bell 1943), but only in 1970 it was attributed to the "fragile site" on the X chromosome by Frederick Hecht, and the name of Fragile X Syndrome was born. Since then research for the cause, the pathophysiology and the treatment for FXS has increased exponentially. It started with searching for the gene involved in FXS. Only in 1991 a gene was identified on X chromosome at position q27.3 and sequenced, and named fragile X mental retardation 1 gene (*FMR1*) (Verkerk et al. 1991). It continued with studying its product, fragile X mental retardation 1 protein (FMRP), with investigating the intellectual disability and behavioral abnormalities in patients, with modeling the disorder in laboratory animals, and, of course, with developing drugs and therapies aimed at improving the life of individuals with FXS.

FMRP was found to play a role in protein synthesis at the neuronal synapse, mediating α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) internalization. According to a theory called "the mGluR theory", in FXS absence of FMRP leads to exaggerated protein synthesis following group 1 metabotropic glutamate receptor (mGluR) activation and consequently to increased AMPAR internalization (Bear et al. 2004), meaning a weakening of the synapse and disruption of its normal functioning. Another theory is that the inhibitory γ-aminobutyric acid (GABA) pathway would also be affected in individuals with FXS, as problems like epileptic seizures, sleep disorders are associated with this pathway (D'Hulst and Kooy 2007), and FMRP has been found to also act as a target for $GABA_A$ receptor subunit mRNAs (Miyashiro et al. 2003). Studies on GABAR levels in *Fmr1* KO indicated that in the cortex of *Fmr1* KO mice reduced levels of mRNA for 8 out of 18 $GABA_A$

receptor subunits can be found (D'Hulst et al. 2006), while in the hippocampus and brainstem decreased levels of $GABA_A$ β subunits were found (El Idrissi et al. 2005). In forebrain of *Fmr1* KO mice $GABA_B$ R1 subunit has been found to be down regulated (Pacey et al. 2011). $GABA_B$ receptors are localized both presynaptically and postsynaptically, the presynaptical ones being involved in glutamate release (Vigot et al. 2006).

In addition to the two major theories, others propose that disruptions at several other levels, among which the matrix metalloproteinase-9 (MMP-9) and striatal-enriched protein tyrosine phosphatase (STEP), could also account for the abnormal phenotypes found in FXS (Bilousova et al. 2008;Park et al. 2008;Utari et al. 2010;Rotschafer et al. 2011;Goebel-Goody et al. 2012a). These theories are offering a starting point for understanding the cause behind the disruptions and alterations observed in individuals with FXS and animal models for FXS, and a base for the search for therapy. Therefore most of the currently developed and under investigation pharmaceutical compounds are acting on at least one of the above named pathways, being either mGluR(5) antagonists (MPEP, fenobam, AFQ056/Mavoglurant, STX107, RO4917523, CTEP, JNJ16259685), GABA agonists (diazepam, allopregnanolone, alphaxalone, ganaxolone, baclofen) or MMP-9 inhibitors (minocycline), just to name a few (Levenga et al. 2010). As epileptic seizures, hyperactivity, language deficit, anxiety, social withdrawal, learning and memory deficits are only a few of the characteristics of FXS, such a broad range of conditions require broad spectrum (pharmaco)therapies or a combination of drugs. Currently, more and more pharmaceutical companies are interested in developing new drugs that could help individuals with FXS; more and more research groups are testing these drugs on different levels for behavioral, molecular, morphological rescue of the abnormalities. Thus, the research field and scientific involvement in FXS is growing by the day.

The current thesis is part of the ongoing search for a cure for FXS. We tested the effect of long-term treatment with three classes of compounds currently used in clinical trials: mGluR5 antagonists, GABA agonists and MMP-9 inhibitors, on abnormal dendritic spine morphology and altered social behavior in *Fmr1* KO mice. These types of substances have been used in previous studies and proven to correct certain altered phenotypes in *Fmr1* KO mice and FXS patients, including: rescue of

neural defects, aberrant behavior, audiogenic seizures, hyperactivity, and improving learning in mice (Yan et al. 2005;Bilousova et al. 2008;De Vrij et al. 2008;Pacey et al. 2009;Su et al. 2010;Burket et al. 2011;Rotschafer et al. 2011;Thomas et al. 2011; Henderson et al. 2012;Heulens et al. 2012;Michalon et al. 2012;Vinueza Veloz et al. 2012). In clinical trials use of these drugs led to decreased irritability and anxiety, improved language performance and better scores on Aberrant Behavior Checklist (ABC) (Berry-Kravis et al. 2009;Paribello et al. 2010;Utari et al. 2010;Berry-Kravis et al. 2012). The compounds we used were AFQ056/Mavoglurant (mGluR5 antagonist), racemic baclofen ($GABA_B$ agonist), and minocycline (MMP-9 inhibitor). We observed a rescue of the immature dendritic spine phenotype in CA1 hippocampal area of 25-week-old *Fmr1* KO male mice and of exaggerated sociability in 15-week-old *Fmr1* KO male mice after long-term treatment with AFQ056/Mavoglurant, but no significant effect of treatment with racemic baclofen and minocycline on the same exaggerated social approach phenotype. However, before discussing more in depth the effect of the treatment, I would like to discuss the abnormal phenotype found in this FXS mouse model in more detail.

Studies on post-mortem brain material from patients with FXS showed abnormal dendritic spine morphology and increased spine density (Rudelli et al. 1985;Hinton et al. 1991;Irwin et al. 2001). Therefore we decided to investigate whether the same phenotype is found in *Fmr1* KO mice, and test the effect of treatment with AFQ056/Mavoglurant on this phenotype. In order to reach our goals, we studied the dendritic spines in pyramidal neurons from CA1 hippocampal area of *Fmr1* KO male mice on C57Bl/6J background at the age of 2 weeks, 10 weeks and 25 weeks (Pop et al. 2013), as the hippocampus plays a major role in learning and memory processing. We found a decreased spine length in 2-week-old *Fmr1* KO mice compared with wild-type (WT) control, which changed into a reverse phenotype by the age of 10 weeks with longer spines in KO mice, and increased by 25 weeks. No difference in spine density between *Fmr1* KO and WT mice was detected at any age. Our results fall at least partially into the findings reported in literature so far, as previous studies observed an altered dendritic spine phenotype, with either higher dendritic spine density in cortex or hippocampus of *Fmr1* KO adult mice (Galvez and Greenough 2005;McKinney et al. 2005), or a decreased one (Braun and Segal 2000), but also an immature dendritic spine morphology either in the cortex or the

hippocampus of *Fmr1* KO mice, compared with WT littermates (Nimchinsky et al. 2001;Irwin et al. 2002;Galvez and Greenough 2005;McKinney et al. 2005;Grossman et al. 2006;Bilousova et al. 2008;Cruz-Martin et al. 2010;Levenga et al. 2011). An overview of these findings has been published recently (Portera-Cailliau 2011).

The inconsistence in findings comes most likely from the mouse background strain, the brain region studied, age of mice and the research method used by each research group. For a more concrete example, we found a decreased length of spines in 2-week-old *Fmr1* KO mice, while previously Nimchinsky et al. (2001) reported longer spines at this age, but they studied a cortical area and in a different strain. An increase in spine length at the age of 10 weeks was reported by other studies too, although again in the cortex (Galvez and Greenough 2005;McKinney et al. 2005). The immature spine phenotype found in CA1 area of the hippocampus of 25-week-old *Fmr1* KO mice was previously also found by Levenga et al. (2011), but accompanied by an increase in spine density in the KO mice. The difference between that study and the current one consisted in the software used for visualizing and quantifying the spines. Even so, given that the spine length phenotype found by Levenga et al. in 25-week-old KO mice was quite strong, we decided to test whether AFQ056/Mavoglurant can rescue this alteration at adult age. Therefore we treated 19-week-old mice with AFQ056/Mavoglurant mixed in food pellets for 6 weeks. This long-term treatment rescued the spine length phenotype observed in 25-week-old *Fmr1* KO mice: the treated KO group showed no difference in spine length compared with untreated WT group, indicative of a reversal to a normal morphology of the spines. So, we managed to prove that AFQ056/Mavoglurant is able to correct an abnormal morphological phenotype at the level of hippocampal synapses in an adult mouse model for FXS, but how does that relate to behavior? Does the altered spine morphology directly influence behavior? If so, in which way? Can aberrant behavior also be reverse by the same compound? There are so many questions arising from answering another.

