EXPERIMENTAL APPROACHES TOWARDS THERAPEUTIC INTERVENTIONS FOR FRAGILE X SYNDROME

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EXPERIMENTELE BENADERINGEN RICHTING THERAPEUTISCHE INTERVENTIES VOOR FRAGIELE X SYNDROOM

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LIST OF ABBREVIATIONS

7TMD	seven-helix containing transmembrane domain
ABC-C	aberrant behaviour checklist-community
ADHD	attention-deficit hyperactivity disorder
AFQ056	mGluR5 antagonist
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APRA	antibody-positioned RNA amplification
Arc	activity-regulated cytoskeleton-associated *protein*
BC RNA	brain cytoplasmic RNA
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
CGI-I	clinical global impressions-improvement
CNS	central nervous system
CREB	cAMP-response element binding
CYFIP	cytoplasmic FMRP-interacting protein
DHPG	(*S*)-3,5-Dihydroxyphenylglycine
DiI	1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
DIV	days *in vitro*
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGFP	enhanced green fluorescent protein
ERK	extracellular signal regulated kinase
FMR1	fragile X mental retardation 1
fMRI	functional magnetic resonance imaging
FMRP	fragile X mental retardation protein
FXR1P	fragile X related protein 1
FXR2P	fragile X related protein 2
FXS	fragile X syndrome
FXTAS	fragile X associated tremor/ataxia syndrome
GABA	gamma-aminobutyric acid
GAD	glutamatic acid decarboxylase
GPCR	G-protein coupled receptor
Gquartet	nucleic acid structure of four guanine residues
GSK3	glycogen synthase kinase 3
i.p. injection	intraperitoneal injection
ID	intellectual disability
KH domain	K-homology domain
KLC	kinesin light chain
KO	knockout
LAC	L-acetylcarnitine

LTD	long-term depression
LTP	long-term potentiation
Map1b	microtubule-associated protein 1b
MDMT	magnetic distance measurement technique
MEK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
miR	microRNA
MMP-9	matrix metalloproteinase-9
MPEP	2-methyl-6-(phenylethynyl)-pyridine hydrochloride
(m)RNA	(messenger) ribonucleic acid
mTOR	mammalian target of rapamycin
NES	nuclear export signal
NLS	nuclear localisation signal
NMDA	N-methyl-D-aspartic acid
NoS	nucleolar targeting signal
NUFIP	nuclear FMRP interacting protein
PAK	p21-activated kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDD (NOS)	pervasive developmental disorder (not otherwise specified)
POI	premature ovarian insufficiency
PP2A	protein phosphatase 2A
PPI	prepulse inhibition of acoustic startle response
Psd95	postsynaptic density protein-95
RBPs	RNA-binding proteins
RGG box	domain containing repeats of an arg-gly-gly (RGG) motif
RGS protein	regulator of G-protein signalling proteins
RISC	RNA-induced silencing complex
RNP	ribonucleoprotein
RRL	reticulocyte lysate
TSC1	tuberous sclerosis protein 1 or hamartin
TSC2	tuberous sclerosis protein 2 or tuburin
UTR	untranslated region
WT	wild type
YAC	yeast artificial chromosome

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THE *FMR1* GENE AND THE FUNCTION OF ITS PRODUCT FMRP

FRAGILE X SYNDROME

Fragile X syndrome (FXS) is one of the most common inherited forms of intellectual disability. It affects on average 1/4000 males and 1/7000 females. FXS was described for the first time in 1943 by Martin and Bell. They reported a family with an inherited form of mental retardation that was linked to a sex chromosome, hence mainly males were affected. In 1969, the syndrome was linked to the X chromosome. Karyotyping of cells from patients revealed a fragile site at the end of the long arm of the X chromosome at position q27.3. Finally, the gene involved in FXS was discovered in 1991. It was called fragile X mental retardation 1 (*FMR1*) gene (Verkerk et al., 1991).

1.1 FRAGILE X MENTAL RETARDATION GENE, *FMR1*

The *FMR1* gene is mapped on the X chromosome at position q27.3 and the entire locus spans approximately 40 kb of genomic sequence (Verkerk et al., 1991). The *FMR1* gene contains 17 exons and its mRNA is ~ 4 kb long. Exons 12, 14, 15 and 17 can be alternatively spliced, resulting in different mRNAs and protein isoforms (Verkerk et al., 1993; Sittler et al., 1996). Cloning of the *FMR1* gene revealed that the fragile site of the X chromosome contains a CGG repeat in the 5' untranslated region (UTR) of the gene (Verkerk et al., 1991). This CGG trinucleotide repeat is unstable, and therefore the repeat length is variable (polymorpyhic) in the normal population, ranging from 6-55 repeats. However, this repeat can become unstable upon maternal transmission, usually resulting in the expansion of the repeat in the next generation. When the repeat expands and the repeat length ranges from 55-200, the individuals are considered premutation carriers (see figure 1.1). Premutation carriers have an increased risk to develop fragile X-associated tremor/ataxia syndrome (FXTAS), and 20% of female carriers manifest premature ovarian insufficiency (POI) (Brouwer et al., 2009a). In patients with FXS, the CGG repeat has expanded above 200 units (full mutation) (Oberlé et al., 1991; Yu et al., 1991). Usually, a full mutation results in hypermethylation of the CpG site in the promoter region of the *FMR1* gene (Bell et al., 1991). Methylation of DNA promoter sequences is associated with gene silencing and can be accompanied by a number of modifications in histone N-tails (Chiurazzi and Neri, 2003). On the other hand, the promoters of actively transcribed genes typically have demethylated DNA and acetylated lysines in the N-tail of histones H3 and H4 (Liang et al., 2002; Iizuka and Smith, 2003). Although histone acetylation generally makes chromatin accessible to the transcription-activating machinery resulting in gene expression, one exception exists in which the acetylation of histone H4 at lysine 12, has been found in regions of silent heterochromatin; therefore histone acetylation is not always associated with active transcription. Pietrobono et al. showed that the earliest events in the cascade leading to the inactivation of an expanded *FMR1* gene seems to be loss of acetylation

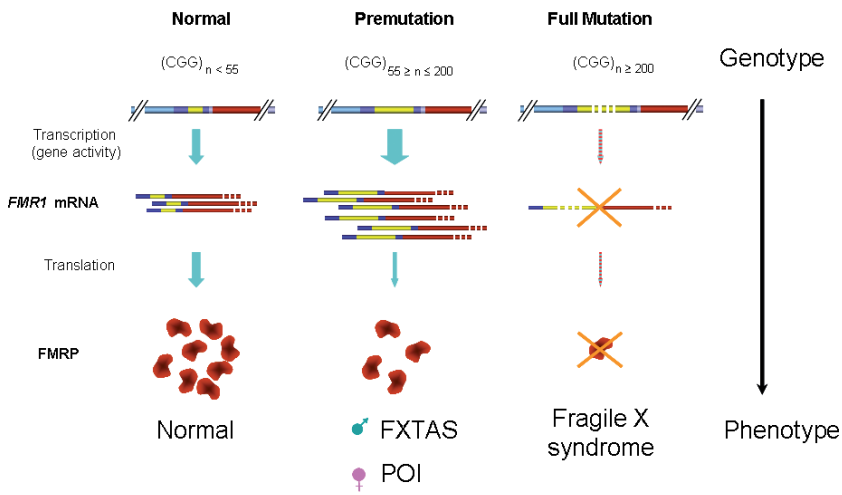

Figure 1.1. CGG repeat length, FMRP expression and clinical outcome. In unaffected individuals, the CGG repeat in the 5' UTR ranges between 5-55, leading to normal *FMR1* mRNA transcription and translation, and normal FMRP expression. Consequences of the expansion of the CGG repeat are depicted in the 2 columns on the right. The premutation (CGG repeat between 55-200 units) results in elevated *FMR1* mRNA transcription, but reduced FMRP expression. This increases the risk of developing FXTAS in males or premature ovarian insufficiency (POI) in females (Brouwer et al., 2009a). A full mutation (CGG repeat over 200 units) leads to silencing of the *FMR1* gene due to hypermethylation. As a consequence, the protein product of this gene, FMRP, is lacking which results in fragile X syndrome.

of histone 3 (H3) and histone 4 (H4), histone deacetylation and an increase in H3-K9 methylation, which are followed by DNA methylation and H3-K4 demethylation (Pietrobono et al., 2005). Additionally, using human embryonic stem cells, it has also been demonstrated that *FMR1* inactivation is initiated by downregulation of transcription and chromatin modifications prior to DNA methylation (Eiges et al., 2007). Typically, hypermethylation of the promoter region of the *FMR1* gene results in sustained transcriptional silencing, leading to fragile X mental retardation protein (FMRP) deficiency and intellectual disability in patients with FXS. Although different hypotheses have been postulated about CGG repeat instability, the exact mechanism is still not fully understood (Brouwer et al., 2009b).

The *FMR1* gene is highly conserved during evolution. The gene has also two autosomal paralogs, called fragile X-related gene 1 and gene 2 (*FXR1* and *FXR2*), which are mapped to chromosomes 3q28 and 17p13, respectively. Together, these three genes belong to the *FXR* family. Sequence analysis of these paralogs revealed high conservation, especially in the functional domains, and an overlap in tissue distribution (Siomi et al., 1995; Zhang et al., 1995; Tamanini et al., 1997; Bakker et al., 2000). To date, the cellular functions of both FXR1P and FXR2P are still less understood, although

it has been suggested that both proteins have a similar function as FMRP. All three paralogs are present in all vertebrates, whereas *Drosophila* only contains one single related gene, called *dFmr1* (or *dFxr1).* FMRP, FXR1P and FXR2P are ubiquitously expressed, although they predominate in brain and testes. Interestingly, FXR1P also seems to play an important role in striated muscle tissue and is the only family member that shows high expression in heart and skeletal muscle tissue (Mientjes et al., 2004).

1.2 COMPOSITION OF THE BRAIN

Lack of FMRP expression causes mental retardation, and therefore it is important to study the function of FMRP in the brain. The brain is mainly composed of two cell types: neurons and glia. Glia cells can be divided in different types, such as astrocytes and oligodendrocytes, each with different functions, including metabolic support and insulation. Neurons are polarised cells, with a dendrite to receive and an axon to transmit signals. Through axons, neurons transmit signals to each other in the form of electrochemical pulses, called action potentials. The junction at which the axon contacts another neuron is called a synapse. The synapse consists of a presynaptic compartment at the end of the axon, and a postsynaptic compartment, the structure which receives the stimulus. When an action potential has travelled along the axon and arrives at the synapse, neurotransmitters will be released to transmit the signal. Neurotransmitters are small molecules, such as glutamate or acetylcholine, that can bind to receptors at the postsynaptic membrane. The binding of the neurotransmitter to the postsynaptic receptor results in signal transmission and can induce synaptic plasticity, which is the ability of a synapse between two neurons to change in strength. Synaptic strength can be defined as the change in transmembrane potential resulting from activation of the postsynaptic neurotransmitter receptors. Several underlying mechanisms exist that cooperate to achieve synaptic plasticity, including changes in the quantity of neurotransmitters released into the synaptic cleft and changes in the effectiveness with which cells respond to those neurotransmitters.

Protein synthesis is necessary to achieve long-term strengthening or weakening of the synapse (Goelet et al., 1986). However, the requirement for local protein synthesis at the synapse, as opposed to transport of cytoplasmic proteins, remained unclear for a long time. In the early 1980s, evidence for dendritic localised RNAs and ribosomes was demonstrated by different laboratories (Colman et al., 1982; Steward and Levy, 1982), revealing that the protein synthesis machinery and mRNAs were not only present in the cell soma, but also in the dendrites and in postsynaptic compartments of neurons. Using translational inhibitors in synaptic plasticity experiments, it has been demonstrated that rapid dendritic protein synthesis is very important to induce long-lasting changes after synaptic activation (Weiler and Greenough, 1993; Kang and

Schuman, 1996). It has been demonstrated that FMRP, the protein that lacks in FXS, plays an important role in local protein synthesis.

In brain tissue, FMRP is mainly present in the cell soma of neurons and is packed together with other proteins and mRNAs in ribonucleoprotein (RNP) particles. Significant quantities of these FMRP-positive RNP particles are transported into the dendrite, and during development a minority of the protein is localised in the axons (Antar et al., 2006; Christie et al., 2009).

FMRP in the neurons

In neurons, many different types of cytoplasmic granules exist, i.e. stress granules, P-bodies and RNA-granules . Granules are small packages within the cell, and FMRP is mainly present in these cytoplasmic granules (reviewed in Kiebler and Bassel (Kiebler and Bassell, 2006)). Briefly, stress granules are cytoplasmic foci where untranslated mRNAs accumulate when cells are subjected to a variety of environmental stressors. Processing bodies (P-bodies) on the other hand are sites where decapping and mRNA decay occur. P-bodies are also important in microRNA processing and contain the ribonucleoprotein RNA-induced silencing complex (RISC). Finally, RNA-granules harbour translationally silenced mRNAs, and in neurons (dendritic) RNA-granules are transported to synapses. These dendritic RNA-granules can be divided in two types: RNA-granules that contain ribosomal subunits (RNP particles) and RNA-granules that do not contain ribosomal subunits (transporting granules) (Kiebler and Bassell, 2006).

Unfortunately, it is still unclear in which types of granules FMRP is exactly present. Several studies showed that FMRP is required for stress granule formation and is also present in stress granules (Mazroui et al., 2002; Didiot et al., 2009), whereas others demonstrated that FMRP is mainly present in RNP particles or RNA-granules (Feng et al., 1997a; De Diego Otero et al., 2002; Antar et al., 2005; Levenga et al., 2009). This suggests that the localisation in the cell or the state of the cell determines in which type of granule FMRP is present. Furthermore, it has been reported in *Drosophila* that dFmrp is present in P-bodies and is involved in microRNA processing (Barbee et al., 2006). Although the exact function of FMRP in these different types of granules is still unknown, FMRP seems to display many different functions in neurons, including repression of mRNA translation during dendritic transport and at the synapse.

Besides in neurons, FMRP is also developmentally expressed in astrocytes (Pacey and Doering, 2007). The presence of FMRP in astrocytes during development may be essential for the role of astrocytes in synaptogenesis. Recently, it has been demonstrated that FMRP expression in astrocytes is indeed important for shaping the dendritic arbours of neurons in FXS (Jacobs and Doering, 2010). These results suggest a functional role of astrocytic FMRP in the neurobiology of FXS.

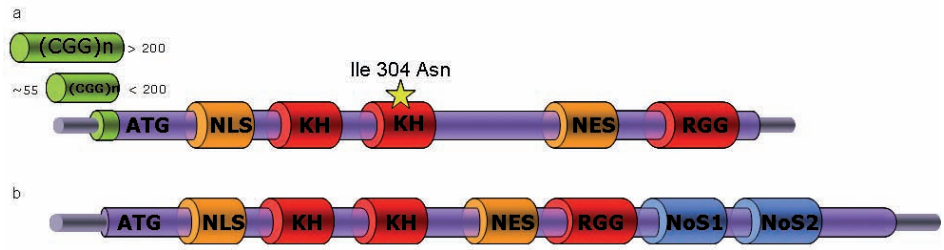

Figure 1.2. Schematic representation of the functional domains of FMRP and its paralogues FXR1P and FXR2P. a) Nuclear-cytoplasmic shuttling activity of FMRP is mediated by its NLS and NES. The two KH domains and the C-terminal RGG box are the three RNA-binding domains of FMRP. A missense point-mutation (yellow star) in the second KH domain has been found in a severe fragile X patient. b) Schematic representation of FXR1P and FXR2P. In contrast to FMRP, both paralogues also code for two Nucleolar-targeting signals (NoS).

1.3 FMRP DOMAINS

FMRP contains several functional domains that partly illustrate the function of the protein (see figure 1.2). In the N-terminus of the protein, a nuclear localisation signal (NLS) has been identified (Eberhart et al., 1996). By studying truncated versions of FMRP, it was demonstrated that the first 167 amino acids were sufficient for nuclear localisation. Soon after it was shown that the NLS activity was localised in the region between amino acid residues 114-150 (Bardoni et al., 1997). FMRP also contains a nuclear export signal (NES) encoded by exon 14 (Eberhart et al., 1996; Fridell et al., 1996; Sittler et al., 1996). The presence of these domains suggests that FMRP can shuttle between the nucleus and cytoplasm.

In addition to the nuclear transport domains, FMRP harbours three RNA-binding domains, two tandem KH-type domains (hnRNP K homology), and an arginine- and glycine-rich RNA-binding domain (RGG box) (Ashley et al., 1993; Siomi et al., 1993). Furthermore, the N-terminal domain of FMRP shows similarities with Tudor/Agenet domains that may also be involved in both RNA-binding and protein-protein interactions (Adinolfi et al., 2003; Mazroui et al., 2003; Ramos et al., 2006; Reeve et al., 2008). The KH domain is an evolutionary-conserved sequence motif, originally identified in heterogeneous nuclear ribonucleoprotein K (hnRNP K). The first KH domain of FMRP is encoded by exons 8-10, the second one by exon 13. It has been demonstrated that the second KH domain binds to an RNA sequence forming the "kissing complex", although to date no target mRNAs have been identified using this KH domain (Darnell et al., 2005). Moreover, the second KH domain is of special interest after the identification of a patient with FXS carrying a missense point mutation (I304N) in the second KH domain (De Boulle et al., 1993). The mutation Ile304Asn concerns a highly conserved hydrophobic amino acid change into an asparagine. This pathogenic mutation seems to result in: (i) complete or partial impairment of the

RNA-binding properties of the protein (Siomi et al., 1994), (ii) loss of association of the protein with actively translating polyribosomes (Feng et al., 1997a) and (iii) failure of binding a specific kissing complex RNA-sequence (Darnell et al., 2005).