Taking it step by step we decided to look at behavior in *Fmr1* KO mice. Choosing which aspect to investigate was not an easy task, as most tests used in humans cannot be applied on mice, and autistic-like behavior could manifest differently in mice than in men. Several studies can be found in literature investigating different behaviors in the *Fmr1* KO mouse model: open-field activity, fear conditioning, learning and memory with the help of mazes, anxiety, locomotor activity

and strength, aggression, social behavior and many more. But just like in the case of spine phenotype not all results point to the same conclusion. Some studies indicated alterations in one behavior, others did not find differences, or found the reverse phenotype. For example: in Morris water maze, some studies found impairment in the reversal trials in *Fmr1* KO mice (Bakker et al. 1994;D'Hooge et al. 1997), while others did not observe any difference between KO and WT mice in the learning and reversal tasks (Paradee et al. 1999); for anxiety on the elevated plus maze some reports found no difference between KO and WT mice (Mineur et al. 2002;Nielsen et al. 2002), while other indicate either an increase or a decrease in open arms time and entries (Peier et al. 2000;Restivo et al. 2005;Spencer et al. 2005;Bilousova et al. 2008; Michalon et al. 2012).

Social behavior was studied by several different methods which led to an abnormal phenotype observed by some groups ranging from increased social preference in KO (Spencer et al. 2005), to no difference (McNaughton et al. 2008), to decreased social interest (Mineur et al. 2002). In the case of behavior the background strain, the age and the testing method used play an even more important role in the outcome of the experiment. Social withdrawal is a core feature of autism spectrum disorders (ASD), therefore we decided to investigate more in depth this aspect in *Fmr1* KO mice, as it is known that many individuals with FXS fulfill some characteristics for ASD. We chose the automated three chamber test, also known as Sociability and Preference for Social Novelty (SPSN) test, as it has been successfully tested on more mouse models for autism, it is not invasive, and it is based on mice natural behavior, being known that rodents are in general social animals. In short, this test measures the time spent by a mouse in proximity of and interacting with a stranger mouse versus time with an empty cage (Nadler et al. 2004). Some mouse models for autism failed to show preference for a conspecific versus an empty cage (DeLorey et al. 2008;McFarlane et al. 2008), or showed significantly decreased interest in the new mouse when compared with WT mice (Tabuchi et al. 2007). For this test too the mouse background is important, as was demonstrated by Moy et al. (Moy et al. 2007;Moy et al. 2009).

As we found that around 10 weeks of age the immature spine phenotype initiated in *Fmr1* KO male mice, we decided to choose 12 weeks as the age for testing mice with the SPSN test (Gantois et al. 2013). Our results indicated an increased

sociability in the *Fmr1* KO mice compared with WT littermates, meaning that although both genotypes spent significantly more time interacting with the new mouse compared with the empty cage, the KO mice interaction time was significantly higher than WT's. This is different from what was seen in individuals with autism and animal models for autism (Hall et al. 2010), but it is similar with findings in some (young) FXS patients who seek more social contact and show interest in communication (Tranfaglia 2011). Next step was to treat *Fmr1* KO and WT mice with AFQ056/ Mavoglurant mixed in food pellets for 3 weeks prior to testing and see if the exaggerated sociability could be reversed. And indeed, long-term administration of AFQ056/Mavoglurant reduced the higher interaction time of *Fmr1* KO mice with the stranger mouse to WT levels, rescuing the abnormal phenotype, without affecting general activity. Thus, we have demonstrated that the mGluR5 antagonist we used managed not only to reverse the abnormal spine phenotype, but also the altered social behavior in adult *Fmr1* KO male mice, proving once again that mGluR pathway is a major pathway affected in FXS. Then how about other pathways like GABA or MMP-9?

In order to investigate the involvement of these pathways in social behavior of *Fmr1* KO mice we treated mice with either minocycline or racemic baclofen for two weeks in drinking water (Chapter 5). Initially we started with minocycline and as this substance has a bitter taste a sweetener was added to the water: sucrose. Surprisingly, mice receiving only sucrose (vehicle treated mice) showed an altered social behavior compared with mice on normal water, therefore we chose aspartame for the experiment with racemic baclofen. Mice receiving only the corresponding sweetened water were used as controls for each treatment. No effect of the treatment on sociability was obtained in either one of the experimental conditions. As the *Fmr1* KO mice drinking sucrose water failed to reproduce the exaggerated sociability found in untreated mice, the lack of the abnormal social behavior in minocycline treated *Fmr1* KO mice could not be related to an effect of the compound. Even more, the treated KO mice spent more time interacting with the stranger mouse than with the empty cage, but relatively equal time in the proximity of the new mouse and of the empty cage. Actually, they spent significant more time in the chamber with the empty cage than the treated WT mice did.

In the case of baclofen treated mice, both genotypes exhibited a preference for the stimulus mouse versus the empty cage, without a significant difference between genotypes. Even though the mice receiving only aspartame water reproduced the abnormal sociability phenotype of the untreated mice, the absence of this phenotype in the baclofen treated groups could not be attributed to a rescuing effect of the drug. The reduction of the difference between the treated *Fmr1* KO and WT mice interaction time with the stranger mouse was due to an increase in sociability of the WT group. The only effect of the baclofen treatment found was an increase in general activity in both genotypes, but especially in WT mice.

The conclusion of the behavioral experiment would be that AFQ056/ Mavoglurant has therapeutic properties on aberrant sociability phenotype in *Fmr1* KO mice, while minocycline and baclofen failed to rescue the same phenotype. This could mean that $GABA_B$ and MMP-9 pathways are not involved in the aberrant sociability behavior in *Fmr1* KO mice, or that the dose we used for baclofen and minocycline treatments was not optimal. The same dosage was previously used in other studies (Bilousova et al. 2008;Henderson et al. 2012), but the age of mice and the readout method differed from the one in our study.

So far, we found an immature hippocampal dendritic spine phenotype and an exaggerated sociability phenotype in the *Fmr1* KO mice, thus the next obvious question would be how they relate to each other. It is known that longer filopodia-like spines are related to weaker synapses, due to less receptors present on the postsynaptic membrane, and it is expected that this impairs the functionality of the synapse, which in turn could affect normal behavior. We observed that the normal social behavior in *Fmr1* KO mice was altered, and the dendritic spine had an immature morphology in hippocampal area CA1, but can we say that this spine phenotype in this particular brain region influenced the behavioral one? Do indeed those spines have less membrane receptors and does this disrupt normal synaptic transmission, expressed as altered behavior? In order to answer these questions we decided to investigate the levels of a few membrane receptors and synaptic protein, such as: AMPA, $GABA_A$ and striatal-enriched protein tyrosine phosphatase (STEP).

Previous studies reported normal levels of AMPA receptors in total protein homogenates and synaptic membrane preparations from forebrain of *Fmr1* KO mice (Giuffrida et al. 2005), but also reduced levels of AMPA receptor subunit GluR1 in

cortical synaptoneurosome (SN) fraction in pups (Till et al. 2012). One study reported increased levels of STEP in hippocampal SN fraction in the same mouse model (Goebel-Goody et al. 2012b). As for the GABA receptors, $GABA_B$ R1 subunit has been found to be down regulated in frontal cortex of *Fmr1* KO mice (Pacey et al. 2011); reduced levels of mRNA for 8 out of 18 $GABA_A$ receptor subunits have been found in the cortex of *Fmr1* KO mice (D'Hulst et al. 2006), while in the hippocampus and brainstem decreased levels of the $GABA_A$ β subunits were found (El Idrissi et al. 2005). Having known all this, we chose to investigate the levels of GluR1 subunit of AMPA receptor, α1 subunit of $GABA_A$ receptor, and STEP in frontal cortex of *Fmr1* KO mice (Chapter 4). We chose the cortex, as earlier studies from the literature indicated alterations in receptor levels in this brain area in *Fmr1* KO mice, and in particular the frontal area as it is known to be involved in social behavior, and to be affected in autism (Pelphrey and Carter 2008;Zikopoulos and Barbas 2010). The mice we used were adult males, of 12-13 weeks of age, as at the same age the immature spine phenotype and exaggerated sociability were observed in the *Fmr1* KO, and the correlation would be more reliable.

We isolated SN fraction from the frontal cortex, as this fraction is enriched in membrane receptors. We resolved the samples on SDS-PAGE and blotted the membranes, incubated with the desired antibodies against GluR1, $GABA_A$ α1 and STEP, imaged and quantified the signal intensity. Unfortunately we found no difference in levels between *Fmr1* KO and WT mice for any of the proteins studied. We used both actin and GAPDH as loading controls, as actin gave a relatively big variation between and within samples, while GAPDH showed more constant values. Even so, we found between 10% and 60% variation in protein levels between the same sample loaded a few times on the same blot and on separate blots, and also between mice belonging to the same genotype. This variation reduced the chance of obtaining a statistically significant difference between genotypes for any protein of interest. Although the protocols we used have been described in other studies, and the experiments were repeated several times, we did not manage to reproduce the results obtained by other research groups (Giuffrida et al. 2005;Till et al. 2012;Goebel-Goody et al. 2012b). Does this mean that in fact no difference in the studied protein levels

exists in frontal cortex of ~12-week-old *Fmr1* KO mice, or that the difference cannot be reliably detected with the methods we used?

The answer to this question can be found by further testing with several different methods. But what if indeed there is no difference in GluR1, $GABA_A$ α1 and STEP levels in frontal cortex SN between *Fmr1* KO and WT mice at 12-13 weeks? As the previous studies finding significant differences looked at different time points in the development, younger and older mice. Would it mean that even in filopodia-like spines membrane receptors and synaptic proteins are at normal levels in *Fmr1* KO mice, or that, although not attached to the membrane but in close vicinity of it, in the postsynaptic density, a normal level of proteins and receptors is found? Could it be that there are normal levels of proteins, but not functioning properly? Or maybe with the isolation protocol we used only synapses formed by the mature spines were captured, and those had unaltered protein levels.

It is true that we examined dendritic spine morphology in hippocampus of *Fmr1* KO mice, and isolated SN from the frontal cortex, but studies described in literature report immature spine phenotype in the cortex of *Fmr1* KO mice too (Galvez and Greenough 2005;McKinney et al. 2005). If there would not be a molecular, functional alteration at the level of the synapses in the brain of *Fmr1* KO mice, what could explain the abnormal behaviors observed in these mice? There are so many questions yet to be answered, and we hope that the future will manage to shed more light on this subject.

As any study ours also had limitations. For instance, the labeling technique we used for investigating the spine morphology did not allow for a precise determination of the distance from the soma at which the selected dendritic segments were located. The limitations for the social behavior study are that sucrose alone influenced sociability in the *Fmr1* KO mice, thus an effect of minocycline on this behavior was not possible to be determined; baclofen increased general activity in both genotypes, but especially in WT mice, which might have affected the social interaction behavior; and last but not least, the mice treated with AFQ056/Mavoglurant were tested at 3 weeks older than the controls, and not at the same time with them. As for the synaptic protein level determination in *Fmr1* KO and WT mice, the variation between the same sample loaded multiple times, and the variation within a group was

too high to give significance to the difference between the values of each genotype for any of the proteins of interest.

Even with the above mentioned limitations our study has a solid scientific base, it brings extra value to the research field of FXS, and opens new avenues towards further research. It supports the clinical trial use of AFQ056/Mavoglurant, and brings a little more hope for a future cure for individuals with FXS.

Concluding remarks

This thesis combines a three level research and therapeutic interventions in a mouse model for FXS, the *Fmr1* KO mouse. Behavioral, morphological and molecular studies were combined in order to identify more pieces of the puzzle named FXS. Abnormal social behavior and altered dendritic spine phenotype were found in the *Fmr1* KO mice, but no anomaly was observed at the molecular level. The innovative aspect of this work is the therapeutic effect of AFQ056/Mavoglurant on both abnormal social behavior and altered spine phenotype in *Fmr1* KO mouse model. Unfortunately two other candidate compounds, baclofen and minocycline, failed to prove effective in correcting the social phenotype of these mice. This could be dose-related or could indicate that sociability is not regulated via GABAergic or MMP-9 pathways. All three compounds are already tested in clinical trials with some promising results. Hopefully the work presented here will contribute to the progress of FXS-related therapeutic intervention studies.

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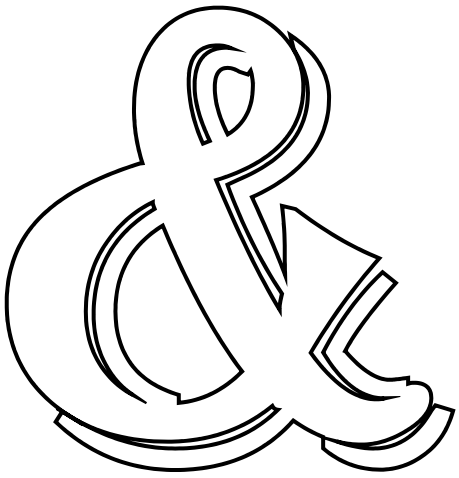

Summary

In a continuously developing society we are still confronted with intellectual disability (ID) and autism around us with quite a high prevalence. 1 in 88 children is diagnosed with autism spectrum disorder (ASD), while 2-3% of the general population is affected by intellectual disability. These two conditions are often comorbid, with approximately 75% of people with autism having a non-verbal Intelligence Quotient (IQ) in the intellectual disability range (below 70). The causes of both ID and ASD are numerous, either genetic or environmental, but the leading inherited single-gene cause of both afflictions if known to be the Fragile X Syndrome (FXS), with approximately 10% of intellectually disabled and 2-6% of autistic individuals being diagnosed with FXS. This syndrome was initially named Martin-Bell syndrome, after the two doctors who first described an X-linked intellectual disability in multiple male members of a family in 1943. Later it was linked to a "fragile site" on the X chromosome, and the name was changed into "fragile X syndrome" in 1970. Only in 1991 the gene responsible for FXS, the fragile X mental retardation 1 gene (*FMR1*), was discovered, and from that moment on research flourished. The apparent cause of FXS is a CGG trinucleotide repeat longer than 200 CGG units, which leads to hypermethylation and consequently silencing of the *FMR1* gene, resulting in the absence of *FMR1*'s protein product, the fragile X mental retardation protein (FMRP).

FMRP is present in all cells of the body, but with a higher expression in brain and testes. High levels are found in neurons from hippocampus and cerebellum, and there is moderate expression in the cerebral cortex. Brain material from patients with FXS and from a mouse model lacking Fmrp, *Fmr1* knockout (KO) mouse, showed no gross morphological abnormalities, but in certain areas of the brain an altered morphological aspect of dendritic protrusions was found: longer and thinner dendritic protrusions than in unaffected controls, consistent with an immature dendritic protrusion phenotype. As mentioned above, individuals diagnosed with FXS present with intellectual disability and autistic-like behavioral alterations, which appears to be a consequence of FMRP absence.

The role of FMRP has been under investigation by many research groups for the last 20 years. The protein's activity has been linked to messenger RNA (mRNA) trafficking and protein synthesis at the level of the synapse, the latter process

regulating the function of the synapse (synaptic plasticity). Two major theories attempting to explain the role of FMRP at the synapse are known as "the mGluR (metabotropic glutamate receptor) theory" and "the GABA (gamma-aminobutyric acid) hypothesis". The first theory states that a specific glutamatergic signaling cascade is disrupted in FXS, while the second theory hypothesizes that the GABA inhibitory pathway is impaired in individuals with FXS. Both theories are behind the rationale for different therapeutic interventions currently under investigation. The current thesis is part of the ongoing search for a cure for FXS. The work presented here was conducted on the *Fmr1* KO mice, and it refers to investigation of the dendritic protrusion phenotype in hippocampus at different ages; study of the social behavior; biochemical analysis of some membrane protein levels; and effectiveness of therapeutic intervention with chemical compounds on the aberrant phenotypes found.