The RGG box is an arginine- and glycine-rich region (RGG) which was first identified in the hnRNPU protein. The RGG box of FMRP is encoded by exon 15 and recognises specific mRNAs containing a G-quartet structure *in vitro* (Darnell et al., 2001; Schaeffer et al., 2001). The G-quartet is a 4-stranded nucleic acid structure formed by the interaction of four guanine residues in one plane by Hoogsteen-type hydrogen bonding. Such G-quartet structures are found in several target mRNAs of FMRP, like *Map1b*, *Psd95* and *Fmr1* (Brown et al., 2001). Recently, it has been demonstrated that another type of RNA-motif might bind to the RGG box, called the triple stem-loop "SoSlip" (Bechara et al., 2009).

Except binding to mRNAs, FMRP also has the capacity to bind several proteins. Using yeast two-hybrid or co-immunoprecipitation techniques, several FMRP-interacting proteins have been identified, including FXR1P, FXR2P, NUFIP (Nuclear FMRP interacting protein 1), CYFIP (cytoplasmic FMRP-interacting protein) and KIF3C kinesin motor protein (Zhang et al., 1995; Bardoni et al., 1999; Schenck et al., 2001; Davidovic et al., 2007). Each interaction seems to have different functions. It has been proposed, for example, that FMRP functions as an adaptor between KIF3C and the cargo of this motor for transport into the dendrite (Davidovic et al., 2007). By contrast, Dictenberg et al. could not confirm binding of FMRP to KIF3C by IP, but reported that FMRP binds directly or indirectly to another cargo adaptor, Kinesin Light Chain (KLC) (Dictenberg et al., 2008). The other FMRP-interacting proteins might function in the stability or affinity of FMRP for different mRNAs.

1.4 FMRP: DIVERSITY OF ACTION

A major challenge in fragile X research is to understand the physiological cellular function of FMRP. Many different hypotheses have been postulated.

RNA binding and nuclear transport

After it was found that FMRP contains nuclear shuttling domains (NLS and NES) and three RNA binding domains, it has been suggested that FMRP plays an important role in binding specific target mRNAs in the nucleus. Using electron microscopy, it has been shown that FMRP is present in the nucleus (Willemsen et al., 1996; Feng et al., 1997b; Bakker et al., 2000) and nuclear export blockade with leptomycin B resulted in accumulation of FMRP in the nucleus (Tamanini et al., 1999). Moreover, abolishing the NES signal of FMRP by two point mutations also resulted in nuclear accumulation of FMRP (Fridell et al., 1996). More recently, it has been shown in amphibian oocytes that FMRP indeed can bind mRNAs in the nucleus (Kim et al., 2009).

Several approaches have been taken to identify specific mRNA targets of FMRP. The first approach revealed that FMRP binds approximately 4% of total brain mRNAs and has preferences for a G-quartet-containing or U-rich sequence (Brown et al., 2001; Schaeffer et al., 2001; Chen et al., 2003). However, the specificity of these methods is subject of debate (will be discussed in chapter 7). To identify specific FMRP mRNA targets, Miyashiro et al. used a new technique called "antibody-positioned RNA amplification" (APRA) (Miyashiro et al., 2003) and identified new specific target mRNAs in cultured hippocampal neurons. Unfortunately, not many similarities were found after comparing target mRNAs identified using different approaches. Therefore, additional research is necessary to elucidate specific mRNA targets of FMRP.

In conclusion, the presence of different RNA-binding domains suggests that FMRP can bind specific mRNA targets, most likely in the nucleus, and is subsequently packaged into ribonucleoprotein (RNP) granules.

Dendritic mRNA transport

In neurons, FMRP has been found in dendritic RNA-granules, but recently axonal RNA-granules positive for FMRP were also identified (Antar et al., 2006; Christie et al., 2009). The role of FMRP in RNA-granules has been studied extensively. The transport of FMRP-containing granules has been demonstrated to be microtubule-dependent, with the same speed as that of general dendritic RNA transport measured by time-lapse confocal microscopy (De Diego Otero et al., 2002; Antar et al., 2005). When microtubules were disrupted by nocadazole treatment, FMRP-positive RNA-granules became immobile, whereas disruption of the actin network had no effect (De Diego Otero et al., 2002). Because FMRP binds mRNAs and is found in dendritic RNA-granules, it is hypothesised that FMRP is important for the transport of its target mRNAs into the dendrite. Electrical stimulation is known to induce transport of specific mRNAs such as *CaMKII* and *Arc*, which are both target mRNAs of FMRP. Therefore, it was suggested that lack of FMRP resulted in differences in the amount of these target mRNAs in the dendrites at basal or after stimulation. However, Steward et al. found no differences in the amount of these target mRNAs in the *Fmr1* KO hippocampus at basal state or after stimulation (Steward et al., 1998). In contrast, Dictenberg et al. did find differences in the amount of dendritic target mRNAs in *Fmr1* KO hippocampal neurons after chemical stimulation of group I metabotropic glutamate receptors (mGluRs) with DHPG (dihydroxyphenylglycine), whereas no differences were observed at basal state (Dictenberg et al., 2008). After stimulation with DHPG, fluorescent *in situ* hybridisation (FISH) showed enhanced signals for *CaMKII, MAP1B, SAPAP4* and $GABA_A$-*R*-δ mRNAs in the dendrites of wildtype hippocampal neurons, while in *Fmr1* KO neurons the signals did not differ compared to unstimulated conditions. It was also shown that the RNA-granules were less motile in *Fmr1* KO neurons (Dictenberg et al., 2008). These

results suggest that FMRP is at least partially involved in activity-dependent dendritic transport of its target mRNAs, however more research is necessary to define the exact function of FMRP in mRNA transport.

FMRP as a translational repressor

The majority of FMRP is present in the cytoplasm, associated with elongated polyribosomes in large messenger ribonucleoprotein (mRNPs) particles (Feng et al., 1997a; Khandjian et al., 2004). The importance of this association of FMRP with polyribosomes has been demonstrated in a severely mentally retarded patient with the missense mutation I304N in the second KH domain of FMRP (De Boulle et al., 1993). As a consequence of this mutation, FMRP (i) is unable to bind to the "kissing-complex" RNA sequence, (ii) can no longer associate with polyribosomes (Darnell et al., 2005), and (iii) is predominantly found in small RNP particles (Feng et al., 1997a; Schrier et al., 2004; Wang et al., 2008; Levenga et al., 2009). It has been postulated that FMRP is associated with polyribosomes to mediate translation of target mRNAs at the synapse. *In vitro* experiments have demonstrated that FMRP acts as a translational repressor in rabbit reticulocyte lysates (RRL) in a dose-dependent manner (Laggerbauer et al., 2001; Li et al., 2001). Moreover, local translation in the spines after mGluR activation seems to be regulated by FMRP as well (Weiler et al., 2004). The exact mechanism of translational repression is not fully understood, and different mechanisms involved in repression of translation have been proposed, including the phosphorylation status of FMRP and the association with *Brain Cytoplasmic* (BC1) RNA and/or the microRNA pathway.

Recently, it has been demonstrated that FMRP acts as a translation repressor in a phosphorylation-dependent mechanism (Narayanan et al., 2007; Narayanan et al., 2008) (figure 1.3). Initially, it was shown that FMRP can be phosphorylated specifically at serine 499 (Ceman et al., 2003). The phosphorylation status of FMRP influences the translation of target mRNAs since phosphorylated FMRP is associated with stalled polyribosomes (repression of translation) and unphosphorylated FMRP with actively translating polyribosomes (Ceman et al., 2003). Subsequently, it has been shown that FMRP is rapidly dephosphorylated by PP2A after specific stimulation at the synapse, probably resulting in local protein synthesis of target mRNAs (Narayanan et al., 2007). Simultaneously, after specific synaptic stimulation, a second cascade involving TSC1/TSC2 and the mTOR pathway is activated, which ultimately results in re-phosphorylation of FMRP by S6 kinase (Narayanan et al., 2008).

Although this hypothesis is an attractive model, it has been proposed as an alternative hypothesis that FMRP regulates translation at the initiation stage. Zalfa et al. has reported that FMRP is associated with light mRNPs, in which polyribosomes are absent (Zalfa et al., 2003). They also found FMRP to be associated with *BC1* RNA,

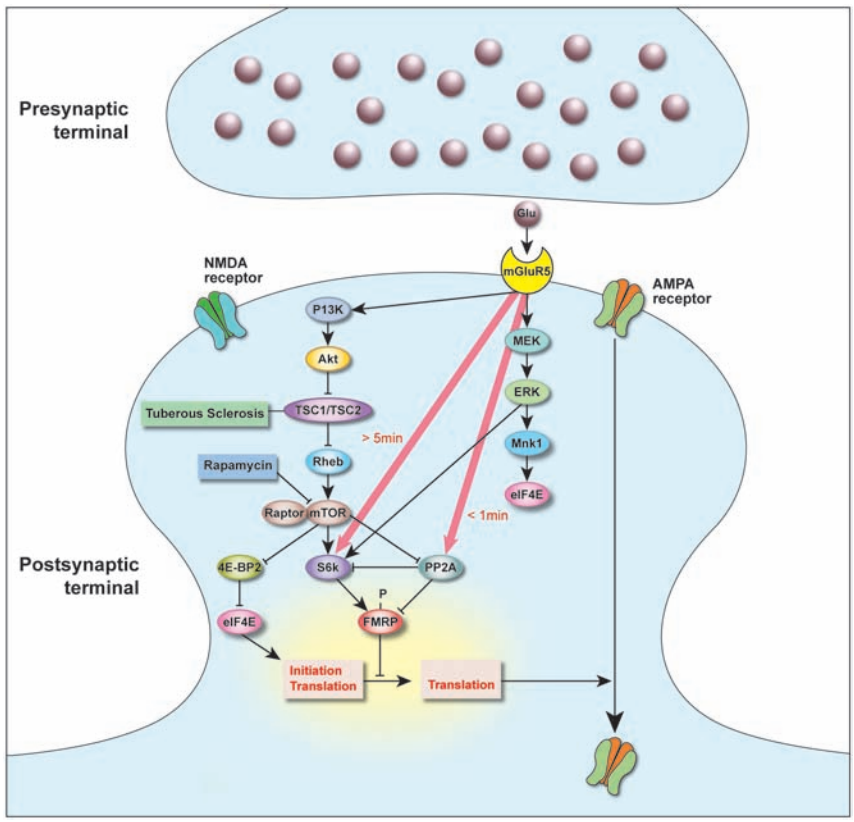

Figure 1.3. The mTOR signalling pathway. The two signalling pathways downstream of mGluR5 that affect translation and the MEK-ERK-Mnk1 and the PI3K-mTOR pathway (Richter and Klann, 2009). Activation of mTOR is one of the primary triggers for the initiation of cap-dependent translation via phosphorylation of 4E-BP and S6K. After stimulation of mGluR5, PI3K phosphorylates the membrane phospholipid PIP2, converting it to PIP3. PIP3 recruits Akt to the membrane where it is phosphorylated and activated by PDK1. Akt activates mTOR by inhibiting TSC, a heterodimer of TSC1 and TSC2. TSC2 contains a GAP domain for the small G-protein Rheb. When TSC2 is phosphorylated, its GAP activity decreases, resulting in Rheb and mTOR activation. Subsequently, mTOR interacts with Raptor, which binds both 4E-BP and S6K. Active mTOR can phosphorylate S6K resulting in the phosphorylation of S6. In addition, active mTOR phosphorylates 4E-BP. Unphosphorylated 4E-BP binds tightly to eIF4e, whereas phosphorylated 4E-BP does not, thereby permitting eIF4F to form a translation initiation complex. Phosphorylation of S6 and 4E-BP finally results in mRNA translation. The role of FMRP in the mTOR pathway is indicated. Upon mGluR5 stimulation, PP2a activity is enhanced (<1 minute), which rapidly dephosphorylates FMRP so mRNAs are translated. Extended mGluR5 activation (>5 minutes) suppresses PP2a activity and FMRP is phosphorylated by S6K. Phosphorylated FMRP represses the translation of target mRNAs. Abbreviations: MEK, Mitogen-activated protein kinase kinase; ERK, Extracellular signal regulated kinase; Mnk1, Mitogen-activated protein kinase interacting serine/threonine kinase 1; PI3K, phosphoinositide-3 kinase; 4E-BP, 4E-binding protein; S6K, S6 kinase; PIP2, phoshatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PDK1, 3-phosphoinositide-dependent kinase 1; TSC, tuberous sclerosis complex; GAP, GTPase-activating protein.

which is a 200 bp long non-coding RNA that is involved in initiation of translation (Kindler et al., 2005). It was proposed that FMRP binds directly to *BC1* RNA to facilitate the binding of target mRNAs to FMRP, resulting in translational suppression. In line with this evidence, it has been shown recently that FMRP regulates synaptic translation at the level of translation initiation (Napoli et al., 2008). FMRP inhibits translation initiation through an interacting factor CYFIP1, which in turn binds to the initiation factor IF4E. In this study, *BC1* RNA increases the affinity of FMRP for the CYFIP1-eIF4E complex in the brain. However, different studies argued against these results: (i) FMRP is mainly present in the polyribosomal fractions (Feng et al., 1997a; Davidovic et al., 2005), (ii) *BC1* did not directly bind to target mRNAs of FMRP (Schuett et al., 2009), and (iii) in absence of *BC1* RNA, FMRP was still able to bind to the target mRNAs (Khandjian et al., 2004; Iacoangeli et al., 2008).

MicroRNA pathway

The last hypothesised mechanism of FMRP-dependent regulation of mRNA translation might be in cooperation with the microRNA pathway. Several studies have suggested that FMRP acts as a translational repressor in a microRNA-dependent way. MicroRNAs (miRs) are small single-stranded RNAs of ~20 nucleotides long that can bind to specific mRNAs. The binding of miRs to mRNAs can result in either degradation or translational silencing of the mRNA (Filipowicz et al., 2008). MiRs are transcribed by RNA polymerase II, resulting in pri-miRs. In the nucleus, pri-MiRs are processed by RNAse III endonuclease Drosha. Drosha cleaves the pri-MiR into pre-miR, which is then exported by exportin5 to the cytoplasm. In the cytoplasm, the pre-miR is processed by Dicer/Argonaute 2 complex to generate a mature miR. Next, the mature miR is selected by the ribonucleoprotein RNA-induced silencing complex (RISC). Subsequently, RISC mediates sequence-specific translational repression or degradation, depending on an imperfect or perfect miRNA:RNA base pairing.

Multiple studies have suggested that FMRP plays a role in the miRNA pathway. The first indications came from *Drosophila* studies, where it was shown that dFmrp interacts directly or indirectly with two components of the RISC complex, Argonaute 2 (AGO2) and Dicer (Caudy et al., 2002; Ishizuka et al., 2002). In mammalian cells, FMRP seems to be incorporated in RNP particles that also contain Ago2, Dicer and miRNAs (Jin et al., 2004), and also in postsynaptic compartments FMRP seems to interact with Dicer and Ago2 (Lugli et al., 2008). Although the exact function of FMRP in the miR pathway is still unclear, it has been suggested that FMRP acts as a miRNA acceptor protein for Dicer, facilitating the miRs' assembly on specific target RNA sequences (Plante et al., 2006). However, in contrast to these results, Didiot et al. could not confirm a direct link between FMRP and RISC (Didiot et al., 2009). The involvement of FMRP in the microRNA pathway might explain the mechanism of translational repression by FMRP.

Furthermore, the phosphorylation status of FMRP also seems to play a role in miR pathway (Cheever and Ceman, 2009b). In this model, however, it has been proposed that miRs are important to activate mRNA translation, as opposed to repressing translation (Cheever and Ceman, 2009a). Unphosphorylated FMRP associates with Dicer, through a protein-protein interaction, and this Dicer-containing complex processes pre-miRs into mature miRs. The mature miRs bind FMRP's target mRNA to induce translation. The FMRP-Dicer binding was found to be abolished by phosphorylation of FMRP, and pre-miRs are not processed into mature miRs. Without activating miRs, translation of the target mRNA cannot occur. This suggests that the phosphorylation of FMRP indirectly suppresses translation by decreasing miRNA production through loss of Dicer binding.

More recently, FMRP was found to interact with specific miRs resulting in translational repression of corresponding target mRNAs (Edbauer et al., 2010). The results show that FMRP interacts with miRNA125b and thus regulates the translation of NMDA receptor subunit 2A (Edbauer et al., 2010). Evidence for the relationship between the miRNA pathway and FMRP is increasing. However, the exact role of FMRP in the miRNA pathway needs to be further investigated.