Chapter 1 of this thesis is an introduction into FXS, with a description of the phenotype in humans and in animal models, the causing factor- the *FMR1* gene and its protein, FMRP-, and the therapeutic interventions applied to date. Some of the symptoms recognized in patients with FXS are macroorchidism, specific facial aspect, accompanied by hand flapping and biting, sleep problems, epileptic seizures, hyperactivity, anxiety, irritability, social deficits, language deficits, working and short-term memory problems, deficits in executive function, and the list can continue. Some of these symptoms have been reproduced in animal models for FXS, like fruit fly, zebrafish, and mouse. The most used animal model for FXS is the *Fmr1* KO mouse, which has been shown to copy more of the FXS characteristics: macroorchidism, immature dendritic protrusion phenotype, hyperactivity, repetitive behavior, susceptibility to audiogenic seizures, altered social behavior, impaired learning and memory. These symptoms appear to be caused by the lack of FMRP, and the disruptions created at the synaptic level. According to the mGluR theory, FMRP is acting as a break on postsynaptic protein synthesis, regulating the endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). These receptors are playing a crucial role in synaptic plasticity. Lack of FMRP leads to an increased protein synthesis following mGluR activation, which in turn leads to a higher internalization rate of the AMPARs. This causes disruption of the synaptic plasticity and elimination of the synapse, with the dendritic protrusion morphology reverting to the immature aspect. Weakening and elimination of the synapses lead to

cognitive impairments and behavioral alterations. As FMRP is also targeting GABA receptor subunits, its absence disrupts the GABAergic pathway in FXS, which could explain epileptic seizures and amygdala dysfunctions. Therefore most of the current therapeutic interventions are focused on mGluR antagonists and GABA agonists, but also on other molecular targets.

Chapter 2 presents the dendritic protrusion phenotype found in CA1 hippocampal area of *Fmr1* KO mice at different ages: pup (2-week-old), young adult (10-week-old) and adult (25-week-old), and the therapeutic effect of an mGluR5 antagonist, AFQ056/Mavoglurant, at adult age on the immature phenotype. We used a diOlistic labeling technique for visualizing the neurons, whereby we filled the neurons with a fluorescent dye and imaged with the confocal microscope. Although at 2 weeks of age the dendritic protrusions in CA1 region of *Fmr1* KO mice were shorter than the ones in the control mice, by the age of 10 weeks the phenotype changed into a more immature one in KO mice, the dendritic protrusions were found to be longer than in controls. This phenotype deepened by adult age. A six week treatment with AFQ056/ Mavoglurant was proved to rescue the immature protrusion phenotype in *Fmr1* KO mice. The length of the dendritic protrusions from CA1 area of treated *Fmr1* KO mice did not differ from the controls.

The therapeutic effect of AFQ056/Mavoglurant was further tested on altered social behavior of *Fmr1* KO mice, as presented in **chapter 3**. Using the automated three chamber test, we investigated the sociability and preference for social novelty of *Fmr1* KO mice. This test consists of three phases: acclimation, sociability, and preference for social novelty; and is performed with the help of a three chamber setup. The test measures a mouse preference for proximity and interaction with another novel mouse compared with an empty cage (sociability), or a familiar mouse (preference for social novelty). To our surprise we found that *Fmr1* KO mice socialized more with the new mouse than control mice did, both groups preferring the new mouse to the empty cage. This exaggerated sociability was corrected by three weeks treatment with AFQ056/ Mavoglurant. As for the preference for social novelty, *Fmr1* KO mice did not preferentially interact with the familiar mouse or the new mouse, while control mice preferred the new mouse versus the familiar one. Treated *Fmr1* KO mice showed increased preference for the newer mouse, although a statistical effect of the treatment

could not be found. Even so, these results advocate for a positive therapeutic effect of AFQ056/Mavoglurant for FXS.

Chapter 4 takes the study to molecular level, as we were interested to find whether the level of synaptic proteins like AMPA, $GABA_A$ and STEP (striatal-enriched protein tyrosine phosphatase) is altered in *Fmr1* KO mice. The role of AMPA and GABA receptors in synaptic plasticity and brain function has been discussed above, whereas for STEP it has been shown in other studies to regulate AMPA receptor endocytosis. Other research groups found lower levels of AMPA receptor subunits GluR1 and GluR2/3 and reduced levels of mRNA for 8 out of 18 $GABA_A$ receptor subunits in cortex of *Fmr1* KO mice, but higher levels of STEP in hippocampus of these mice compared with unaffected controls. We investigated by immunoblotting the levels of GluR1 subunit, $GABA_A$ $\alpha 1$ subunit and STEP in synaptoneurosome fraction from frontal cortex of adult *Fmr1* KO mice. The synaptoneurosome is an enriched fraction of brain homogenate containing mainly synaptic components. Unfortunately, we were not able to find any significant difference in protein levels between frontal cortex of *Fmr1* KO and of control mice. Whether no difference exists or the method used was not sensitive enough can be proven only by further research.

In **chapter 5** we studied the therapeutic effect of other two compounds on the exaggerated sociability found with the automated three chamber test. As the effect of an mGluR antagonist has been proven positive on altered social behavior in *Fmr1* KO mice, this time we chose a GABA agonist, more precisely a $GABA_B$ agonist: racemic baclofen; and a compound acting on other targets, in this case an MMP-9 (matrix metalloproteinase-9) inhibitor: minocycline. Both compounds have been used for a long time as therapeutic intervention for other medical conditions, but administration to *Fmr1* KO mice has proven beneficial in correcting immature dendritic protrusion phenotype, audiogenic seizures susceptibility, and some behavioral alterations. We treated *Fmr1* KO mice and unaffected controls for two weeks with one of the two compounds, and then we tested the mice only for sociability as this aspect was corrected by AFQ056/Mavoglurant in previous experiment. Unfortunately both treatments failed to show an effect on sociability. The explanation could be in the dose

used, although it has proven efficient in other studies, or it could mean that social behavior is not $GABA_B$ or MMP-9 dependent.

The thesis ends with an overview and discussion of our findings in conjunction with other studies, in **chapter 6**. We showed that *Fmr1* KO mice have an altered dendritic protrusion phenotype during development that can be corrected even at adult age by treatment with an mGluR5 antagonist, AFQ056/Mavoglurant. The same compound corrected an exaggerated sociability phenotype of these mice, but not the same effect was obtained with racemic baclofen and minocycline. We did not find any alteration of GluR1, $GABA_A$ α1 or STEP levels in frontal cortex of *Fmr1* KO mice. In conclusion, current work brings extra value to the FXS research field, especially by proving the positive therapeutic effect of AFQ056/Mavoglurant on morphological and behavior alterations in *Fmr1* KO mice, but also by opening new avenues towards future research for new therapies.

Samenvatting

In een constant ontwikkelende samenleving worden we nog steeds geconfronteerd met een vrij hoge prevalentie van verstandelijke beperking (intellectul disability (ID)) en autisme. Bij kinderen die gediagnosticeerd zijn met autistisch spectrum disorder (ASD) is dat 1 op de 88, terwijl 2-3% van de algemene populatie is aangedaan met een verstandelijke beperking. Deze twee zijn vaak comorbide, ongeveer 75% van de mensen met autisme hebben een non-verbale Intelligentie Quotiënt (IQ) in het bereik van een verstandelijke beperking (onder 70). Er zijn veel oorzaken voor zowel ID en ASD, zowel genetisch als omgevingsfactoren, maar het Fragile X syndroom (FXS) staat bekend als de meest voorkomende genetische enkel-gens oorzaak van beide aandoeningen. Ongeveer 10% van de mensen met een verstandelijke beperking en 2-6% van de autistische individuen zijn gediagnosticeerd met FXS. Van oorsprong stond dit syndroom bekend als het Martin-Bell syndroom, vernoemd naar de twee artsen die in 1943 als eerste een X-linked verstandelijke beperking beschreven bij meerdere mannen uit één familie.

Later is het “fragiele stukje” aan het X-chromosoom aan het syndroom gekoppeld en de naam werd in 1970 veranderd in ”fragiele X syndroom”. Pas in 1991 werd het gen, het fragiele X mentaal retardatie 1 gen (*FMR1*), ontdekt en van af dat moment bloeide het onderzoek op. De oorzaak van FXS is een CGG trinucleotide repeat langer dan 200 CGG eenheden, die leidt tot hypermethylering en als consequentie daarvan het uitschakelen van het *FMR1* gen, resulterend in een afwezigheid van het FMR1 eiwit product, het fragiele X mentale retardatie proteïne (FMRP).

FMRP is aanwezig in alle lichaamscellen, maar met een hogere expressie in de hersenen en de testis. In de hersenen wordt de hoogste expressie gevonden in de zenuwcellen van de hippocampus en het cerebellum en wordt een matige expressie in de cerebraal cortex waargenomen. Hersenmateriaal van FXS patiënten en van muizen die geen *FMR1* expressie hebben, *Fmr1* knock-out (KO) muis, laten geen grove morfologische afwijkingen zien, maar in bepaalde gebieden van het brein werd een veranderde morfologie van de dendritische uitstekels (spines) waargenomen: de uitstekels zijn langer en dunner dan in controle materiaal, overeenkomend met een ongerijpte dendritisch uitsteeksel fenotype. Zoals eerder genoemd, individuen

gediagnosticeerd met FXS met een verstandelijke beperking en autistisch-lijkende gedragsverandering, blijken een gevolg te zijn van het ontbreken van FMRP.

De functie van FMRP is de afgelopen 20 jaar door vele researchgroepen onderzocht. De functie van het eiwit is gekoppeld aan messenger RNA (mRNA) transport en eiwit synthese bij de synaps, dit laatste reguleert de functie van de synaps (synaptische plasticiteit). Twee belangrijke theorieën proberen de rol van FMRP in de synaps te verklaren en zijn bekend als "de mGluR (metabotropic glutamate receptor) theorie" en "de GABA (gamma-aminobutyric acid) hypotheses". De eerste theorie stelt dat een specifiek glutamaterge signalerings cascade is verstoord in FXS, en de tweede theorie veronderstelt dat het remmende GABA pad verzwakt is bij personen met FXS. Beide theorieën liggen ten grondslag voor verschillende therapeutisch interventies die op dit moment worden onderzocht. Dit proefschrift is een onderdeel van een nog voortdurende zoektocht naar een genezing en/of behandeling van FXS. Het werk dat hier wordt gepresenteerd is verricht op de *Fmr1* KO muis en behandelt onderzoek van het dendritische uitloper fenotype in de hippocampus bij verschillende leeftijden; een studie naar het sociale gedrag; een biochemische analyse van niveaus van enkele membraan eiwitten; en de effectiviteit van de therapeutische toepassing van chemische stoffen bij afwijkende fenotypes die zijn gevonden.

Hoofdstuk 1 van dit proefschrift is een inleiding in FXS en beschrijft het fenotype zowel in de mens als in diermodellen, het gen dat ten grondslag ligt aan het syndroom, *FMR1*, en het eiwit product, FMRP, en de therapeutische toepassingen tot nu toe. Enkele symptomen/kenmerken die worden gezien bij patiënten met FXS zijn macroorchidisme, specifieke gezichtskenmerken, vergezeld met handen klappen en bijten, slaapproblemen, epileptische aanvallen, hyperactiviteit, angsten/onrust, prikkelbaarheid, sociale tekortkomingen, taal tekortkomingen, problemen met werk- en korte termijn geheugen, tekortkomingen in uitvoerende functies, en de lijst kan nog worden voortgezet. Enkele van de symptomen zijn nagebootst in FXS-diermodellen, zoals in de fruitvlieg en de muis. Voor FXS is de *Fmr1* KO muis het meest gebruikt als diermodel, die meerdere karakteristieken van FXS heeft: macroorchidisme, immature dendritische uitloper fenotype, hyperactiviteit, repetitief gedrag, gevoeligheid voor audiogeen geïnduceerde epileptische aanvallen, tekortkomingen in sociaal gedrag, gepaard gaand met problemen met leren en het geheugen. Deze symptomen worden veroorzaakt door het ontbreken van FMRP in de zenuwcellen en

de verstoringen op het synaptisch niveau. Volgens de mGluR theorie fungeert FMRP als een rem op postsynaptische eiwitsynthese na mGluR5 stimulatie en daardoor de endocytose van α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) reguleert. Deze receptoren spelen een cruciale rol in de synaptische plasticiteit. Het ontbreken van FMRP leidt tot verhoogde eiwitsynthese na mGluR activering, met als gevolg een hogere internalisatie snelheid van de AMPARs. Dit kan leiden tot verstoring van de synaptische plasticiteit en het verdwijnen van de synaps, waarbij de dendritische uitloper een immature vorm terugkrijgt of verdwijnt. Het verzwakken of verdwijnen van de synaps kan leiden tot cognitieve stoornissen en gedragsveranderingen. FMRP bindt ook mRNAs van GABA receptor subunits en het ontbreken van FMRP verstoort ook het GABAerge pad in FXS, dit kan leiden tot epileptische aanvallen en het disfunctioneren van de amygdala. Daarom zijn de meeste therapeutische toepassingen gericht op mGluR antagonisten en GABA agonisten.

In **hoofdstuk 2** wordt het dendritische immature uitloper fenotype gevonden in de CA1 regio van de hippocampus van *Fmr1* KO muizen op verschillende leeftijden beschreven: pup (2-weken-oud), jong volwassen (10-weken-oud) en volwassen (25-weken-oud) en het therapeutisch effect van een mGluR5 antagonist, AFQ056/ Mavoglurant, op volwassen leeftijd. We hebben een diOlistic labeling techniek gebruikt voor het visualiseren van de zenuwcellen, waarbij de zenuwcellen worden gevuld met een fluorescerende kleurstof en worden bestudeerd met een confocale microscoop. Hoewel de dendritische uitlopers in de CA1 regio van *Fmr1* KO muizen bij een leeftijd van twee weken korter waren dan bij de controle muizen, veranderde het fenotype in een meer immature vorm in *Fmr1* KO muizen bij een leeftijd van tien weken, dendritische uitlopers werden langer dan in de controles. Dit fenotype werd versterkt bij volwassen leeftijd. Met een behandeling van zes weken met AFQ056/ Mavoglurant werd aangetoond dat het dendritische uitlopers fenotype in *Fmr1* KO muizen resulteerde in een verbetering. De lengte van de dendritische uitlopers van het CA1 gebied van behandelde *Fmr1* KO muizen geven geen verschil met de controles.

Het therapeutisch effect van AFQ056/Mavoglurant werd verder getest op het afwijkende sociale gedrag van de *Fmr1* KO muizen en wordt behandeld in **hoofdstuk 3**. We onderzochten het sociaal gedrag en de voorkeur voor sociale vernieuwing van *Fmr1* KO muizen. De test bestaat uit drie fasen: acclimatiseren, sociaal vermogen, en voorkeur voor sociale vernieuwing. De test wordt uitgevoerd met behulp van een drie

kamer opzet. De test meet de voorkeur van de muis voor nabijheid en interactie met een andere nieuwe muis vergeleken met een lege kooi (sociaal vermogen), of met een bekende muis (voorkeur voor sociale vernieuwing). Tot onze verrassing vonden we dat *Fmr1* KO muizen socialer waren ten opzichte van nieuwe muizen dan de controle muizen, beide groepen prefereren de nieuwe muis boven de lege kooi. Het overdreven sociaal vermogen werd gecorrigeerd door drie weken te behandelen met AFQ056/ Mavoglurant. Wat betreft de voorkeur voor sociale vernieuwing, vertonen *Fmr1* KO muizen geen voorkeur in interactie met de bekende muis of de nieuwe muis, terwijl controle muizen de voorkeur geven aan een nieuwe muis boven een bekende muis. Behandelde *Fmr1* KO muizen vertonen een toegenomen voorkeur voor de nieuwe muis hoewel een statistisch effect van de behandeling uit blijft. Evengoed pleiten deze resultaten voor een positief therapeutisch effect van AFQ056/ Mavoglurant voor FXS.