In conclusion, deficiency of FMRP seems to result in aberrant protein synthesis at the synapse and, as a consequence, in altered signal transmission between neurons. Although it is still unclear which specific proteins show elevated expression, there are some indications, such as Map1b, Psd95 and Nr1 (Lu et al., 2004; Zalfa et al., 2007; Schuett et al., 2009). All together, it seems that FMRP is important for stimulus-dependent transport and silencing of target mRNAs into the dendrite, and for repressing translation of the target mRNAs at the synapse until the corresponding proteins are needed (overview figure 1.4).

1.5 FMRP AND SPINE ABNORMALITIES

On pyramidal neurons in the cerebral cortex, excitatory synapses terminate at spines, which are short protrusions joined to a dendrite by a thin neck. Spines are specialised dendritic protrusions, implicated in learning and memory through synaptic plasticity (Kasai et al., 2010). Protrusions can be divided into two main classes: i) immature spines or filopodia, and ii) mature spines. Filopodia are long and thin protrusions that typically lack "heads" (or have very small heads), whereas spines have a distinct head and neck.

Microscopic analysis of autopsy material from patients with FXS revealed no gross morphological abnormalities in brain tissue. However, it was found that in some brain areas the dendrites of neurons show a higher protrusion density than neurons of control individuals and the dendritic spines exhibited a more immature phenotype (Hinton et al., 1991; Irwin et al., 2001). Due to the increased number and abnormal

morphology of the protrusions, the balance in signal transmission is believed to be altered, resulting in the FXS phenotype. Interestingly, several mental retardation syndromes show an abnormal protrusion phenotype, including Down syndrome and Rett syndrome (Purpura, 1974; Kaufmann and Moser, 2000; Fiala et al., 2002). Generation of the first *Fmr1* KO mouse model finally allowed examination of *in vivo* protrusion morphology in different brain areas at different ages (the *Fmr1* KO mouse model will be introduced in the next paragraph). Comery et al. quantified dendritic protrusions of layer V pyramidal neurons in the visual cortex of adult *Fmr1* KO mice (Comery et al., 1997). These neurons have more elongated protrusions and fewer shorter protrusions. Unfortunately, the genetic FVB background of the mice used in this study might interfere with the protrusion morphology in the visual cortex, since FVB mice usually carry a mutation that causes retinal degeneration. Therefore, Greenough and colleagues extended this examination by classifying eight classes of protrusions, ranging from immature to mature, and quantified the protrusions in mice in a C57BL/6 background (Irwin et al., 2002). The visual cortex of *Fmr1* KO mice in the Bl6 background also showed protrusion abnormalities, although less dramatic than *Fmr1* KO mice in the FVB background. Furthermore, the number of protrusions was not significantly different between WT and *Fmr1* KO mice in Bl6 background.

Nimchinsky et al. showed that elongated protrusions in the *Fmr1* KO mice were only present in the somatosensory cortex at young ages, i.e. 1 week and 2 weeks postnatal, whereas the abnormal morphology disappeared at 4 weeks of age (Nimchinsky et al., 2001). *Fmr1* KO mice also showed significantly more protrusions in young animals. These data strongly suggest that differences in spine morphology and number are related to development. Therefore, the spine morphology was examined again in younger and older *Fmr1* KO and wildtype mice in a C57BL/6 background (25 days and 75 days, respectively) (Galvez and Greenough, 2005). It was demonstrated that at P25 minimal protrusion abnormalities (expressed in length, morphology and number) were present in the somatosensory cortex of *Fmr1* KO mice, whereas the abnormalities "reappeared" at 75 days of age, in accordance with Nimchinsky et al. Thus, at 75 days, dendritic protrusions of *Fmr1* KO neurons in the somatosensory cortex are significantly longer, have a more immature morphology, and show increased density per apical dendrite. Subsequently, the group of Greenough also investigated the CA1 region of the hippocampus (Grossman et al., 2006). Pyramidal neurons in the hippocampus of *Fmr1* KO mice showed more immature protrusions and fewer shorter protrusions, but the number of protrusions was not significantly different. Furthermore, cultured hippocampal primary neurons of *Fmr1* KO showed protrusion abnormalities, illustrated by an increased number of immature protrusions and a decreased number of synapses (Antar et al., 2006), more filopodia (immature protrusions) (De Vrij et al., 2008), and increased length of protrusions (chapters 5 and 6).

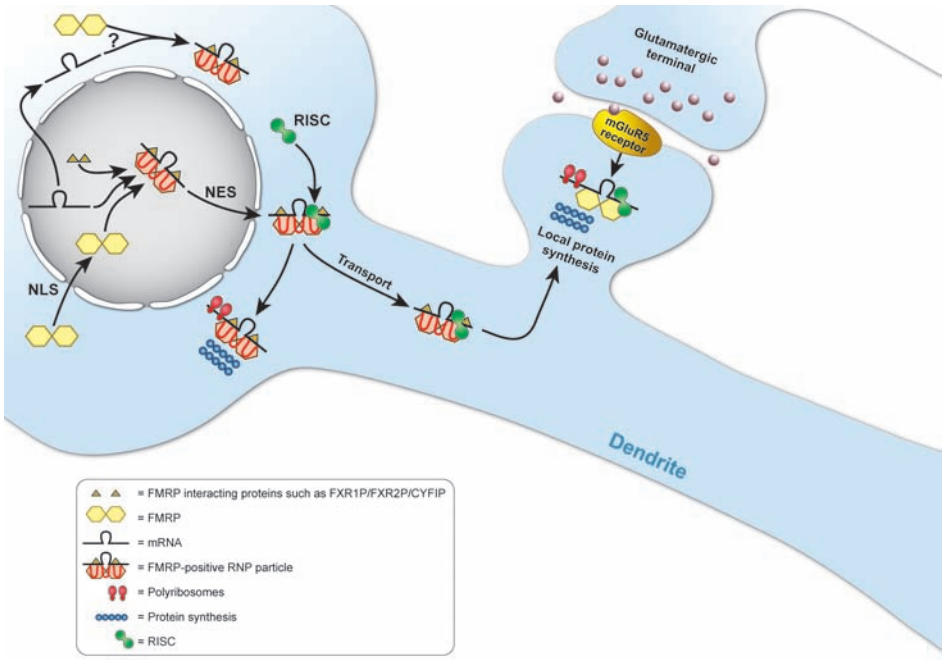

Figure 1.4. Schematic model of the function of FMRP in neurons. FMRP is synthesised in the cell soma. FMRP (yellow hexagons) enters the nucleus via the NLS domain. In the nucleus, FMRP will bind to target mRNAs and other proteins, forming an RNP particle. The FMRP-positive RNP particle is transported back to the cell soma, probably via the NES of FMRP. Once in the cell soma, the FMRP-positive RNP particle may interact with members of the RNA-induced silencing complex (RISC; green circle) and also associate with ribosomes (red ovals). FMRP regulate protein synthesis (string of blue circles) in the cell soma of the neuron. Furthermore, a significant proportion of the FMRP-positive RNP particles is packed in RNA-granules which subsequently are transported into the dendrites. During transport, FMRP fulfils its major role as a translational repressor of target mRNAs. Upon synaptic stimulation of group I mGluRs, FMRP allows local translation at the synapse of its mRNA targets. The proteins that are locally synthesised seem to be involved in the internalisation and recycling of AMPA receptors, proposing a role for FMRP in learning and memory.

Although there is some inconsistency concerning the protrusion morphology due to the use of different methods and mouse strains, overall the combined data show an immature spine phenotype in *Fmr1* KO mice (table 1.1). The protrusion phenotype found in *Fmr1* KO mice has been used to examine genetic (Dolen et al., 2007; Hayashi et al., 2007) and pharmacological therapeutic interventions (Bilousova et al., 2008; De Vrij et al., 2008) (chapter 2).

1.6 ANIMAL MODELS FOR FXS

Different FXS animal models have been generated to study the effects of a lack of FMRP on behaviour, mRNA translation, and pharmacological interventions. The animal models are not identical to the human genotype, because in the latter case the silencing of the gene is due to methylation of an expanded CGG repeat, whereas the animal models were generated using genetic modification resulting in a (almost) complete loss of *Fmr1* transcription and therefore total lack of Fmrp expression.

The *Fmr1* KO mouse model

After cloning and identification of the *FMR1* gene and its mouse orthologue, it became feasible to generate an *Fmr1* KO mouse model. This is the most widely used animal model to study FXS. The first *Fmr1* KO mouse model was created by interrupting exon 5 with a neomycin cassette (Bakker et al., 1994). The neomycin insertion results in almost complete loss of *Fmr1* mRNA transcription and total lack of Fmrp expression. Although numerous studies have shown that these *Fmr1* KO mice do not express Fmrp, the *Fmr1* promoter remains intact and aberrant transcription, presumably driven by the Fmr1 promoter, can be found, producing an abnormal RNA species (Bakker et al., 1994 ; Mientjes et al., 2006). Therefore, a second *Fmr1* KO mouse model was created by deleting the promoter and the first exon, and therefore this mouse lacks detectable *Fmr1* mRNA transcripts and Fmrp expression (Mientjes et al., 2006).

The *Fmr1* KO mice have been studied extensively and are found to be a useful animal model to study FXS. The *Fmr1* KO mice show increased testicular weight, similar to male patients with FXS (Bakker et al., 1994; Mientjes et al., 2006). In addition, numerous behavioural tests have been performed to investigate behavioural deficits in the *Fmr1* KO mice. Although not all studies show consistent results, it has been reported that *Fmr1* KO mice show deficits in spatial learning, defect of prepulse inhibition of acoustic startle response (PPI), and increased locomotor activity (Bakker and Oostra, 2003; Kooy, 2003; De Vrij et al., 2008). Anxiety is also a main symptom of patients with FXS, but this phenotype is more difficult to study in mice. Some studies suggest that *Fmr1* KO mice are less anxious in the open field test (Bakker et al., 1994; Restivo et al., 2005), whereas other contributions propose that they are more anxious in a social context (Mineur et al., 2002). As mentioned above, *Fmr1* KO mice seem to exhibit abnormal spine morphology in different brain areas, similar to what is found in patients with FXS. Moreover, this animal model has offered the opportunity to study synaptic plasticity in the brain. As will be discussed in chapter 2, the synaptic plasticity is altered in many brain areas, such as hippocampus, cortex, and cerebellum.

The *Drosophila Melanogaster* FXS model

In the beginning of this century, the *Drosophila Melanogaster* orthologue of FMRP, dFmrp, was cloned and characterised (Wan et al., 2000; Zhang et al., 2001). dFmrp displays considerable amino acid sequence similarities with the vertebrate FMRP, FXR1P and FXR2P. It exhibits many functional properties comparable to the function of FMRP, such as RNA-binding capacity and repression of translation, and it is required for modelling neurons in the nervous system (Wan et al., 2000). However, it is debated whether the functional properties of dFmrp are more comparable to FMRP or to one of the two orthologues, FXR1P or FXR2P. Sequence comparison showed that dFmrp has 56% overall similarity to FMRP, 65% to FXR1P, and 65% to FXR2P (Zhang et al., 2001). This suggests that the function of dFmrp is more related to FXR1P and FXR2P than to FMRP. However, Coffee et al. recently demonstrated that dFmrp has a conserved neuronal function which is not shared with FXR1P and FXR2P (Coffee et al., 2010), which suggests that *FMR1* developed a specific neuronal function in evolution that cannot be compensated by either *FXR1* or *FXR2*.

After identification of the fly orthologue, *dFmr* null mutant fly models were generated and shown to be viable (Zhang et al., 2001). The *dFmr* null mutant flies are anatomically normal, but show altered behaviour. It has been demonstrated that dFmrp deficiency results in abnormal circadian rhythm (Bushey et al., 2009), locomotor defects (Zhang et al., 2001), and altered courtship behaviour (McBride et al., 2005). A closer look at the central nervous system also revealed morphological abnormalities of neurons. Loss of dFmrp resulted in mushroom body defects and altered axonal branching (Zhang et al., 2001).

The zebrafish FXS model

Danio Rerio, or zebrafish, is a very convenient animal model to study embryonic development in a vertebrate system. Within 24 hours, all organs are developed, and in 90 days the zebrafish will mature. In addition, the embryos develop outside the mother and are transparent, allowing direct observation of their development. The zebrafish genome codes for all three orthologues of FMRP, FXR1P and FXR2P (Engels et al., 2004; Tucker et al., 2004; van 't Padje et al., 2005). To study the function of FMRP during development, one of the approaches which can be used is the morpholino antisense oligonucleotide knockdown technique. Injecting an antisense oligonucleotide morpholino in fertilised eggs will result in a transient knockdown of the target gene, i.e. *Fmr1*. Using this approach, an FXS phenotype has been observed including abnormal axonal branching, neuronal guidance, and defasciculation defects (Tucker et al., 2006). However, these results are under debate since a genetic *Fmr1* KO zebrafish model, developed with ENU-mutation screening, shows no obvious phenotype at all (den Broeder et al., 2009). This suggests that the phenotype of morpholino injected *Fmr1*

knockdown fish appears to result from potential experimental artefacts and therefore seems not suitable to be used as an FXS disease model.

In conclusion, animal models are valuable in elucidating the molecular mechanisms underlying the clinical symptoms in FXS.

The aim of this PhD thesis was to study the role played by functional domains of FMRP in dendritic transport and to investigate potential therapeutic strategies for FXS. To achieve this goal we used the *Fmr1* KO mouse model. Cultured hippocampal neurons gave us the opportunity to study the function of different FMRP variants in granule formation and mRNA transport. In addition, using these cultured hippocampal neurons we were able to study the effect of different drug treatments on the spine morphology. To investigate the effects of drug treatment on behaviour, we developed a new model to test the prepulse inhibition of startle response in mice. Finally, to study the effect of treatment on the spine morphology *in vivo*, we used a DiOlistic labelling protocol to analyse the spine morphology in the hippocampus of adult *Fmr1* KO mice.

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2

POTENTIAL THERAPEUTIC INTERVENTIONS FOR FRAGILE X SYNDROME

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ABSTRACT

Fragile X syndrome (FXS) is caused by a lack of the fragile X mental retardation protein (FMRP); FMRP deficiency in neurons of patients with FXS causes intellectual disability (IQ<70) and several behavioural problems, including hyperactivity and autistic-like features. In the brain, no gross morphological malformations have been found, although subtle spine abnormalities have been reported. FXS has been linked to altered group I mGluR-dependent and independent forms of synaptic plasticity. Here, we discuss potential targeted therapeutic strategies developed to specifically correct disturbances in the excitatory metabotropic glutamate receptor (mGluR) and the inhibitory gamma-aminobutyric (GABA) receptor pathways that have been tested in animal models and/or in clinical trials with patients with FXS.

Keywords: fragile X syndrome, therapy, group I metabotropic glutamate receptors (mGluR), gamma-aminobutyric (GABA) receptor, mGluR theory, GABA hypothesis

INTRODUCTION

Intellectual disability (ID; IQ<70), affecting 1-3% of the population, is represented by an IQ less than 70 and can be caused by nongenetic and genetic factors. Fragile X syndrome (FXS) is one of the most common inherited forms of intellectual disability affecting approximately 1 in 4000 males and 1 in 8000 females (Hagerman, 2002). In 1991, an expanded trinucleotide repeat in the *FMR1* gene, located on the long arm of the X chromosome, was identified as the genetic cause of FXS (Verkerk et al., 1991). The gene contains a CGG repeat in its 5' untranslated region (UTR) that is normally shorter than 55 repeats. However, this repeat can be unstable upon maternal transmission resulting in lengthening of the CGG repeat in the next generation. Individuals with repeat sizes between 55 and 200 units long are considered premutation carriers (Brouwer et al., 2009). If the repeat size exceeds 200 units, referred to as a full mutation, the CGG repeat and the neighbouring CpG island in the *FMR1* promoter region are hypermethylated (Naumann et al., 2009). Typically, hypermethylation results in silencing of the *FMR1* gene, leading to fragile X mental retardation protein (FMRP) deficiency an intellectual disability in patients with FXS.

2.1 FRAGILE X SYNDROME: COGNITIVE SYMPTOMS

Intellectual disability is identified in most fully mutated patients, with an average IQ of 40 (Merenstein et al., 1996). In addition to intellectual disability, patients with FXS usually develop neurodevelopmental problems including attention-deficit hyperactivity disorder (ADHD) and disruptive and autism-like behaviour (Hagerman, 2002). Autism spectrum disorder is clearly associated with FXS, since 15-33% of patients with FXS display autism (Rogers et al., 2001; Hatton et al., 2006; Harris et al., 2008). Autism has a broad spectrum of diagnosis, and in patients with FXS it seems to reflect mainly an impairment in social interaction that is expressed with variable severity (Kaufmann et al., 2004). Additional classical features of FXS are a long face and prominent ears, although these are usually not visible before puberty. Approximately 79% of patients show long, wide or protruding ears, and approximately 64% of patients show a long and narrow face (Merenstein et al., 1996). After puberty, macroorchidism is a common symptom too, with approximately 92% of the male patients having enlarged testes (Merenstein et al., 1996).

Epilepsy is another feature found in approximately 13-18% of patients with FXS (Musumeci et al., 1999; Berry-Kravis and Potanos, 2004). In relation to epilepsy, patients show increased sensitivity to sensory stimuli, like loud noise and smell. Other problems related to FXS involve sleeping. Patients show a shorter sleep duration, greater variation in total sleep time, longer night waking episodes and sleep timing problems (Gould et al., 2000).

2.2 PATHOGENESIS OF FRAGILE X SYNDROME

Microscopic analysis of brain material of patients with FXS revealed no gross morphological abnormalities (Bakker et al., 1994; Reyniers et al., 1999). However, in some brain areas it was found that the dendrites of neurons have a higher spine density than controls and that the dendritic spines exhibited a more immature phenotype (Hinton et al., 1991; Irwin et al., 2001). Immature spines typically lack "heads" (or have very small heads) and only consist of necks, while matured spines maintain both head and neck. Examination of different brain areas from the *Fmr1* knockout (KO) mouse model also revealed similar spine abnormalities (Comery et al., 1997; Irwin et al., 2002; McKinney et al., 2005; Grossman et al., 2006). The discovery of a morphological spine phenotype indicates a possible defect in synaptic plasticity in FXS. The balance in signal transmission is believed to be altered, owing to the abnormal morphology of the spines. However, whether the abnormal spine morphology is a cause or consequence of altered signal transmission is currently unknown. Interestingly, several intellectual disability syndromes including Down syndrome and Rett syndrome syndromes also show an altered spine phenotype (Purpura, 1974; Kaufmann and Moser, 2000).

Functional magnetic resonance (fMRI) neuroimaging studies also demonstrate changes in brain areas in patients with FXS, like enlargement of the caudate nucleus and a reduced cerebellar vermis (Lightbody and Reiss, 2009). The caudate nucleus is important for movement as well as learning and complex behaviour. The cerebellar vermis is important for visual-spatial processing, learning and language. These areas correlate with FMRP expression and cognitive and behavioural symptoms in patients.

2.3 NEURONAL RECEPTORS IMPLICATED IN FRAGILE X SYNDROME

Intellectual disability is the main symptom of FXS and it is thought that the signal transmission between neurons is disturbed. In the brain, many different types of receptors are present at the synaptic membranes which can be divided in two major classes of neurotransmitter receptors: ionotropic and metabotropic receptors. Ionotropic receptors are ligand-gated ion channels, and binding of a specific ligand induces a conformational change which leads to the opening of the receptor pore. The open receptor permits ionic influx across the cell membrane resulting in a change of excitability of the neuron. Metabotropic receptors have a seven helix-containing transmembrane domain (7TMD) region and are coupled to G-proteins and hence also referred to as G-protein-coupled receptors (GPCR). G-proteins extrapolate extracellular signals into an intracellular response, and often an intracellular linkage to ionotropic channels is present. The signal transduction of metabotropic receptors is regulated by "regulator of G-protein signalling proteins" (RGS proteins). RGS proteins are GTPase-activating proteins for heterotrimeric

G-protein α subunits that can modulate GPCR signalling. RGS proteins are important for regulation of GPCR signalling, since altered RGS protein expression has been implicated in many diseases (Muma et al., 2003; Liu et al., 2006).

In the brain, many different types of neurotransmitters exist, however in FXS mainly two neurotransmitters seem to play a major role in the FXS phenotype, i.e. glutamate and gamma-amino butyric acid (GABA). Glutamate receptors are expressed throughout the brain and are essential for excitatory neurotransmission and synaptic plasticity. Glutamate receptors are found both at the postsynaptic and presynaptic membranes. AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-aspartic acid) and kainate receptors form the major class of ionotropic glutamate receptors in the brain, mediating fast excitatory neurotransmission. The family of metabotropic glutamate receptors (mGluRs) comprises eight different subtypes (mGluR1-8), that are divided into three distinct groups (i.e. group I, II and III) on the basis of sequence similarities and pharmacological properties. Group I includes mGluR1 and mGluR5 receptors, which couple to the Gq protein and activate phospholipase C (PLC) (Ferraguti et al., 2008). Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, Glu7 and Glu8) receptors couple to Gi/Go protein and inhibit adenylyl cyclase (Conn and Pin, 1997; Gerber et al., 2007). Group I mGluRs are mainly localised at the postsynaptic membrane, whereas group II and III are mainly localised at the presynaptic membrane.

In addition to excitatory signalling, neurons also receive inhibitory signals. GABA is the most abundant inhibitory neurotransmitter in the central nervous system (CNS). GABA is synthesised from glutamate by the enzyme Glutamic Acid Decarboxylase (GAD), which is found exclusively in GABAergic neurons. These neurons also make presynaptic contacts with excitatory neurons. In contrast to glutamatergic synapses, GABAergic synapses are usually not located on spines but are found directly on the dendritic shaft (Reiss et al., 1988; Megias et al., 2001). The GABA receptors are classified in two different groups: $GABA_A$ and $GABA_B$ receptors ($GABA_AR$ and $GABA_BR$), of which $GABA_AR$ are ionotropic receptors and $GABA_BR$ are metabotropic receptors. The ionotropic $GABA_AR$ are heteropentameric assemblies of five subunits. To date, 19 $GABA_AR$ subunits are identified in mammals, which are all encoded by different genes. Sequence analysis resulted in eight subunit classes: α (1-6), β (1-3), γ (1-3), ρ (1-3), ε, δ, θ and π.

There are two subunits of $GABA_BR$: the $GABA_{B1}$ and $GABA_{B2}$. Functional $GABA_BR$ are formed as heterodimers. The $GABA_{B1}$ subunit can further be divided into $GABA_{B1A}$ and $GABA_{B1b}$ subclasses (Bettler et al., 2004). It has been demonstrated that $GABA_{B1a}$ is predominantly localised at presynaptic glutamatergic terminals, whereas $GABA_{B1b}$ was found mainly in postsynaptic sites (Huang, 2006). Activation of the GABA receptors can mediate inhibitory neurotransmission by hyper- or depolarisation of the postsynaptic membrane.

The proposed basis of the underlying molecular mechanisms of FXS involves defects in the signalling cascade of group I mGluRs and GABA inhibitory pathway, which will be explained in more detail in paragraph 5.

2.4 LEARNING, MEMORY AND SIGNAL TRANSDUCTOIN

Long-term potentiation and long-term depression implicated in FXS

Long-term potentiation (LTP) and long-term depression (LTD) are considered to be the major cellular mechanisms underlying synaptic plasticity, a molecular mechanism implicated in learning and memory. Since the generation of the *Fmr1* KO mouse, researchers have investigated synaptic plasticity in this model to understand the underlying molecular mechanisms of the intellectual disability in patients with FXS.

Generally, the most extensively studied form of LTP is in the hippocampal CA1 region and is NMDA-receptor-dependent (Malenka and Bear, 2004). LTP is the strengthening of the connection between a presynaptic and postsynaptic neuron for a long period. Glutamate is one of the neurotransmitters that can induce LTP. Due to the binding of glutamate to the NMDA receptor, the receptor pore will open and Ca^{2+} flows into the cell. This rise of Ca^{2+} in the cell triggers short-lasting activation of proteins, such as CaMKII. Active CaMKII can phosphorylate AMPA receptors resulting in more AMPA receptors in the postsynaptic membrane. LTP results in more AMPA receptors at the postsynaptic membrane, which improves the signalling transmission from the presynaptic neurons and thus strengthens the connection.

LTD is the antithesis of LTP and is defined as the weakening of the synapse, and is mainly reflected by a reduced number of ionotropic AMPA receptors at the postsynaptic membrane (Malenka and Bear, 2004). There are different types of LTD, such as NMDAR, mGluR or endocannabinoids-dependent LTD. Most studies examined the NMDAR-dependent LTD in the hippocampus and this type of LTD is induced after a small, slow rise in postsynaptic Ca^{2+}. In FXS, another important type of LTD, the group I mGluR LTD, seems to be altered. It was demonstrated that both a paired-pulse low-frequency stimulation (PP-LFS) protocol or stimulation of group I mGluRs agonist by DHPG ((RS)-3,5-dihydroxyphenylglycine) results in LTD that is independent of NMDA receptors (Huber et al., 2001). This form of LTD is dependent on local protein synthesis at the synapse and also results in a net loss of AMPA receptors at the postsynaptic membrane.

Two signalling pathways that are involved in translation initiation during mGluR-LTD are the MEK-ERK-Mnk1 and the PI3K-mTOR pathways (Richter and Klann, 2009). Briefly, activation of mTOR is one of the primary triggers for the initiation of cap-dependent translation via phosphorylation of 4E-BPs (4E-binding protein) and S6K. After stimulation of the mGluR1 or mGluR5, phosphoatidylinositol-3 kinase (PI3K) phosphorylates the membrane phospholipid phoshatidylinositol-4,5-bisphosphate

(PIP2), converting into PIP3. PIP3 then recruits Akt to the membrane where it is phosphorylated and activated by PDK1. Akt activates mTOR by inhibiting the tuberous sclerosis complex (TSC), a heterodimer of TSC1 (hamartin) and TSC2 (tuburin). TSC2 contains a GAP (GTPase-activating protein) domain for the small G-protein Rheb. When TSC2 is phosphorylated, its GAP activity decreases, resulting in Rheb and mTOR activation. Subsequently, mTOR interacts with Raptor, which binds both 4E-BP and S6K. Active mTOR can i) phosphorylate S6K, resulting in phosphorylation of S6, and ii) phosphorylate 4E-BP. Unphosphorylated 4E-BP binds tightly to eIF4e, whereas phosphorylated 4E-BP does not, thereby permitting eIF4F to form an initiation complex. Phosphorylation of S6 and 4E-BP finally results in mRNA translation. In parallel, mGluR-LTD also triggers the activation of the MEK-ERK-Mnk1 signalling pathway (Gallagher et al., 2004).

GABA signalling pathway

Besides synaptic plasticity via LTP and LTD, neurons also receive inhibitory input through $GABA_AR$ or $GABA_BR$ signalling (Bettler et al., 2004; D'Hulst et al., 2009a). The GABA receptors are involved in controlling the excitability of the brain.

Binding of GABA to the $GABA_AR$ opens channels that are selectively permeable to Cl^- and mediate the influx of Cl^-. The influx of negatively charged Cl^- results in hyper- or depolarizing of the postsynaptic membrane, which is able to induce an inhibitory postsynaptic potential (IPSP). An IPSP decreases the probability of firing and thus it can regulate the excitatory synaptic response.

Presynaptic metabotropic $GABA_BR$ modulate excitability through second-messenger systems that regulate the activity of Ca^{2+} channels and can inhibit glutamate release (Scanziani et al., 1992; Isaacson and Hille, 1997). On the other hand, postsynaptic $GABA_BR$ activation leads to increased outward K^+ current, resulting in hyperpolarisation. In addition, $GABA_BR$ activation also leads to activation of the ERK pathway, resulting in CREB phosphorylation which induces CREB-mediated gene transcription (Tu et al., 2007). The transcription of these genes seems to result in long-term changes in the brain.

Dysfunction of GABA-mediated synaptic transmission in the CNS is believed to underlie various nervous system disorders. Epilepsy, spasticity, anxiety, stress, sleep disorders, depression, addiction and pain are all linked to hypoactivity of the GABA system. A significant number of patients with FXS show some of these symptoms.

2.5 HYPOTHESIS EXPLAINING THE PATHOGENESIS OF FXS

Two key milestones in fragile X research were the isolation of the *FMR1* gene and the generation of the *dFmr1* mutant fly and *Fmr1* KO mouse model (Verkerk et al., 1991;

Bakker et al., 1994; Wan et al., 2000). These animal models made it possible to study the molecular basis of FXS.

mGluR theory

In 2004, Bear et al. proposed the mGluR theory to explain many aspects of the clinical symptoms found in patients with FXS and in the *Fmr1* KO mouse including: (i) higher density of spines and more immature spines as compared to controls, (ii) electrophysiological deficits in *Fmr1* KO mice, (iii) exaggerated dendritic protein synthesis in *Fmr1* KO mice after the activation of mGluR5, and (iv) behavioural phenotypes in patients and *Fmr1* KO mice (Bear et al., 2004). This mGluR theory states that AMPA receptor internalisation, triggered by group I mGluR stimulation (mGluR1 or mGluR5) is exaggerated in FXS (figure 2.1). Many experiments indeed showed defects in synaptic signal transmission in the *Fmr1* KO mice. In 1997, the group of Greenough found that Fmrp was synthesiszed in synaptosomes after stimulation of

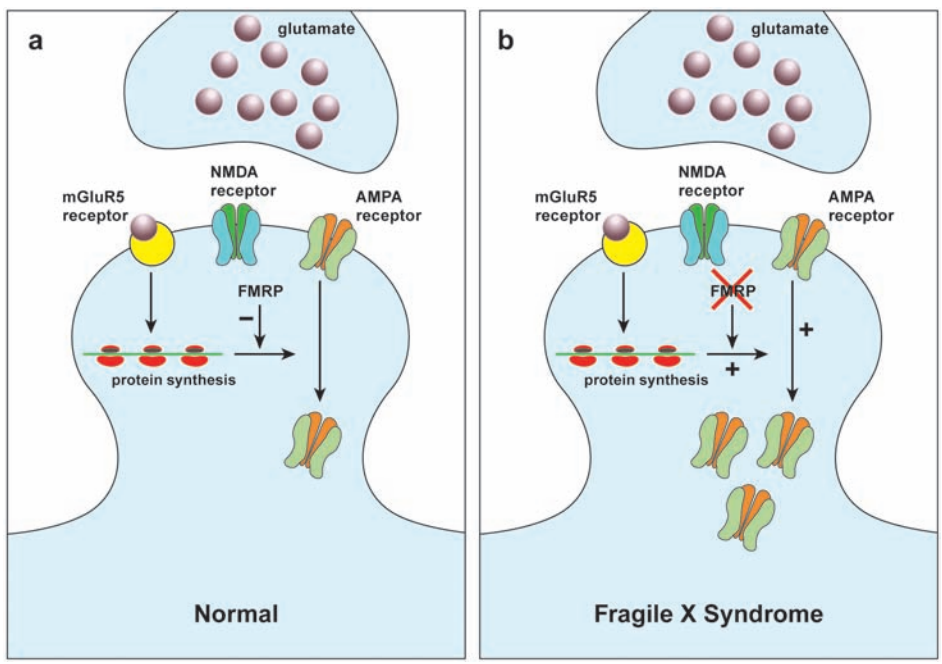

Figure 2.1. The mGluR theory. (a) Stimulation of mGluR5, a metabotropic glutamate receptor, by glutamate induces local mRNA translation at the synapse. This results in local novel protein synthesis that stimulates the internalisation of ionotropic AMPA receptors, which are essential for long-term plasticity. FMRP acts as a negative regulator of transcription, reducing the internalisation of ionotropic AMPA receptors. (b) In neurons from patients with FXS the absence of FMRP leads to an increase internalisation of ionotropic AMPA receptors which results in weakening of the synapse.

group I mGluRs with DHPG, a specific group I mGluR agonist (Weiler et al., 1997). From this perspective, it was hypothesised that group I mGluR signalling pathway might be disturbed in *Fmr1* KO mice and in patients with FXS. Finally, in 2002, a defect in group I mGluR mediated long-term depression (LTD) was reported (Huber et al., 2002). In short, group I mGluR LTD depends on local postsynaptic protein synthesis in the hippocampus, and this form of LTD is prevented when protein synthesis is blocked. In absence of FMRP, group I mGluR LTD can be induced and can persist while protein synthesis is blocked. This suggested that due to the absence of FMRP, proteins that are important for the maintenance of mGluR-LTD are abundantly present in *Fmr1* KO hippocampal slices. These results are in line with the hypothesis that FMRP is a translational repressor (Laggerbauer et al., 2001). Defects in LTD as a result of elevated protein levels might be responsible for increased AMPA receptor internalisation at the postsynaptic membrane, resulting in more immature spines in different brain areas of patients with FXS and *Fmr1* KO mice (Nakamoto et al., 2007; Park et al., 2008).

As mentioned above, the mTOR signalling pathway links the mGluR-LTD to translation initiation. Recently, it has been shown that mTOR signalling pathway in the hippocampus is dysregulated in *Fmr1* KO mice (Sharma et al., 2010). It was found that in the *Fmr1* KO mice phosphorylation status of different kinases, such as AKT, TSC1 and mTOR, were elevated, implicating overactive signalling of the mTOR pathway after activation of group I mGluRs. As a consequence of lacking FMRP, specific mRNA translation is elevated, resulting in dysregulation of the mTOR pathway. Interestingly, as mentioned in chapter 1 (paragraph 1.4), FMRP itself has also been implicated in the mTOR pathway (see figure 1.3) (Narayanan et al., 2007; Narayanan et al., 2008).

GABA hypothesis

In addition to the mGluR theory, it is also hypothesized that GABA signalling is altered in patients with FXS (D'Hulst and Kooy, 2007). Many patients suffer from epilepsy and sleeping problems, which are linked to GABA signalling. Identification of target mRNAs, revealed that mRNAs encoding $GABA_AR$ subunits are targets of FMRP (Miyashiro et al., 2003). *Fmr1* KO mice express decreased mRNA and protein levels of several $GABA_AR$ subunits ($GABA_AR$ α5, β2 and δ) compared to wildtype littermates (D'Hulst et al., 2006; Gantois et al., 2006; Curia et al., 2009; D'Hulst et al., 2009b). In addition, reduced GABA synthesizing enzyme glutamate decarboxylase (*GAD67)* mRNA expression has been reported in *Fmr1* KO mice compared to wildtype mice, although, another study showed increased GAD67 protein expression in *Fmr1* KO mice (El Idrissi et al., 2005). Altered expression of GABA signalling components in *Fmr1* KO mice reflects: (i) decreased GABAergic signalling efficiency in the hippocampus of *Fmr1* KO mice (D'Antuono et al., 2003), (ii) downregulation of tonic GABAergic inhibition (Curia et al., 2009) and (iii) morphological defects of GABA releasing interneurons in the neocortex as compared to wildtype animals (Selby et al., 2007).