Hoofdstuk 4 brengt de studie naar een moleculair niveau, daar we geïnteresseerd zijn of de expressie van synaptische eiwitten zoals AMPA, $GABA_A$, en STEP (striatal-enriched protein tyrosine phosphatase) is veranderd in *Fmr1* KO muizen. De rol van AMPA en GABA receptoren in synaptische plasticiteit en in het brein is hierboven al besproken, terwijl voor STEP uit eerdere studies is gebleken dat het de endocytose van AMPA receptoren reguleert. Andere onderzoeksgroepen vonden lagere niveaus van AMPA receptor subunits GluR1 en GluR2/3 en gereduceerde niveaus van mRNA voor 8 van de 18 $GABA_A$ receptor subunits in de cortex van *Fmr1* KO muizen, maar hogere niveaus van STEP in de hippocampus van deze muizen vergeleken met de niet aangedane controle muizen. Door middel van immunoblotting onderzochten we de niveaus van GluR1 subunit, $GABA_A$ α1 subunit en STEP in de synaptoneurosomale fractie van frontale cortex van volwassen *Fmr1* KO muizen. Het synaptoneurosoom is een verrijkte fractie van brein homogenaten die voornamelijk synaptische componenten bevat. Helaas waren we niet in staat om een significant verschil aan te tonen in eiwit niveaus tussen de frontale cortex van *Fmr1* KO muizen en van de controle muizen. Of er geen verschil is of dat de gebruikte methode niet gevoelig genoeg is zal moeten blijken uit verder onderzoek.

In **hoofdstuk 5** bestudeerden we het therapeutisch effect van nog twee andere stoffen op het overdreven sociaal vermogen dat gevonden is in de automatische drie kamer test. Van een mGluR antagonist is een positief effect aangetoond op het sociaal gedrag van *Fmr1* KO muizen (hoofdstuk 3), maar nu gaat het om een GABA agonist,

en wel om een $GABA_B$ agonist: racemisch baclofen; en een stof die op andere targets werkt, in dit geval een MMP-9 (matrix metalloproteinase-9) inhibitor: genaamd minocyline. Beide stoffen worden al voor langere tijd gebruikt als therapeutische toepassing voor andere aandoeningen en worden momenteel getest in klinische studies voor FXS. Eerder is aangetoond dat de behandeling van *Fmr1* KO muizen met deze stoffen goed blijkt te werken op het corrigeren van het immature uitloper fenotype, gevoeligheid voor audiogeen geïnduceerde epileptische aanvallen en enkele gedragsveranderingen. *Fmr1* KO muizen en onaangedane controles werden twee weken behandeld met één van de twee stoffen, vervolgens werden muizen getest op sociaal vermogen, daar dit aspect werd gecorrigeerd met AFQ056/Mavoglurant in vorige experimenten.

Helaas konden voor beide stoffen geen herstellende effecten worden aangetoond. De gebruikte dosis zou een verklaring kunnen zijn, hoewel die in andere studies efficiënt is gebleken, of het zou kunnen zijn dat andere pathways het sociaal gedrag reguleren op het overdreven sociaal vermogen.

In **hoofdstuk 6** wordt het proefschrift afgerond met een overzicht en discussie over onze bevindingen in samenhang met andere studies. We hebben aangetoond dat *Fmr1* KO muizen gedurende de ontwikkeling een veranderd uitloper fenotype hebben en dat dit fenotype gecorrigeerd kan worden op volwassen leeftijd door behandeling met een mGluR5 antagonist, AFQ056/Mavoglurant. Deze stof corrigeert ook een overdreven sociaal vermogen fenotype van de *Fmr1* KO muizen, maar dit effect werd niet waargenomen met racemisch baclofen en minocycline. We konden geen verandering aantonen van GluR1, $GABA_A$ α1 of STEP eiwit concentraties in de frontale cortex van *Fmr1* KO muizen. In conclusie heeft dit onderzoek toegevoegde waarde voor het FXS onderzoeksveld, vooral door een positief therapeutisch effect van AFQ056/Mavoglurant aan te tonen in *Fmr1* KO muizen op morfologie en gedrag, maar ook door het identificeren van nieuwe wegen voor toekomstig onderzoek naar nieuwe therapieën voor het FXS.

Rezumat

Într-o societate în continuă dezvoltare încă ne confruntăm în jurul nostru cu handicap intelectual (intellectual disability-ID) şi autism, cu o prevalenţă destul de ridicată. 1 din 88 de copii este diagnosticat cu tulburare de spectru autist (autism spectrum disorder-ASD), pe când 2-3% din populaţia generală este afectată de handicap intelectual. Aceste două condiţii sunt deseori comorbide, cu aproximativ 75% dintre persoanele cu autism având un coeficient intelectual non-verbal (IQ) în intervalul handicapului intelectual (sub 70). Cauzele ID şi ASD sunt numeroase, de origine genetică sau determinate de mediul înconjurător, dar cauza principală monogenică ereditară cunoscută este sindromul X fragil (FXS), aproximativ 10% dintre indivizii cu handicap intelectual şi 2-6% dintre cei cu autism fiind diagnosticaţi cu FXS. Acest sindrom a fost iniţial numit Martin-Bell, după cei doi doctori care au descris pentru prima dată în 1943 o formă de handicap intelectual X-dependent la mai mulţi membri de sex masculin ai unei familii. În 1970, acea formă de handicap intelectual a fost corelată cu un „site fragil" al cromozomului X, şi a dus la schimbarea numelui în „sindromul X fragil". Abia în 1991 a fost descoprită gena responsabilă pentru FXS, gena retard mintal X fragil 1 (*FMR1*), şi din acel moment cercetarea ştiinţifică a înflorit. Cauza aparentă a FXS este o repetiţie a trinucleotidei CGG cu o lungime mai mare de 200 unităţi, ceea ce duce la hipermetilarea şi silenţierea genei *FMR1*, rezultând în absenţa proteinei normale, proteina retard mintal X fragil (FMRP).

FMRP este prezentă în toate celulele organismului, dar predomină în creier şi testicule. FMRP se găseşte la un nivel ridicat în neuroni hipocampici şi cerebeloşi, pe când în cortexul cerebral exprimarea ei este moderată. Material prelevat din creierul pacienților cu FXS şi de la un model de şoarece în care FMRP este absentă, *Fmr1* knockout (KO), nu a indicat prezenţa unor abnormalităţi morfologice majore, dar în anumite regiuni ale creierului au fost descoperite alterări ale morfologiei protruziilor dendritice: protruzii mai alungite şi mai subţiri decât la subiecţii neafectaţi, indicativul unui fenotip imatur al protruziilor dendritice. După cum am menţionat mai sus, indivizii diagnosticaţi cu FXS pot prezenta de asemenea handicap mintal şi comportament de tip autist, aspecte care par a fi o consecinţă a lipsei proteinei FMR.

Rolul FMRP face de peste 20 de ani obiectul cercetărilor mai multor grupuri ştiinţifice. Activitatea proteinei a fost corelată cu traficul ARN-ului mesager şi cu

sinteza proteică la nivelul sinapsei neuronale, procesul din urmă reglând funcţionarea sinapsei (plasticitatea sinaptică). Două teorii majore care încearcă să explice rolul FMRP la nivelul sinapsei sunt cunoscute sub numele de „teoria mGluR (receptorul metabotropic de glutamat)" şi „ipoteza GABA (acid γ-aminobutiric)". Prima teorie susţine că o cascadă glutamatergică de semnalizare este perturbată în FXS, pe când a doua teorie propune alterări ale căii inhibitorii GABA. Ambele teorii stau la baza intervenţiilor terapeutice în curs de investigare la ora actuală. Teza curentă face parte din continua căutare a unui remediu pentru FXS. Studiul prezentat aici a avut ca subiecţi şoareci *Fmr1* KO, şi cuprinde investigarea fenotipului protruziilor dendritice în hipocamp la diferite vârste, studierea comportamentului social, analiza biochimică a unor proteine membranare şi eificienţa terapeutică a unor compuşi farmaceutici asupra dereglărilor descoperite.