In addition to the $GABA_AR$, the $GABA_BR$ might also play a role in FXS and therefore might be a therapeutic target. $GABA_BR$ agonists inhibit presynaptic glutamate release and inhibit the postsynaptic signalling cascade downstream of mGluR5. Recently, $GABA_B$ deficits were linked to FXS in a study that showed reduced audiogenic seizures in *Fmr1* KO mice after the administration of a $GABA_B$ agonist compared to untreated animals (Pacey et al., 2009).

Also, in light of exaggerated excitatory mGluR5 signalling in FXS, stimulation of the inhibitory pathway might be a good therapeutic strategy to restore the balance between inhibitory and excitatory signalling.

2.6 THERAPEUTIC INTERVENTIONS IN FXS

To date, treatment of patients with FXS is symptomatic. Importantly, the number of patients who receive treatment may vary between continents viz. high in the USA and low in Europe. Only 25% of patients with FXS in the USA do not use medication (Berry-Kravis and Potanos, 2004). The two most widely used medications are stimulants that help with attention and hyperactivity, and a selective serotonin reuptake inhibitors (SSRIs) that can reduce aggression associated with anxiety (see list for overview of medication at *http://www.fragilex.org/html/medications.htm*). Patients with FXS are not only treated with pharmacological agents but also seem to benefit from behavioural therapy addressing speech and emotional problems. As also demonstrated in mouse studies, an enriched environment can improve behaviour (Restivo et al., 2005; Meredith et al., 2007). Current therapeutic strategies, both medication and non-pharmacological treatment, impact symptoms only and do not improve cognitive functioning. Recently, new strategies for therapeutic intervention have been developed based on the mGluR and GABA theories (figure 2.2). Most treatments are first tested in the FXS animal models, like the *dFmr1* mutant (KO) fly or *Fmr1* KO mice. Several clinical tested a variety of existing drugs and new drugs that are designed to correct the abnormal activity of the mGluR or GABA pathways in patients with FXS. In this review we will combine all available data to date (table 2.1).

Negative mGluR5 modulators

MPEP

Since the formulation of the mGluR hypothesis, scientists are searching for a therapy that might partially cure some of the features in patients with FXS. The *Drosophila* genome only contains a single homologue of the *FMR1* gene, called *dFmr1*. Besides the *Fmr1* KO mouse model, a *Drosophila* model of FXS have been generated and also exhibits phenotypes that show similarities to FXS symptoms (Dockendorff et al., 2002; Lee et al., 2003). Studies of the *dFmr1* mutant uncovered alterations in circadian rhythm, synaptic branching and courtship behaviour.

The first study showing pharmacological rescue with a negative mGluR5 modulator was performed using MPEP (2-methyl-6-(phenylethynyl)-pyridine hydrochloride) in the *Drosophila* FXS model. MPEP is one of the first negative modulators of mGluR5, but is often referred as an mGluR5 antagonist or inverse agonist. MPEP interacts within the 7TMD of the mGluR5 receptor to stabilise the inactive state of the receptor (Gasparini et al., 2002). Treatment of *dFmr1* mutant flies with MPEP rescued synaptic plasticity, courtship behaviour and mushroom body defects (McBride et al., 2005). Interestingly, mushroom body defects were only rescued when MPEP was administrated to *dFmr1* mutant flies from birth. However, treatment at later ages could not induce rescue of mushroom body defects, but could rescue the altered courtship behaviour in *dFmr1* mutant flies.

One of the first experiments to test whether mGluR5 antagonists might have effects on *Fmr1* KO mice was performed by Yan et al. (Yan et al., 2005). They studied the effect of MPEP on both audiogenic seizures and open field behaviour using *Fmr1* KO mice. It is well known that *Fmr1* KO mice show a higher susceptibility to audiogenic

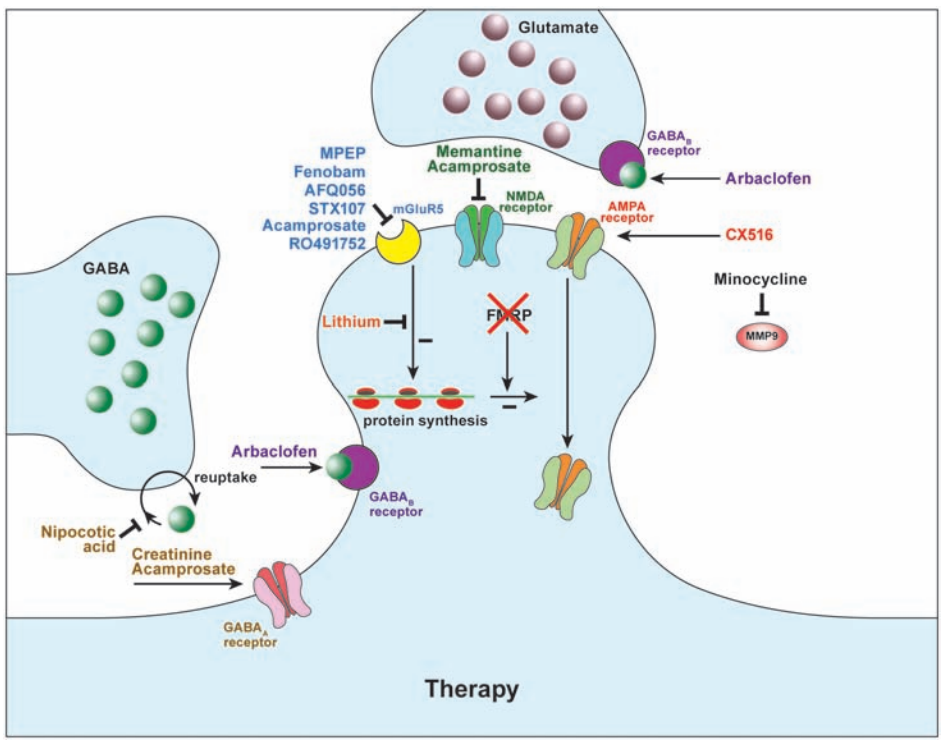

Figure 2.2. Therapeutic strategies for FXS. Schematic representation of a glutamatergic excitatory and GABAergic inhibitory synapse lacking FMRP. Several types of drugs can interact with different type of neuronal receptors which may result in rescue of the disturbed synaptic transmission found in FXS.

seizures compared to wildtype animals (Musumeci et al., 2000; Chen and Toth, 2001). Injections with 20 mg/kg MPEP resulted in suppression of audiogenic seizures in *Fmr1* KO mice. Moreover, MPEP could also rescue abnormal behaviour in the open field test: *Fmr1* KO mice show less anxiety, revealed by enhanced centre field behaviour in an open field compared to wildtype mice (Yan et al., 2005). After MPEP administration, behaviour of *Fmr1* KO mice in open field was indistinguishable from wildtype mice. These experiments demonstrated for the first time that MPEP might have beneficial effects on some specific behaviour deficits in *Fmr1* KO mice.

In 2007, biochemical analysis on FMRP deficient cells was performed to examine the effect of MPEP treatment on the AMPA receptor internalisation (Nakamoto et al., 2007). Reduced FMRP expression indeed resulted in an increased AMPA receptor subunit GluR1 internalisation (Nakamoto et al., 2007). Treatment of FMRP deficient neurons with MPEP showed reduced GluR1 internalisation, suggesting that MPEP treatment results in more AMPA receptors at the postsynaptic membrane of spines. The decreased number of AMPA receptors at the spine surface has been linked to an altered spine phenotype found in patients and mice.

Recently it has been demonstrated that MPEP treatment results in more mature spines in cultured hippocampal neurons (De Vrij et al., 2008). Examination of cultured hippocampal neurons revealed more filopodia (immature spines) in *Fmr1* KO neurons compared to hippocampal wildtype neurons. MPEP treatment of these primary neurons from *Fmr1* KO mice rescued the immature phenotype of the filopodia, and those filopodia seemed to evolve into mature spines. Also a rescue effect of MPEP in a behavioural test named prepulse inhibition of startle (PPI) was demonstrated (De Vrij et al., 2008). PPI is an operational measure of sensorimotor gating whereby a weaker pre-stimulus (the prepulse) inhibits the motor response to a strong, startling stimulus. Interestingly, one of the most prevalent symptoms of patient with FXS is heightened sensitivity to sensory stimuli. De Vrij et al. showed that PPI is also reduced in *Fmr1* KO mice, similar to that what is found in patients with FXS (Frankland et al., 2004; De Vrij et al., 2008; Hessl et al., 2009). After MPEP administration, the percentage of PPI is indistinguishable from wildtype mice and therefore MPEP rescues the deficits in PPI. However, it has been demonstrated that MPEP may give unwanted adverse reactions because it is not completely mGluR5-specific (Popoli et al., 2004; Lea et al., 2005). Furthermore is the use of MPEP in clinical trials for patients with FXS not feasible, due to toxicity and a short half life time.

Fenobam

During a functional high-throughput screen, fenobam was identified as a highly potent, selective mGluR5 antagonist (Porter et al., 2005). In the early 1980s, fenobam was studied as an anxiolytic drug in several clinical phase II trials. Like MPEP, the effect of fenobam on the spine morphology was examined in cultured hippocampal

Table 2.1. Current and future potential therapy for FXS.

New therapeutic interventions	Drug	Model	Results	References
mGluR5	MPEP	*dFmr1* fly	Rescue courtship behavior Partial rescue mushroom body defects Memory defects	McBride S.M., *et al,* 2005
		Fmr1 KO mice	Rescue audiogenic seizure and open field behavior Rescue deficit in PPI of startle response	Yan Q.J., *et al,* 2005 de Vrij F.M., *et al,* 2008
		Fmr1 KO cultured hippocampal neurons	Rescue enhanced AMPA receptor internalisation Rescue abnormal spine morphology cultured neurons	Nakamoto M., *et al*, 2007 de Vrij F.M., et al, 2008
	Fenobam	*Fmr1* KO mice	Rescue abnormal spine morpholgoy cultured neurons Rescue deficit in PPI of startle response	de Vrij F.M., e*t al*, 2008 Unpublised data
		FXS patients	6 out of 12 patients improve in PPI	Berry-Kravis E.M., *et al, 2009*
	AFQ056	*Fmr1* KO mice	Rescue abnormal spine morphology cultured neurons deficit in PPI of startle response	Levenga J., *et al, in preparation*
	STX107 and RO4917523	unknown	unknown	www.seasidetherapeutics.com http://www.clinicaltrials.gov
$GABA_A$ receptor	Nipocotic acid	*dFmr1* fly	Rescue lethality after increased levels of glutamate in food	Chang S., *et al,* 2008
	Creatinine	*dFmr1* fly	Rescue lethality after increased levels of glutamate in food	Chang S., *et al,* 2009
$GABA_B$ receptor	(R) baclofen/Arbaclofen	*Fmr1* KO mice	Rescue audiogenic seizures	Pacey L.K., *et al,* 2009
NMDA receptor	Memantine	FXS patients	No significant effects	Erickson C.A., *et al*, 2009
AMPA receptor	CX516	FXS patients	No significant effects	Berry-Kravis E., *et al,* 2006

Table 2.1. Continuation

New therapeutic interventions	Drug	Model	Results	References
Other interventions	Lithium	*dFmr1* fly	Rescue courtship behavior Partial rescue mushroom body defects Memory defects	McBride S.M., *et al,* 2005
		Fmr1 KO mice	Rescue audiogenic seizure and open field behavior	Min W.W., *et al*, 2009
		FXS patients	Improvement in behaviour, hardly in cognitive function	Berry-Kravis E., et al, 2008
	Minocycline	*Fmr1* KO mice	Rescue abnormal spine morphology Rescue of anxiety	Bilousova T., *et al*, 2008
	Acamprosate	FXS patients	3 out of 3 FXS patients improve in language	Erickson C.A., *et al,* 2010
Genetic interventions	Introducing *FMR1* gene using YAC	*Fmr1* KO mice expressing human FMRP	Rescue audiogenic seizures and PPI deficit	Paylor R., et al, 2008 Musumeci S.A., *et al*, 2007
	50% reduction of mGlu5 receptor	*Fmr1* KO mice, heterozygous for mGluR5	Rescue spine morphology, behavior, elevated protein synthesis and LTD deficit	Dölen G., *et al*, 2007
	Inhibition of P21-activated kinase	*Fmr1* KO mice expressing dnPAK TG	Rescue abnormal spine morphology, partially behavioral abnormalities and cortical LTP deficit	Hayashi M.L., *et al*, 2007
Epigenetics	5-azadeoxycytidine	Cell lines of FXS patients	Modest reactivation of *FMR1* gene	Chiurazzi P., *et al.*, 1998
	4-phenylbutyrate, sodiumbutyrate	Cell lines of FXS patients	Modest reactivation of *FMR1* gene	Chiurazzi P., *et al.*, 1999
		FXS patients with ADHD	Improvement of behavior and reduction of hyperactivity*	Torrioli M.G., et al, 2008
	Valproic acid	Cell lines of FXS patients	Histone modifications, no reactivation of *FMR1* gene	Tabolacci E, *et al*, 2008

* Improvement is not likely due to reactivation of the *FMR1* gene, but rather to other effect on carnitine metabolism

neurons from *Fmr1* KO and WT mice and proved to have similar maturation effects on spine morphology (De Vrij et al., 2008). In addition, fenobam was also able to rescue PPI deficits in *Fmr1* KO mice (chapter 5).

Fenobam was the first mGluR5 antagonist tested in patients with FXS (Berry-Kravis et al., 2009). Twelve patients (six males and six females) received a single oral dose of 50-150 mg fenobam. Measuring the plasma concentration of fenobam revealed that 150 mg resulted in a peak value at 180 minutes, which was taken as the optimum dose. To test if fenobam had any significant effect on the phenotypes of patients, PPI was measured before and after the administration of the drug. Patients generally showed a decreased PPI compared to healthy control individuals (Frankland et al., 2004; Hessl et al., 2009). Response criteria for fenobam on PPI levels were based on an improvement of at least 20% over baseline with a 95% confidence interval. With these criteria, six out of twelve patients showed an improvement after fenobam, ranging from 23.7% to 44.2%. No significant adverse reactions to fenobam were noticed and no safety concerns were found. Although these results look promising, the trial was not placebo-controlled, patients only received a single dose of fenobam and the number of participants is too limited to draw final conclusions.

AFQ056

Novartis recently developed a new specific mGluR5 antagonist, named AFQ056. The effects of AFQ056 on PPI and hippocampal spine morphology have been studied in *Fmr1* KO mice (chapter 5). Similar to MPEP and fenobam, AFQ056 rescues the PPI deficits and abnormal spine morphology *in vitro* using cultured primary neurons from *Fmr1* KO mice. Short-term and long-term treatment of mice with AFQ056 produced no adverse side effects (chapter 6). These results show that AFQ056 might be an effective treatment for patients with FXS. Indeed, a clinical phase II trial has been finished and these results will soon be published (http://www.clinicaltrials.gov).

STX107 and RO4917523

Both Merck and Hoffmann-LaRoche have also designed specific mGluR5 antagonists. One compound from Merck STX107, licensed to Seaside Therapeutics (*seasidetherapeutics.com*), is a small, selective mGluR5 antagonist which has been tested in *Fmr1* KO mice and is in phase I clinical trials and in preparation for trials in FXS (http://www.clinicaltrials.gov). One compound from Hoffman-LaRoche called RO4917523 is currently in phase II clinical trial (*http://www.clinicaltrials.gov*). Unfortunately, no data has yet been published about the effects of STX107 or RO4917523 on behaviour or spine deficits in FXS.

$GABA_A$ treatment: Nipocotic acid and creatinine

D'Hulst et al. postulated a hypothesis that the GABA signalling in the *Fmr1* KO mouse brain is altered (D'Hulst and Kooy, 2007). As discussed above, dysfunction of the GABA receptor signalling pathway also seems to be involved in the pathogenesis of the FXS and therefore a target for therapeutic intervention.

Until recently, only one pharmacological experiment shows evidence for the $GABA_AR$ theory. Chang et al. reported that GABA-related treatment had beneficial effects on *dFmr1* mutant flies (Chang et al., 2008). It was discovered that *dFmr1* mutant *Drosophila* flies died when reared on food that contained increased levels of glutamate. This lethal phenotype was used to screen pharmacological compounds that were able to rescue it. Two compounds, nipocotic acid and creatinine, were able to rescue the glutamate-induced toxicity of *dFmr1* mutant flies. The abnormal mushroom body structure and abnormal courtship behaviour seen in *dFmr1* mutant flies was also restored. Nipocotic acid acts as a GABA reuptake inhibitor and creatinine seems to be a potential activator of the $GABA_AR$. In addition, both drugs and $GABA_AR$ treatment also resulted in rescue of (i) Futsch overexpression, an orthologue of mammalian *Map1b* in *Drosophila*, and (ii) abnormalities in mushroom body structure.

$GABA_AR$ agonists are currently in use as anticonvulsants, antidepressant and anxiolytic compounds. Benzodiazepines, which enhance GABA receptor function, are the best known GABAergic drugs. Although they have proved to have anxiolytic effects and are used as a therapy for patients with FXS, they exhibit unwanted side effects, such as sedation, ataxia and cessation of treatment can cause withdrawal symptoms (Nemeroff, 2003). Currently, more selective $GABA_AR$ agonists that lack unwanted side effects are being investigated (Atack et al., 2006). In addition to selective $GABA_AR$ agonists, neuroactive steroids that allosterically modulate $GABA_AR$ might be effective; for instance, ganaxolone has a favourable safety profile and this drug might eventually be evaluated in patients with FXS (Cornish et al., 2008). However, this class of compounds also has other expected properties of $GABA_AR$ modulators, including anticonvulsant activity and the (unwanted) ability to cause sedation (Nohria and Giller, 2007).

$GABA_B$ treatment: Arbaclofen

Audiogenic seizures are one of the most robust and reproducible phenotypes in the *Fmr1* KO mouse and approximately 13-18% of patients with FXS. G-protein-coupled-receptors (GPCRs), including group I mGluRs and $GABA_BR$ are implicated as causative mechanism factors of audiogenic seizures (Faingold, 2002; Yan et al., 2005). As mentioned in paragraph 3, RGS proteins are important regulators of GPCRs and different types of RGS proteins are identified. RGS4 is a potent inhibitor of both Gq and Gi/o-coupled signal transduction pathways. It is highly expressed in developing as well as adult brain in which they inhibit the signalling of group I mGluRs and it also associates with $GABA_BR$ (Saugstad et al., 1998). *Fmr1* KO mice showed decreased

RGS4 mRNA expression in the hippocampus and cortex after postnatal development (Tervonen et al., 2005). In 2009, Pacey et al. studied a double *Rgs4*/*Fmr1* KO model to examine the effect of *Rgs4* deficiency on *Fmr1* KO mouse behaviour (Pacey et al., 2009). It was expected that a simultaneous lack of *Rgs4* expression would cause an exaggerated audiogenic seizure phenotype. However, in contrast to what was expected, double *Fmr1*/*Rgs4* KO mice showed a rescue in the audiogenic seizure phenotype (Pacey et al., 2009) and treatment of *Fmr1* KO mice with $GABA_BR$ agonist arbaclofen also reduced the incidence of audiogenic seizures (Pacey et al., 2009). All together, stimulation of the inhibitory signalling pathway in *Fmr1* KO mice appears to reduce audiogenic seizures.

Arbaclofen (STX209), a novel baclofen isomer, will be studied in a phase II randomised, double-blind placebo-controlled crossover trial in patients with FXS (www.seasidetherapeutics.com). Racemic baclofen is already used for many years in clinical practice for spasm, pain and addiction (Bowery et al., 2002). Another advantage of $GABA_B$ receptor agonist treatment might be the reduction in anxiety in patients with FXS, since it has been shown that $GABA_B$ receptor plays an important role in anxiety (Cryan and Kaupmann, 2005). However, similar to most intervention based on the GABA receptor, there is a chance that patient will show withdrawal symptoms after discontinuation of arbaclofen (Leo RJ, 2005). However, similar to most intervention based on the GABA receptor, there is a change that patient will show withdrawal symptoms after discontinuation of arbaclofen (Mann et al., 2004).

AMPA positive modulator: Ampakine CX516

The increased internalisation of AMPA receptors in neurons of *Fmr1* KO neurons is thought to play a major role in the altered signal transmission, for example the enhanced hippocampal mGluR-LTD. CX516 is an ampakine that acts as an AMPA receptor positive allosteric modulator. It binds to the AMPA receptor-channel complex, inducing slower receptor deactivation that results in a longer opening time, slower excitatory postsynaptic potential (EPSP) decay and enhanced hippocampal LTP (Arai et al., 1996). Consequently, it potentiates AMPA receptors after synaptic activation by glutamate, and therefore, it is expected to increase synaptic strength in the presence of glutamate activation. CX516 was tested in a phase II randomised double-blind, placebo-controlled four-week safety trial in adult patients with FXS (Berry-Kravis et al., 2006). Patients underwent a detailed assessment to score cognitive and behavioural outcome. However, four weeks of treatment with CX516 did not result in significant improvement in either cognitive or behavioural measures. This might be due to low doses, the short halflife of CX516 in humans or the short period of treatment. In this study, there were minimal side effects and no serious adverse effects were noted.

Unfortunately, to our knowledge the effect of CX516 treatment has never been examined in *Fmr1* KO mice. Therefore, targeting the AMPA receptor might improve

behaviour in FXS, although beneficial effects of ampakines have not yet been confirmed either in animal studies, or in clinical trials.

NMDA receptor antagonist: Memantine

Memantine is an uncompetitive NMDA receptor antagonist, which can retard the progression of Alzheimer's disease and is tested for treatment of pervasive developmental disorder (PDD) (Reisberg et al., 2003). Binding of memantine to the NMDA receptor blocks receptor signalling at low synaptic glutamate levels; this block is released at high glutamate levels (Parsons and Gilling, 2007). Beside treatment of Alzheimer's disease patients, memantine is also used in several clinical trials to study the effect on subjects with idiopathic PDDs or autistic PDD not otherwise specified (PDD-NOS). Results of these clinical trials showed improvement in use of language and social behaviour (Chez et al., 2004; Chez et al., 2007).

Pilpel et al. found that juvenile *Fmr1* KO mice displayed a significantly lower AMPA to NMDA ration compared with wildtype mice (Pilpel et al., 2008). The difference in ratio at P14 is caused by an up-regulation of the NMDA receptor component and a down-regulation of the AMPA receptor component. Furthermore, several groups have reported LTP deficits in specific brain areas of *Fmr1* KO mice, linked to dysregulated NMDA receptor signalling, although conflicting results are published (Li et al., 2002; Larson et al., 2005; Lauterborn et al., 2007; Shang et al., 2009). In addition to excessive signalling via mGluR5 that is linked to increased AMPA receptor internalisation, dysregulation of NMDA receptor activity may also be involved, and therefore memantine might have positive effects on the behavioural phenotypes of patients with FXS.

Erickson et al. started a small clinical trial with memantine in six patients with FXS who have a comorbid diagnosis of PDD (Erickson et al., 2009). The effect of treatment was determined by clinical assessment of a Clinical Global Impressions-Improvement (CGI-I) score during the treatment period (the average treatment period 34.7 weeks). No significant improvement was shown after treatment with memantine, but four out of six patients trended towards improvement. Unfortunately, this study is not a placebo-controlled randomised trial and, thus, it is difficult to draw conclusions from it.

Additional therapeutic interventions

Lithium

Lithium has been used for many years as a mood stabiliser (Jope, 1999). Lithium influences several pathways including: (i) the inositol(myo-1(or 4)-monophosphatase (IMPase) and inositol-depletion pathway, (ii) the glycogen synthase kinase-3 (GSK3) pathway, and (iii) the β-arrestin-2-Akt complex. Most studies that have linked Lithium to FXS focused on the GSK3 pathway (Min et al., 2009; Yuskaitis et al., 2009). Lithium inhibits activity of glycogen synthase kinase-3 (GSK-3), which in turn inhibits

phosphorylation of microbulule-associated-protein 1B (Map1B). *Map1B* is one of the major mRNA targets that binds to and is translationally regulated by FMRP (Lu et al., 2004). Different brain areas of *Fmr1* KO mice show increased GSK-3 activity, and Lithium and the mGlu5 antagonist MPEP similarly inhibit the activity has of GSK-3 (Min et al., 2009). In 2005, a study reported that Lithium can restore short-term memory deficits in *dFmr1* mutant flies, whereas in *Fmr1* KO mice, Lithium reduced audiogenic seizures and hyperactivity in an open field test (McBride et al., 2005; Min et al., 2009). In summary, these results suggest a positive effect on behaviour and memory in several *Fmr1* KO animal models.

In 2008, an open-label treatment trial of Lithium was published on patients with FXS (Berry-Kravis et al., 2008). Although the trial was not a placebo-controlled randomised trial, Lithium seems to have had beneficial effects on FXS patients (*i.e.* decreased responses of aggression, abnormal vocalisations, self-abuse and anxiety). The outcomes were measured by rating scales and tests, such as the Aberrant Behaviour Checklist-Community (ABC-C) caregiver-rated scale and the CGI-I scale. Significant improvement of cognitive function, assessed by Vineland Adaptive Behaviour Scales (VABS) and Repeatable Battery for the Assessment of Neuropsychological status (RBANS) List Learning, was found. This suggests that the behavioural improvement associates with functional improvements in daily life. However, it remains difficult to study improvement in cognitive function in patients with FXS, since exploratory tasks are proved to be difficult for lower-functioning patients. In conclusion, Lithium seems to have beneficial effects on behaviour and in some cognitive functions, but it will be important to investigate more precisely the effects of Lithium by means of a long-term placebo-controlled trial in patients with FXS.

Minocycline

Minocycline is a tetracycline analogue that can inhibit matrix metalloproteinase-9 (MMP-9) and reduces inflammation in the central nervous system. MMP-9 is an extracellular endopeptidase that cleaves extracellular matrix (ECM) that impact synaptogenesis and spine morphology (Ethell and Ethell, 2007). Minocycline has been tested in clinical trials for treatments of multiple neurological disorders, including stroke, multiple sclerosis and autism. Recently, minocycline was shown to have beneficial effects on spine maturation of cultured hippocamapal neurons and in organotypic slices of *Fmr1* KO mice. It can also rescue the anxiety in *Fmr1* KO mice as assessed by an elevated plus maze test (Bilousova et al., 2008). Minocycline is believed to impact the mGluR pathway. DHPG, a group I mGluR agonist, induces MMP-9 expression and activation upon stimulation of hippocampal neurons. Therefore, it is possible that MMP-9 activation is enhanced in neurons from *Fmr1* KO mice owing to increased group I mGluR signalling. This might contribute to abnormal dendritic spine development in *Fmr1* KO hippocampal neurons.

Following the studies performed in mice, clinical trials have started for patients with FXS. Children 4-13 old years with FXS will be evaluated in a placebo-controlled trial for the efficacy of minocycline. Although the results in mice are promising, treatment of minocycline might have some adverse side effects when used for a longer time period, such as teeth discoloration, a blue-graying of the skin and an autoimmune response (i.e. drug-induced lupus with elevated blood antinuclear antibody levels).

Acamprosate

Acamprosate (calcium acetylhomotaurine) is a commercially available drug used for the maintenance of alcohol abstinence. Acamprosate seems to have many modes of action including an mGluR5 antagonist, a weak NMDA receptor antagonist and a $GABA_AR$ agonist (Gupta et al., 2008; Mann et al., 2008). Although the exact mechanism of action of acamprosate is not completely understood, Erickson et al. conducted a small clinical trial with three patients with FXS (Erickson et al., 2010). To evaluate response to acamprosate treatment, the CGI-I scale was used. After a minimal 16 weeks of treatment, all three patients improved. Strikingly, all three patients showed an improvement in language skills. Two subjects experienced nausea with no other adverse effects. Although this results seem promising, this trial was not placebo-controlled and the number of participants was too limited.

Genetic interventions

In the future, it might be possible to treat and cure patients with FXS with genetic intervention strategies instead of with medication. Although we can only speculate, theoretically it should be possible to introduce the unmethylated *FMR1* gene into the patient genome. It is already known from mouse studies that genetic intervention might improve behaviour, spine and electrophysiological deficits.

Studies in *Fmr1* KO mice expressing a Yeast Artificial Chromosome (YAC) containing the human *FMR1* gene, show that reintroduction of FMRP expression is able to partially rescue the behavioural symptoms found in *Fmr1* KO mice, including susceptibility of audiogenic seizures or deficits in PPI (Peier et al., 2000; Musumeci et al., 2007; Paylor et al., 2008). However, (over)expression of FMRP is likely to cause a phenotype by itself (Peier et al., 2000). Introducing an active *FMR1* gene in cells of patients likely leads to a suitable treatment, although it is important to take the gene dosage into account.

Therefore, reactivation of the endogenous *FMR1* gene seems to be a better strategy. It has been demonstrated that individuals with a full mutation (CGG >200) that escaped methylation exhibit a normal phenotype (Hagerman et al., 1994; Smeets et al., 1995). This suggests that the inactivation of the promoter region is responsible for the FXS phenotype. Modulation of the inactivation in the *FMR1* promoter region seems

to be a logical strategy. It has been proven that treatment with 5-azadeoxycytidine (5-azadC) indeed resulted in partial demethylation and transcriptional reactivation (Chiurazzi et al., 1998). Hyperacetylation might be another strategy to reactivate the *FMR1* gene because it has been shown that transciptionally active chromatin appears to contain more acetylated core histones than tightly packaged heterochromatin. Histone hyperacetylation with 4-phenylbutyrate, sodiumbutyrate and trichostatin A results indeed in DNA demethylation and reactivation of the *FMR1* gene (Chiurazzi et al., 1999). Moreover, combining these drugs with 5-azadC showed a synergistic effect, resulting in 2-5 fold increase of *FMR1* mRNA levels compared to 5-azadC alone. Although *FMR1* mRNA was clearly present, FMRP could not be detected in many cells. The effects on epigenetic modifications of three other drugs, L-carnitine, acetyl-L-carnitine (LAC) and valproic acid, were tested (Pascale et al., 2003; Tabolacci et al., 2008). All compounds show effects on histone modifications but little or no effect could be observed on reactivation of the *FMR1* gene.

The effect of L-acetylcarnitine (LAC) on ADHD in boys with FXS has also been examined in a double-blind, parallel, placebo-controlled and multicenter study (Torrioli et al., 2008). The results showed that both placebo and LAC-treated groups improved their behaviour, indicating that psychosocial intervention has a significant therapeutic effect as well. However, a stronger reduction of hyperactivity and improvement of social behaviour in patients treated with LAC was determined. Genetic studies performed in 50% of the patients demonstrated that the effect of LAC on behaviour was not due to reactivation of the *FMR1* gene, but probably resulted from other effects in carnitine metabolism. Unfortunately, most DNA demethylation or histone hyperacetylation drugs are not specific for the *FMR1* promoter region and will demethylate other promoter regions as well, with unknown consequences.

Other genetic interventions in *Fmr1* KO mice that result in (partial) rescue of the FXS phenotype in *Fmr1* KO mice include the reduction of mGluR5 receptors. An elegant study by Dölen et al. demonstrated that *Fmr1* KO mice with 50% reduced mGluR5 expression could correct specific deficits, including immature spine phenotype, elevated protein synthesis at the synapse, and audiogenic seizures (Dolen et al., 2007). Another experiment demonstrated that p21-activated kinases (PAK) are important proteins involved in the FXS phenotype. Synaptic plasticity is dependent on the structural regulation of actin cytoskeleton in dendritic spines. Rho-GTPases have emerged as the key regulators in the control of actin filament assembly in the dendritic spines. PAKs are important effectors of Rho-GTPase signalling. There are three different groups of PAKs, and mutations in the PAK3 gene can lead to mental retardation (Allen et al., 1998; Kreis et al., 2007). Strikingly, inhibition of all three PAKs could rescue several phenotypes of *Fmr1* KO mice (Hayashi et al., 2007). Mice that expressed a dominant negative PAK in an *Fmr1* KO background showed a rescue of spine abnormalities, the electrophysiological phenotype and partially the behavioural

phenotype of *Fmr1* KO mice. Overall, genetic interventions will plausibly be the best option to cure FXS, although, many obstacles need to be overcome, including technical and ethical ones.

Eventually, all therapeutic interventions aim to improve behaviour and cognitive function by restoring aberrant signal transmission in the brain. As mentioned above, many compounds appear to have positive effects on behaviour and spine morphology in *dFmr1* flies or *Fmr1* KO mice, and some even in patients with FXS. The consequences of normal signal transmission after treatment, however, have yet to be studied. The most sustainable electrophysiological deficit in *Fmr1* KO mice is enhanced mGluR LTD, and in contrast to wildtype mice, this type of LTD is protein synthesis independent in *Fmr1* KO mice. Unfortunately, it has not been investigated that (long-term) treatment with an mGluR antagonist can restore this specific LTD deficit. Other electrophysiological deficits, such as the LTP defects in the ACC, are also not shown to be rescued by therapeutic interventions. Only the two genetic studies, i.e. inhibition of P21-activated kinase in *Fmr1* KO mice and reduced mGluR5 expression, did show electrophysiological changes. Reduction of mGluR5 by 50% in *Fmr1* KO mice resulted in a partial rescue of mGluR LTD (Dolen et al., 2007). Inhibition of P21-activated kinase rescued the reduced cortical LTP in *Fmr1* KO mice (Hayashi et al., 2007).

2.7 THERAPEUTIC CONSIDERATION

The mGluR theory is focused on group I mGluRs, mGluR1 and mGluR5, and their distribution is different throughout the brain. mGluR1 is mainly present in the cerebellum, while mGluR5 is predominantly expressed in the forebrain, including cerebral cortex, hippocampus, basal ganglia and amygdala (Shigemoto et al., 1993; Spooren et al., 2001). Therefore, negative modulators of mGluR5 will most likely target all these brain regions. Currently it is not known of all these brain regions contribute to the FXS phenotype and whether negative mGluR5 modulators will act on all these brain regions. Pharmacological studies will be necessary to investigate the efficacy and specificity of negative modulators of mGluR5 on different brain regions using the FXS mouse model.