Capitolul 1 al acestei teze cuprinde o introducere in FXS, cu o descriere a fenotipului la oameni şi în modele animale, a factorului cauzator- *FMR1* şi proteina sa, FMRP- şi a intervenţiilor terapeutice utilizate în prezent. Unele dintre simptomele identificate la pacienţi cu FXS sunt: macroorchidism, aspecte faciale specifice, acompaniate de scuturări şi muşcări ale mâinii, probleme cu somnul, crize epileptice, hiperactivitate, anxietate, iritabilitate, deficienţe sociale, lingvistice, probleme ale memoriei de lucru şi de scurtă durată, deficienţe în funcţii executive, şi lista poate continua. Unele dintre aceste simptome au fost reproduse în modele animale pentru FXS, precum musculiţa de oţet şi şoarecele. Cel mai des folosit model este şoarecele, acesta fiind capabil să copieze/reproducă mai multe dintre caracteristicele FXS: macroorchidism, fenotip imatur al protruziilor dendritice, hiperactivitate, comportament repetitiv, susceptibilitate pentru crize epileptice audiogenice, comportament social aberant, afectarea memoriei şi învăţării. Aceste simptome sunt cauzate de lipsa FMRP şi de dereglările induse la nivel sinaptic. În conformitate cu teoria mGluR, FMRP acționează ca frână asupra sintezei proteice din densitatea postsinaptică indusă de activarea receptorilor mGlu5, sinteză ce coordonează internalizarea receptorilor AMPA (acid α-amino-3-hidroxy-5-methyl-4-isoxazole-propionic). Aceşti receptori joacă un rol crucial în plasticitatea sinaptică. Lipsa FMRP duce la intenstificarea sintezei proteice indusă de activarea receptorilor mGlu, care la rândul său determină o creştere a ratei de internalizare a receptorilor AMPA. Acest

fenomen poate cauza întreruperea plasticităţii sinaptice şi eliminarea sinapsei în sine, cu protruziile dendritice fie revenind la o morfologie imatură, fie dispărând. Slăbirea şi eliminarea sinapselor poate duce la alterări cognitive şi comportamentale. Deoarece FMRP leagă şi ARN-ul mesager al unor subunităţi ale receptorului GABA, absenţa proteinei afectează de asemenea calea GABA în FXS, putând astfel oferi o explicaţie pentru crizele epileptice şi disfuncţiile amigdalei. Aceste teorii au dus la canalizarea actualelor intervenţii terapeutice pe antagonişti ai receptorilor mGlu5 şi pe agonişti ai receptorilor GABA.

Capitolul 2 prezintă fenotipul imatur al protruziilor dendritice găsit în regiunea CA1 a hipocampului la şoareci *Fmr1* KO de diferite vârste: pui (2 săptămâni), juvenil (10 săptămâni) şi adult (25 de săptămâni), precum şi efectul terapeutic al antagonistului receptorului mGlu5, AFQ056/Mavoglurant, la vârsta adultă. Am utilizat o tehnică diOlistică de colorare pentru vizualizarea neuronilor, ce constă în umplerea neuronilor cu un colorant fluorescent şi vizualizarea la microscopul confocal. Cu toate că la vârsta de 2 săptămâni protruziile dendritice din regiunea CA1 a şoarecilor *Fmr1* KO erau mai scurte decât la şoarecii control, la 10 săptămâni fenotipul s-a transformat într-unul imatur în *Fmr1* KO, protruziile dendritice fiind mai lungi decât ale şoarecilor control. Acest fenotip s-a adâncit la vârsta adultă. Şase săptămâni de tratament cu AFQ056/Mavoglurant s-a dovedit a fi eficient în corectarea fenotipului imatur în şoarecii *Fmr1* KO. Lungimea protruziilor din regiunea CA1 a şoarecilor *Fmr1* KO trataţi nu a diferit de cea a animalelor control.

Efectul terapeutic al AFQ056/Mavoglurant a fost testat în continuare asupra comportamentului social al şoarecilor *Fmr1* KO, după cum este prezentat în **capitolul 3**. Utilizând testul automat cu trei camere, am investigat sociabilitatea şi preferinţa pentru noutate socială a şoarecilor *Fmr1* KO. Acest test consistă din trei faze: aclimatizare, sociabilitate şi preferinţa pentru noutate socială; este aplicat cu ajutorul unei instalaţii cu trei camere. Testul măsoară preferinţa unui şoarece pentru proximitatea şi interacţiunea cu un şoarece necunoscut în comparaţie cu o cuşcă goală (sociabilitate) sau cu un şoarece familiar (preferinţa pentru noutate socială). Spre surprinderea noastră am observat că şoarecii *Fmr1* KO socializează chiar mai mult decât şoarecii control cu noul şoarece, dar ambele grupuri experimentale au preferat noul şoarece faţă de cuşca goală. Această sociabilitate exagerată a fost corectată prin tratarea pentru trei săptămâni cu AFQ056/Mavoglurant. În ce priveşte preferinţa

pentru noutate socială, şoarecii *Fmr1* KO nu au interacţionat prefenţial cu noul şoarece sau cu cel familiar, pe când şoarecii control au preferat noul şoarece. Şoarecii *Fmr1* KO trataţi au prezentat o creştere a preferinţei pentrul noul şoarece faţă de cel familiar, deşi un efect semnificant statistic al tratamentului nu a putut fi determinat. Chiar şi aşa, aceste rezultate susţin efectul terapeutic pozitiv al AFQ056/Mavoglurant pentru FXS.

Capitolul 4 mută studiul la un nivel molecular, fiind interesaţi să aflăm dacă nivelul proteinelor sinaptice precum AMPA, $GABA_A$ şi STEP (striatal-enriched protein tyrosine phosphatase) este alterat în şoarecii *Fmr1* KO. Rolul receptorilor AMPA şi GABA în plasticitatea sinaptică şi în funcţionarea creierului a fost discutat mai sus, pe când alte studii implică proteina STEP în reglarea endocitozei receptorilor AMPA. Alte grupuri de cercetare au găsit niveluri reduse ale subunităţilor GluR1 şi GluR2/3 ale receptorului AMPA, şi niveluri reduse de ARN mesager pentru 8 din cele 18 subunităţi ale receptorului $GABA_A$ în cortexul şoarecilor *Fmr1* KO, dar nivel ridicat al STEP în hipocampul acestor şoareci, comparativ cu animalele de control. Prin imunocolorare am studiat nivelurile subunităţii GluR1, subunităţii $\alpha 1$ ale receptorului $GABA_A$ şi al proteinei STEP în fracţiunea sinaptoneurozomală din cortexul frontal al şoarecilor *Fmr1* KO adulţi. Sinaptoneurozomul este o fracţiune îmbogăţită a homogenatului cerebral conţinând în majoritate componente sinaptice. Din păcate, nu am reuşit să identificăm diferenţe semnificative între nivelurile proteinelor în cortexul frontal al şoarecilor *Fmr1* KO şi cel al şoarecilor control. Doar investigări ulterioare pot dovedi dacă diferenţa într-adevăr nu există sau dacă metoda utilizată nu este suficient de sensibilă.