Patients with FXS are treated with drugs to help with the disease symptoms or behavioural deficits, including hyperactivity and anxiety. Unfortunately, at present, a specific pharmaceutical treatment to correct underlying molecular abnormalities is not available. Nevertheless, an increased attempt to discover new types of drugs to treat patients with FXS is underway, owing to improved insights into the molecular pathways involved in the pathogenesis of FXS. Testing new drugs in mouse models will be essential before they can be used in human clinical trials. Pretesting in mouse models is, however, no guarantee for success in clinical trials. Several new potentially therapeutic drugs have been tested directly in patients with FXS, such as acamprosate

or memantine. If a drug is approved for use in other diseases and is considered to be safe for humans, the effects of this drug can be examined off-label in patients.

Several clinical trials have been conducted to study the effect of different types of drugs in patients with FXS that revealed preliminary data about safety and efficacy. However, to establish definitive efficacy we would like to emphasise that it is crucial to set up an accurate clinical trial that: (i) is randomised and placebo-controlled: (ii) is double-blind; (iii) includes adequate number of patients; and (iv) utilises reliable objective readouts to determine therapeutic efficacy. Most clinical trials use distinct psychological questionnaires to determine the therapeutic efficacy of new treatments. However, improvement of behaviour is subjectively scored by caregivers and teachers. To guarantee objectivity, it is important to include other reliable and objective readouts to determine the efficacy of new treatments. A good candidate for such a test might be the PPI test.

In conclusion, in fewer than 20 years since the discovery of the underlying gene defect of FXS, targeted therapeutic strategies are being seriously studied. It is likely that in the near future a treatment for FXS, based on intervention in mGluR or GABA receptor pathways or a combination of both, will become available and could improve the lives of individuals with FXS significantly.

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3

ULTRASTRUCTURAL ANALYSIS OF THE FUNCTIONAL DOMAINS IN FMRP USING PRIMARY HIPPOCAMPAL MOUSE NEURONS

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ABSTRACT

Fragile X syndrome is caused by lack of the protein FMRP. FMRP mediates mRNA binding, dendritic mRNA transport and translational control at spines. We examined the role of functional domains of FMRP in neuronal RNA-granule formation and dendritic transport using different FMRP variants, including the mutant FMRP_I304N and the splice-variant FMRP_Iso12. Both variants are absent from dendritic RNA-granules in *Fmr1* knockout neurons. Co-transfection experiments showed that wildtype FMRP recruits both FMRP variants into dendritic RNA-granules. Co-transfection of *FXR2*, an FMRP homologue, also resulted in redistribution of both variants into dendritic RNA-granules. Furthermore, the capacity of the variants to transport their mRNAs and the mRNA localisation of an *FMR1* construct containing silent point-mutations affecting only the G-quartet-structure was investigated. In conclusion, we show that wildtype FMRP and FXR2P are able to recruit FMRP variants into RNA-granules and that the G-quartet-structure in *FMR1* mRNA is not essential for its incorporation in RNA-granules.

Keywords: Fragile X syndrome, FMRP, *FMR1*, mRNA transport, FXR2P, RNA-granules

3.1 INTRODUCTION

Fragile X syndrome (FXS) is the most prevalent form of inherited mental retardation (Turner et al., 1996, Imbert et al., 1998). The syndrome is characterised by moderate to severe mental retardation, macroorchidism, mild facial abnormalities and behavioural manifestations (Hagerman et al., 1996). The main cause of the syndrome is an expansion of the CGG repeat in the 5'-untranslated region of the *FMR1* gene. If the expansion exceeds 200 CGG repeats, the adjacent CpG island and promoter region of the *FMR1* gene are methylated, resulting in transcriptional silencing of the gene. The lack of *FMR1* protein (FMRP) is responsible for the FXS phenotype (de Vries et al., 1998).

FMRP is expressed abundantly in the brain and testes. It has several conserved functional domains, containing three RNA-binding motifs -two KH-domains and a RGG-box-, a nuclear localisation sequence (NLS) and a nuclear export sequence (NES). The importance of the second KH-domain was illustrated by the study of a patient with a missense mutation in the second KH-domain (Ile304Asn) who has been diagnosed with a severe phenotype of FXS (De Boulle et al., 1993). This mutation results in the expression of mutant FMRP that no longer associates with translating polyribosomes, and loses its function as a translational repressor (Siomi et al., 1994, Laggerbauer et al., 2001). The RGG-coding region in FMRP can bind intramolecular G-quartet structures in target mRNAs (Schaeffer et al., 2001).

FMRP has two autosomal homologues, FXR1P and FXR2P (Fragile X-related proteins). These proteins are very similar to FMRP and contain the same conserved functional domains in addition to two Nucleolar Targeting Signals (NoS). The precise function of FXR2P is still unknown, although the *Fxr2* KO mice show some behavioural abnormalities similar to *Fmr1* KO mice (Bontekoe et al., 2002). FXR1P is mainly expressed in striated muscle, testis and brain and the *Fxr1* KO mice displays neonatal lethality (Mientjes et al., 2004).

FMRP appears to mediate transport and local translation of several mRNA targets at postsynaptic sites in neurons (Devys et al., 1993, Feng et al., 1997b, Bakker et al., 2000, De Diego Otero et al., 2002, Wang et al., 2008). Moreover, FXS patients and *Fmr1* KO mice both show structural malformations of dendritic protrusions, (Hinton et al., 1991, Comery et al., 1997, Irwin et al., 2001, McKinney et al., 2005, De Vrij et al., 2008) and aberrant synaptic plasticity (Huber et al., 2002, Koekkoek et al., 2005, Nosyreva and Huber, 2006). Clearly, dendritic mRNA transport and local protein synthesis are critical for synaptic plasticity and are widely studied in FXS. However, the exact mechanism of mRNA binding, transport kinetics and regulation of translation by FMRP is still largely unknown. FMRP has been suggested to transport target mRNAs from the nucleus, using its NES and NLS, to the cytoplasm. Although the presence of a NLS and NES suggests a role for FMRP in the nucleus, it has never been shown that it

is necessary for FMRP to associate with target mRNAs in the nucleus before it can be incorporated in dendritic RNA-granules.

To learn more about FMRP and its incorporation in RNA-granules, we studied a naturally occurring isoform of FMRP (FMRP_Iso12) and FMRP with the pathogenic mutation Ile304Asn (FMRP_I304N). The localisation of FMRP-positive RNA-granules containing either normal or the FMRP variants was studied in cultured primary *Fmr1*-knockout (KO) mouse neurons with and without co-expression of wildtype FMRP. In addition, we also studied *FMR1* mRNA localisation in transfected *Fmr1*-knockout neurons expressing different variants of FMRP, including FMRP_Iso12, FMRP_I304N and an *FMR1* construct that has silent point-mutations that affect the G-quartet-structure in the mRNA.

3.2 MATERIALS AND METHODS

Primary hippocampal neuron culture

Primary hippocampal neurons were cultured as described by De Vrij et al. (De Vrij et al., 2008). Hippocampi of *Fmr1* KO mice (Mientjes et al., 2006) were dissected from E18 mouse brain and placed in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL). After dissection, the hippocampi were dissociated using trypsin and mechanical treatment. The neurons were plated on coverslips coated with poly-D-lysine (100 µg/ml, Sigma) and laminin (50 µg/ml, Sigma). In a drop of Neurobasal medium (Gibco) containing penicillin/streptomycin (Gibco), Glutamax (Gibco) and B-27 (Gibco) supplements, 100,000 cells were allowed to attach to the substrate. After 2 h the medium volume was adjusted to 2 ml per coverslip in a six-well plate. After 20 days *in vitro,* the hippocampal neurons were transfected with several variants and wildtype human *FMR1* constructs under control of a chicken βactin promoter.

Expression vectors and transfection

MCherry or *EGFP* coupled fusion constructs were constructed by cloning the EcoR1 fragment containing *FMR1* from pCMV-*EGFP-FMR1* or pCMV-*EGFP-FMR1_I304N* (Castren et al., 2001) into the EcoR1 site of the βactin-*Cherry* or βactin-*EGFP* vector. To clone the natural splice variant of *FMR1* missing exon 12 and exon 14 (encoding for FMRP_Iso12) (Sittler et al., 1996) into βactin-*EGFP*, we digested CMV-*EGFP-FMR1_Iso12* (Tamanini et al., 1999b) with EcoRI and ligated *FMR1_Iso12* fragment into the βactin-*EGFP* that had also been digested with the same restriction enzyme. The G-quartet mutated construct (*FMR1_ΔG1+2*) has been described before (Didiot et al., 2008). This construct contains point-mutations in the G-quartet structure of *FMR1* mRNA without changing the amino acid sequence of the protein. The G-quartet mutant was also cloned behind *EGFP* to create a fusion protein. Finally, βactin-*FXR2* fusion constructs were created using human cDNA and the following FXR2 primers: forward

– cggactcagatctgagctcaagcttcgaat- and reverse – gagaagtactagtcgactggatcctgaatt-. The PCR product encoding for FXR2P was digested by *BglII* and *SalI* and the fragment was cloned in frame behind βactin-*EGFP* or βactin-*mCherry,* also digested with *BglII* and *SalI*. All fusion constructs were tested by sequencing (figure 3.1). Correct expression of all fusion proteins was tested using Western Blot on transfected HEK 293 cells showing that all fusion proteins were expressed as the expected molecular weight (supplemental figure S3.1).

After ~14 days *in vitro* (DIV), cells were transfected (1 µg DNA) or co-transfected (0.5 µg of each construct) with different *FMR1*-fusion constructs by Lipofectamine 2000 (Invitrogen). One day after transfection, cells were fixed with 4% paraformaldehyde in PBS, washed in PBS and mounted in Mowiol mounting solution (Mowiol 4-88, Hoechst).

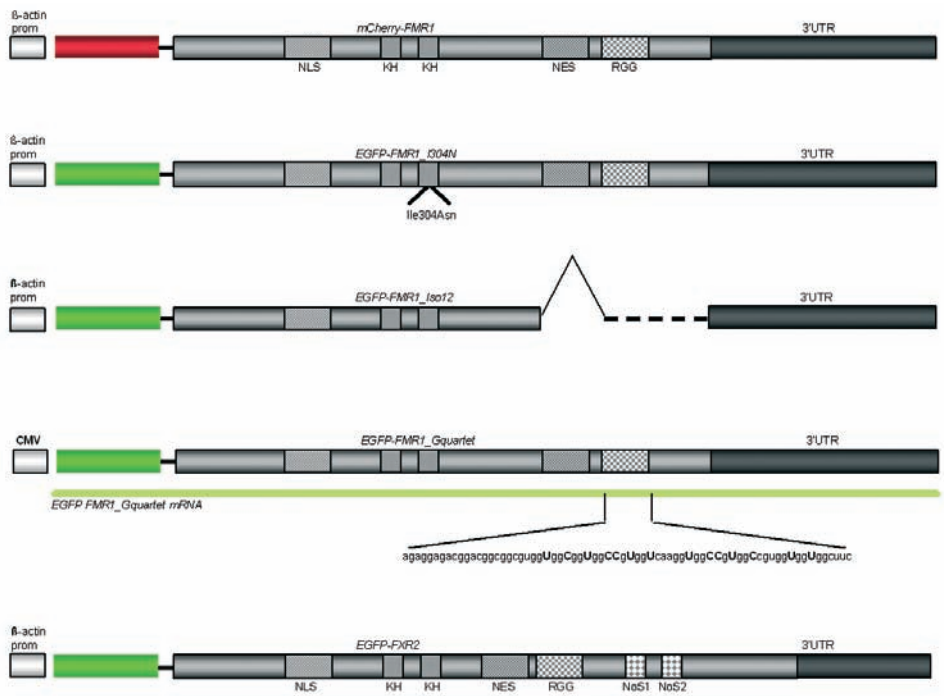

Figure 3.1. Schematic overview of constructs used for transfection experiments. All constructs, except *FMR1_G-quartet*, are driven by the chicken βactin-promoter to promote neuronal expression. The *EGFP-FMR1_I304N* has a pathogenic point-mutation in the second KH-domain. The *EGFP-FMR1_Iso12* is a naturally occurring splice variant lacking exon 12 and 14 and the C-terminal part of the protein is frame shifted due to alternate splicing. The *FMR1*_G-quartet construct harbors silent point-mutations in the mRNA resulting in disruption of the G-quartet structure of *FMR1* mRNA without affecting the amino acid sequence of FMRP. Finally, a construct encoding FXR2p fused to EGFP was used. For several constructs, both *mCherry* and *EGFP* fusion constructs were developed for co-transfection experiments. No differences between EGFP or mCherry coupled fusion proteins were observed.

Immunocytochemistry and antibodies

Neurons cultured for 14 days *in vitro* (DIV) were fixed with 4% paraformaldehyde in PBS and washed in PBS. For blocking and permeabilization we used "staining buffer" containing 0.05M Tris, 0.9% NaCl, 0.25% gelatin, and 0.5% Triton-X-100, pH 7.4. The following antibodies were used: human anti-ribosomal P antigen (RLP0, 1:2000; Immunovision) and polyclonal anti-FXR2P (Ab1937, 1:200) (Tamanini et al., 1999a) and mouse anti-FMRP (T1A, 1:200). Primary antibodies were diluted in *staining buffer* and were incubated overnight at 4 °C. The next day, the cells were washed in PBS and incubated with donkey anti-human-Cy3 antibody (1:200; Jackson Immunoresearch) and donkey anti-rabbit Cy2 antibody (1:200; Jackson Immunoresearch) or donkey anti-mouse Cy3 antibody (1:200; Jackson Immunoresearch) and diluted in *staining buffer* for 1 h at room temperature. Finally, the coverslips were washed in PBS and mounted in Mowiol mounting solution (Mowiol 4-88, Hoechst).

Riboprobes

The *FMR1* cDNA used to generate riboprobes for *in situ* hybridisation is an 800 bp fragment complementary to the 3' UTR of *FMR1*. To clone the 3' UTR fragment, we used cDNA of total human RNA and the following primers: forward – GTGAATGGAGTACCCTAAACTGCA- and reverse – CCTTCCTATCTCTCCAAAATAAGCATT-. The cDNA was then cloned into the TOPOII vector with a dual promoter (Invitrogen) and linearized. *FMR1* sense and antisense probes were synthesized by *in vitro* transcription in the presence of the appropriate RNA polymerase, and digoxigenin (DIG)-conjugated UTP according to the manufacturer's protocol (Roche).

In situ hybridisation

Fmr1 KO neurons were transfected with the different *FMR1* fusion constructs (described above). The following day, neurons were fixed with 4% PFA for 20 min. The coverslips were thoroughly washed with PBS and permeabilised with PBS-Triton X (0.1%), rinsed in 2x SSC for 5 min, dehydrated in an ethanol series, and air-dried. The coverslips were hybridized overnight at 55 °C in hybridisation mix (50% formamide, 5X SSC, 5x Denhardts, 250 µg/ml bakers yeast RNA, 500 µg/ml salmon sperm DNA) with the antisense or sense riboprobe concentration of 500 ng/ml. The *FMR1* sense probe was used as a negative control. After hybridisation, the coverslips were washed in 4x SSC and treated with RNase (10 µg/ml) for 30 min at 37 °C. The coverslips were then subjected to subsequent washing steps with 2x SSC/50% formamide, 1x SSC/50% formamide at 55 °C and 0.1x SSC.

Immunodetection of the DIG-labelled riboprobe was preceded by preincubating the sections for 30 min in buffer 1 (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 2% blocking reagent (Roche). Subsequently, the coverslips were incubated for 2 h at

room temperature with Cy3-conjugated mouse anti-DIG (Jackson ImmunoResearch 1:500) and polyclonal anti-EGFP (Abcam 1:1000) in buffer 1 containing 2% blocking reagent. After thorough washing in buffer 1 containing 0.5% Triton X, the coverslips were incubated with the secondary antibodies donkey anti-mouse Cy3 and donkey anti-rabbit Cy2 in buffer 1 for 1 h at room temperature. Finally the coverslips were washed in buffer 1 and mounted in Mowiol mounting solution (Mowiol 4-88, Hoechst).

Quantification of dendritic granules

Images of neurons transfected with different constructs were acquired using a Zeiss LSM510 confocal microscope. To quantify the number of macromolecular granules in dendrites after transfection of different FMRP constructs, detailed images of two dendrites of at least 5 different neurons in three independent experiments were quantified for each construct. Granules (defined as larger or equal to 0.1 um^2) were counted in equally sized regions of each dendrite using ImageJ software (developed by the National Institutes of Health). The average number of granules was statistical compared using the unpaired tailed Student's *T*-test.

3.3 RESULTS

To study the function of the conserved functional domains of FMRP in mRNA binding and dendritic transport, we used hippocampal primary neurons of *Fmr1* KO mice. Cultures were grown for 10 to 21 days which allowed us to study granule formation and transport into the dendrites and spines. The use of *Fmr1* KO neurons provided an Fmrp background-free cell system, preventing misinterpretation of effects caused by interactions between mutant and endogenous Fmrp.