În **capitolul 5** am studiat efectul terapeutic a doi compuşi asupra sociabilităţii exagerate găsite cu testul automat cu trei camere. Deoarece efectul unui antagonist al receptorilor mGlu asupra comportamentului social aberant al şoarecilor *Fmr1* KO s-a dovedit a fi unul pozitiv (capitolul 3), de această dată am ales un agonist al receptorilor GABA, mai exact al $GABA_B$: baclofen racemic, şi un compus care acţionează asupra altor ţinte, în acest caz un inhibitor al MMP-9 (matrix metalloproteinase-9): minociclină. Ambii compuşi au fost utilizaţi timp îndelungat în alte afecţiuni, şi sunt în prezent în testări clinice. Administrarea lor la şoareci *Fmr1* KO s-a dovedit benefică în corectarea fenotipului imatur al protruziilor dendritice, în corectarea susceptibilităţii la

crize audiogenice şi la unele comportamente aberante. Am tratat două săptămâni şoareci *Fmr1* KO şi control neafectaţi cu unul dintre cei doi compuşi şi apoi i-am testat doar pentru sociabilitate, deoarece acest aspect a fost corectat cu AFQ056/ Mavoglurant în experimentul anterior. Din păcate ambele tratamente au eşuat în a demonstra un efect pozitiv asupra sociabilităţii exagerate. Explicaţia ar putea consta în doza utilizată, deşi în experimente anterioare s-a dovedit eficientă, sau ar putea indica implicarea altor căi moleculare în controlul comportamentului social.

Această teză se încheie cu o recapitulare şi discutare a rezultatelor obţinute în corelație cu alte studii din literatură, în **capitolul 6**. Am arătat că şoarecii *Fmr1* KO au un fenotip alterat al protruziilor dendritice pe parcursul dezvoltării ce poate fi corectat chiar şi la vârstă adultă prin tratarea cu un antagonist al receptorului mGlu5, AFQ056/Mavoglurant. Acelaşi compus a corectat fenotipul de sociabilitate exagerată a acestor şoareci, dar nu acelaşi efect a fost obţinut cu baclofen racemic şi minociclină. Nu am găsit alterări ale nivelului de GluR1, $GABA_A$ $\alpha 1$ sau STEP în cortexul frontal al şoarecilor *Fmr1* KO. În concluzie, studiul curent aduce extra valoare domeniului de cercetare a FXS, în special prin dovedirea efectului terapeutic pozitiv al AFQ056/ Mavoglurant asupra alterărilor morfologice şi comportamentale în şoarecii *Fmr1* KO, dar şi prin deschiderea de noi căi spre viitoare cercetări pentru noi terapii.

Curriculum Vitae

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Born: 28.07.1980 in Cluj-Napoca, Romania.

Education: Primary and secondary school- (1987-1990) General School Nr.17, Cluj-Napoca;
- (1990-1995) National College "George Cosbuc", Cluj-Napoca;
Highschool- (1995-2000) Pedagogical College "Gheorghe Lazar", Cluj-Napoca;
University- (2000-2002) Faculty of Psychology and Education Sciences, field- Special Psycho-pedagogy (Psychology and pedagogy of the deficient/disabled child), "Babes-Bolyai" University, Cluj-Napoca;
- (2002-2006) Bachelor Degree in Biology at Faculty of Biology and Geology, "Babes-Bolyai" University, Cluj-Napoca;
- (2006-2007) Master Degree in Cellular Biotransformations at Faculty of Biology and Geology, "Babes-Bolyai" University, Cluj-Napoca;
- (2006-2008) Master of Neuroscience at Erasmus Medical Center, Rotterdam, the Netherlands;

Work experience: (2000-2006) Primary school teacher at National College "Emil Racovita", Cluj-Napoca, Romania;
(September- December 2008)-scientific researcher, Department of Neuroscience, Erasmus MC, Rotterdam, the Netherlands;
(2008- 2013) PhD program in the Department of Clinical Genetics, Erasmus Medical Center, Rotterdam.

List of publications

1. Levenga J, de Vrij FM, Buijsen RA, Li T, Nieuwenhuizen IM, **Pop A**, Oostra BA, Willemsen R, **Subregion-specific dendritic spine abnormalities in the hippocampus of *Fmr1* KO mice**, Neurobiology of Learning and Memory, 2011

2. Levenga J, Hayashi S, de Vrij FM, Koekkoek SK, van der Linde HC, Nieuwenhuizen I, Song C, Buijsen RA, **Pop AS**, Gomez-Mancilla B, Nelson DL, Willemsen R, Gasparini F, Oostra BA, **AFQ056, a new mGluR5 antagonist for treatment of fragile X syndrome**, Neurobiology of Disease, 2011

3. Gantois, I, **Pop AS**, de Esch CE, Buijsen RA, Pooters T, Gomez-Mancilla B, Gasparini F, Oostra BA, D'Hooge R, Willemsen R, **Chronic administration of AFQ056/Mavoglurant restores social behaviour in *Fmr1* knockout mice,** Behavioral Brain Research**,** 2013

4. **Pop AS,** Levenga J, de Esch CEF, Buijsen RAM, Nieuwenhuizen IM, Li T, Isaacs A, Gasparini F, Oostra BA, Willemsen R, **Rescue of dendritic spine phenotype in *Fmr1* KO mice with the mGluR5 antagonist AFQ056/ Mavoglurant,** Psychopharmacology, 2013

5. **Pop AS**, Gomez-Mancilla B, Neri G, Willemsen R, Gasparini F, **Fragile X syndrome: A preclinical review on metabotropic glutamate receptor 5 (mGluR5) antagonists and drug development,** Psychopharmacology, 2013, submitted

PhD portfolio

Summary of PhD training and teaching

Name PhD student: Andreea Simona Pop Erasmus MC Department: Clinical Genetics Research School: MGC	PhD period: 01.12.2008-30.04.2013 Promoter(s): Prof. dr. B.A. Oostra Co-promoter: Dr. R. Willemsen		
1. PhD training	**Year**	**Workload (Hours)**	**Workload (ECTS)**
General courses			
- Biomedical English Writing and Communication	2011		4 ECTS
- Laboratory animal science - Erasmus MC, Rotterdam	2009		3 ECTS
- Classical methods for Data-analysis Methodology	2010		5.7 ECTS
- Didactic skills	2010		1 ECTS
- Molecular and Cell Biology	2009		6 ECTS
Specific courses			
- From Development to Disease - Erasmus MC, Rotterdam	2009		1 ECTS
- Biomedical Research Techniques VIII- Erasmus MC, Rotterdam	2009		1.5 ECTS
- SPSS: Statistic Course for Novices - Erasmus MC, Rotterdam	2009		0.8 ECTS
- Safely working in the laboratory - LUMC, Leiden	2010	8 h	
Seminars and workshops			
- PhD workshop - Brugge	2009		1 ECTS
- Workshop on Photoshop and Illustrator CS4	2010		0.3 ECTS
- PhD Workshop - Cologne (oral presentation)	2010		2 ECTS
- Get out of your lab (GOL) days	2011		0.6 ECTS
- PhD Career Day (EPAR and EUR)	2012	4 h	
- Loopbaanorientatie workshop	2012	17h	
Presentations			
- In the department (2/year)	2008-2013	16 h	
- In the group (4-7/year)	2008-2013	48 h	

(Inter)national conferences			
- Endo-Neuro-Psycho meeting, Doortwerth, the Netherlands	2009		1 ECTS
- Conferences Jacques Monod "Mental Retardation: from genes to synapses, functions and dysfunctions" (poster presentation), Roscoff, France	2010		1 ECTS
- FRAXA 2011 Investigators Meeting, Boston, USA (oral presentation)	2011		2 ECTS
- FENS Meeting 2012, Barcelona, Spain (poster presentation)	2012		1 ECTS
Other			
- Weekly Wednesday Morning Meetings (Clinical Genetics Dept.)	2008-2013	200 h	
- Group work discussions (weekly)	2008-2013	400 h	
2. Teaching	**Year**	**Workload (Hours)**	**Workload (ECTS)**
Supervising practical and excursions, tutoring			
- Supervising HLO internship student	2009-2010	9 months	
Total		9 months & 693 h	31.9 ECTS

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Here I am after 4 years of PhD: thesis is finished and student time is over, or at least my time in the Department of Clinical Genetics, in Erasmus MC. But this is not the end, it's just a new beginning. New roads, new opportunities are hiding around the corner, and who knows what new adventures are waiting for me. The journey for self-accomplishment is never over, and each step we take just brings us closer. This is why I would like to thank every person I came across in my life so far for marking my development in one way or another. In the following pages I would like to express my thanks to some of these special people.

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Andreea