FMRP is known to be present in RNA-granules trafficking into the dendrites. These granules consist of mRNAs, ribosomal subunits, motor-proteins and other RNA binding proteins (RBPs). In *Fmr1* KO neurons, Fmrp expression is totally absent and therefore the number of RNA-granules or the structure of these granules could be affected (Aschrafi et al., 2005). To study this possibility, *Fmr1* KO neurons and wildtype neurons were immunostained for two proteins that are known to be present in RNA-granules: ribosomal subunit RLP0 and a homologue of Fmrp, Fxr2p. Both wildtype and *Fmr1* KO hippocampal neurons showed P0 positive and Fxr2p positive granules (supplemental figure S3.2). The dendritic RNA-granule population is known to be heterogeneous (reviewed in (Kiebler and Bassell, 2006)), which is reflected in the staining pattern of P0 and Fxr2p in these neurons. Some dendritic RNA-granules contained P0 ribosomal subunit, but did not contain Fxr2p and vice versa. No obvious differences in RNA-granule size or number was observed between wildtype and *Fmr1* KO neurons with both markers.

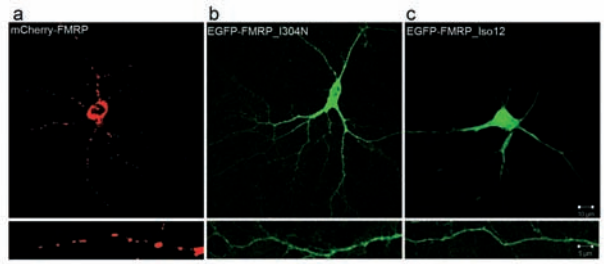

Figure 3.2. Localisation patterns of different FMRP constructs in Fmr1 KO hippocampal neurons. Wildtype mCherry-FMRP (a) showed a granular pattern in the dendrite. In contrast, EGFP-FMRP_I304N (b) and EGFP-FMRP_Iso12 (c) showed a more diffuse dendritic pattern. Moreover, EGFP-FMRP_Iso12 showed accumulation of the protein in the nucleus.

Consistent with the endogenous localisation of Fmrp in wildtype primary neurons, mCherry-FMRP, transiently transfected into *Fmr1*-KO primary hippocampal neurons was present in the cell soma and in a granular pattern within the dendrites (figure 3.2a). Several mutant *FMR1* constructs were developed to study the conserved functional domains of FMRP. The expression pattern of the construct carrying the pathogenic missense mutation I304N (EGFP-FMR1_I304N) showed EGFP-FMRP_I304N localisation in the cell soma and in the dendrites. However, the localisation pattern of EGFP-FMRP_I304N in the dendrites was more diffuse than the granular pattern of wildtype mCherry-FMRP in dendrites (figure 3.2b). We quantified this difference between EGFP-FMRP_I304N expression and wildtype mCherry-FMRP expression in *Fmr1* KO neurons by counting the number of FMRP-positive RNA-granules in distal dendrites. We defined dendritic RNA-granules as having a size of 0.1 μm^2 or more. As figure 3.3 shows, EGFP-FMRP_I304N was less abundant in RNA-granules than wildtype mCherry-FMRP.

In addition, we studied the localisation of EGFP-FMRP_Iso12, a natural splice variant of FMRP that lacks exon 12 and 14, the latter of which contains the NES. Importantly, the C-terminal part of the protein is frame shifted due to alternative splicing. *EGFP-FMR1_Iso12* transfected neurons showed a predominantly nuclear localisation of the protein, with slightly diffuse cytoplasmic expression (3.2c). Also protein seems not to be incorporated into dendritic RNA-granules. In contrast to wildtype FMRP expression, this natural splice variant does not show EGFP-FMRP_Iso12-positive dendritic RNA-granules, as quantified in figure 3.3.

Effect of wildtype FMRP on localisation of FMRP variants

In vitro studies have demonstrated that FMRP_I304N has lost the ability to form homo-oligomers (Laggerbauer et al., 2001) and shows an abolished binding to elongating polyribosomes (Feng et al., 1997a). Laggerbauer et al showed *in vitro*

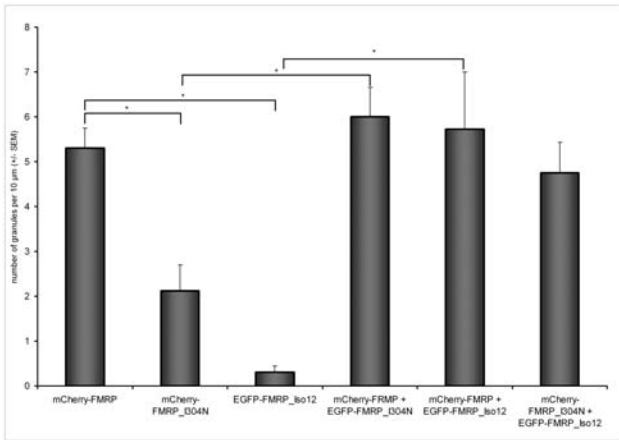

Figure 3.3. Quantification of FMRP-positive granules in dendrites of transfected Fmr1 KO neurons. *Fmr1* KO neurons transfected with wildtype *FMR1* showed 5 granules per 20 µm, while neurons transfected with *Cherry-FMR1_I304N* and *EGFP-FMR1_Iso12* show significantly less granules per 20 µm ($P<0.05$). *EGFP-FMR1_I304N* or *EGFP-FMR1_Iso12* co-transfection with wildtype *Cherry-FMR1* revealed more dendritic granules than with either one of the protein variant alone, similar to wildtype levels (counted number of granules containing the variant) ($P<0.05$). Surprisingly, when mCherry-FMRP_I304N and EGFP-FMRP_Iso12 were co-expressed, both proteins were also incorporated in granules and the number of RNA-granules positive for both variants was not significantly different from the number of granules after wildtype *Cherry-FMR1* transfection.

that FMRP_I304N was still able to form dimers with wildtype FMRP, but this was never shown *in vivo*. Therefore, additional transfection experiments were carried out with the presence of wildtype mCherry-FMRP to test whether wildtype FMRP can recruit FMRP_I304N in RNA-granules. First, we studied the expression pattern of EGFP-FMRP_I304N in *Fmr1* KO primary hippocampal neurons when co-transfected with wildtype mCherry-FMRP. These co-transfected neurons showed dendritic RNA-granules that were positive for wildtype mCherry-FMRP and most of these granules showed co-localisation with EGFP-FMRP_I304N (figure 3.4a). In addition, we quantified the number of RNA-granules positive for EGFP-FMRP_I304N in distal dendritic regions. In these co-transfection studies the number of EGFP-FMRP_I304N-positive dendritic RNA-granules was similar as in wildtype mCherry-FMRP transfected neurons (quantified in figure 3.3), demonstrating that EGFP-FMRP_I304N can be incorporated in RNA-granules and co-localises with wildtype FMRP. These results suggest that the two proteins can hetero-dimerise.

In addition, we also tested whether the presence of wildtype mCherry-FMRP affects the predominantly nuclear and diffuse dendritic distribution of EGFP-FMRP_Iso12. Co-transfection of *EGFP-FMR1_Iso12* and wildtype *Cherry-FMR1* in *Fmr1*-KO primary hippocampal neurons resulted in mCherry-FMRP positive RNA-granules in

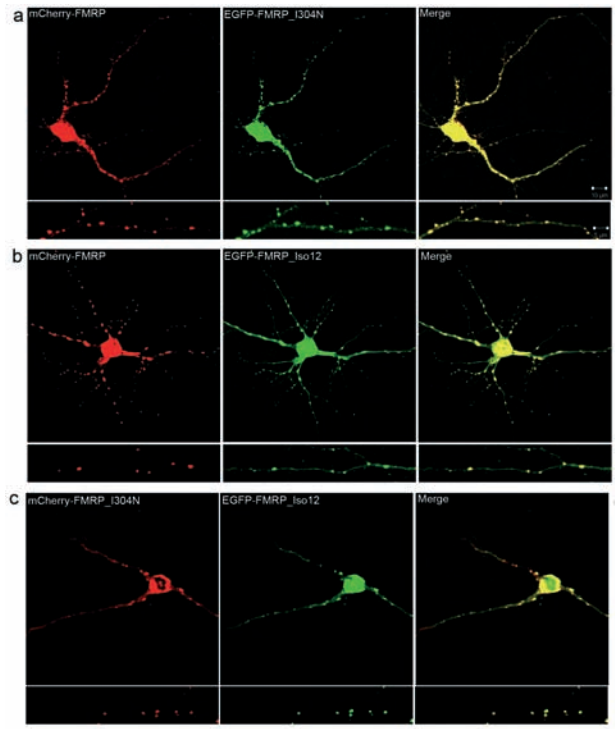

Figure 3.4. Localisation patterns of FMRP variants and mCherry-FMRP after co-transfection in Fmr1 KO hippocampal neurons. (a)*EGFP-FMR1_I304N* and *Cherry-FMR1* co-transfection resulted in co-localisation of the two proteins in dendritic RNA-granules. (b) EGFP-FMRP_Iso12 also co-localised with wildtype mCherry-FMRP and was incorporated in RNA-granules. Although EGFP-FMRP_Iso12 was incorporated in dendritic granules, some EGFP-FMRP_Iso12 remained in the nucleus. (c) Localisation patterns of EGFP-FMRP_Iso12 and mCherry-FMRP_I304N after co-transfection in *Fmr1* KO hippocampal neurons. EGFP-FMRP_Iso12 co-localised with mCherry-FMRP_I304N and both proteins were incorporated in RNA-granules. Although EGFP-FMRP_Iso12 was incorporated in dendritic granules, a small proportion was still present in the nucleus.

the dendrite that were also positive for EGFP-FMRP_Iso12 (figure 3.4b). Quantification of the number of EGFP-FMRP_Iso12 RNA-granules revealed a number of granules similar to wildtype mCherry-FMRP-positive granules in a single transfection (Fig 3), showing that also FMRP_Iso12 can be incorporated in RNA-granules together with wildtype FMRP. Finally, when *EGFP-FMR1_Iso12* and *Cherry-FMR1_I304N* were co-transfected, a small proportion of both proteins was also incorporated together in a significant number of dendritic RNA-granules (figure 3.3). Colocalisation of both FMRP variants in dendritic RNA-granules is shown in figure 3.4c.

Role of FMRP-homologue FXR2P in dendritic granule formation

FXR2P is a homologue of FMRP that contains the same functional domains as FMRP in addition to two Nucleolar Targeting Signals (NoS) in the 3' terminal of the protein. We were interested to see whether FXR2P is capable of recruiting FMRP_I304N or FMRP_Iso12 in dendritic RNA-granules. First we studied EGFP-FXR2P expression in *Fmr1* KO hippocampal neurons. Figure 3.5 illustrates the presence of EGFP-FXR2P in both the cell soma of neurons and dendritic RNA-granules (figure 3.5a). To visualize spines of EGFP-FXR2P transfected neurons, mCherry protein was co-expressed, which clearly showed that EGFP-FXR2P is also present in dendritic spines (figure 3.5a, merge). *EGFP-FXR2* and *Cherry-FMR1* co-transfection showed that all dendritic RNA-granules contained FXR2P and FMRP (figure 3.5b).

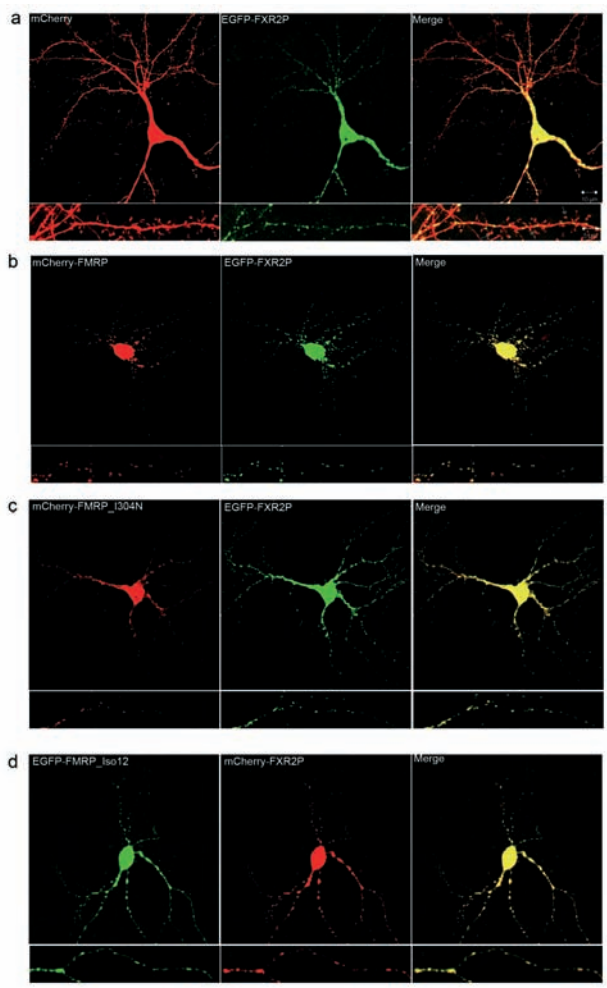

Figure 3.5. (a) *Fmr1* KO neurons co-transfected with βactin-*Cherry* and *EGFP-FXR2* showed cytoplasmic EGFP-FXR2P and EGFP-FXR2P-positive dendritic RNA-granules. Note that a few spines also contained EGFP-FXR2P (examples indicated by arrow). (b) *Fmr1* KO neurons co-transfected with *EGFP-FXR2* and wildtype *Cherry-FMR1* showed dendritic RNA-granules containing both proteins. Like FMRP, FXR2P was able to incorporate mCherry-FMRP_I304N *(c)* and EGFP-FMRP_Iso12 *(d)* in dendritic RNA-granules.

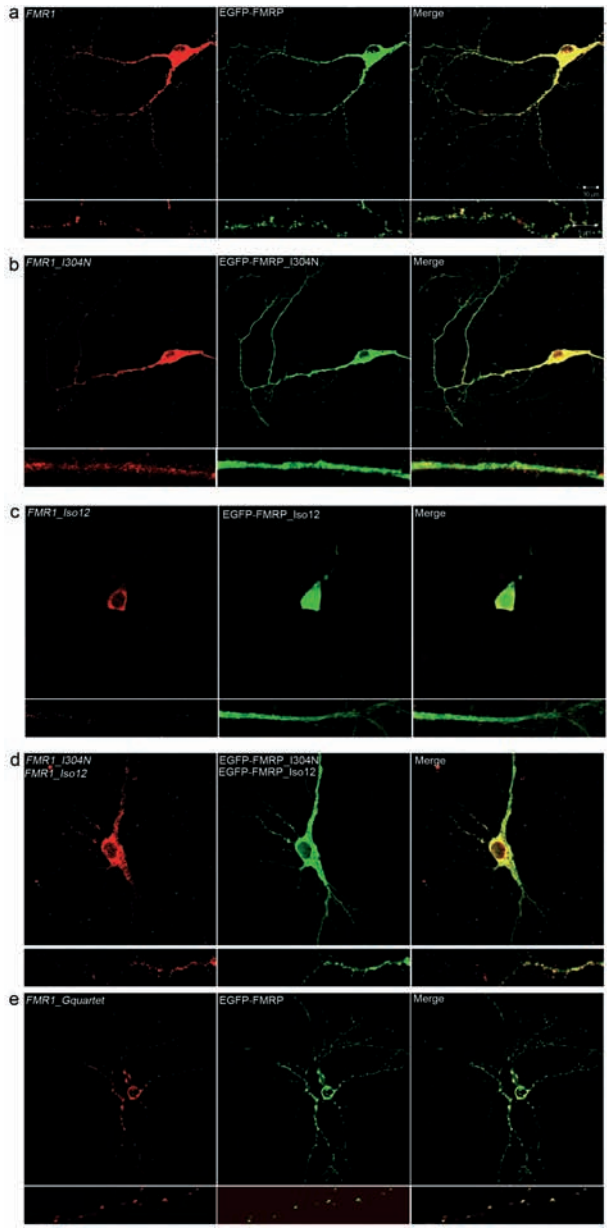

Figure 3.6. *In situ* hybridisation for *FMR1* on transfected *Fmr1* KO neurons. (a) In neurons transfected with *EGFP-FMR1*, *FMR1* mRNA was localised in dendritic RNA-granules co-localising with EGFP-FMRP. (b) *FMR1_I304N* mRNA was more diffusely localised in the dendrite compared to wildtype *FMR1* mRNA. (c) *FMR1_Iso12* mRNA was mainly localised in the cell soma with only some RNA in RNA-granules distributed in the proximal dendrite. (d) *FMR1_Iso12* and *FMR1_I304N* mRNAs (both in red) were incorporated in dendritic RNA-granules in neurons co-transfected with both variants (both in green). (e) *EGFP-FMR1_ΔG1+2* mRNA was localised in dendritic RNA-granules co-localising with EGFP-FMRP.

To study possible recruitment of mutant FMRP by FXR2P in dendritic RNA-granules, *EGFP-FXR2* and *Cherry-FMR1_I304N* were co-transfected in *Fmr1* KO neurons. The results indeed showed dendritic RNA-granules that contained EGFP-FXR2P as well as mCherry-FMRP_I304N (figure 3.5c). This suggests that EGFP-FXR2P is able to recruit mCherry-FMRP_I304N in dendritic RNA-granules. Also the

natural splice variant EGFP-FMRP_Iso12 was recruited into dendritic RNA-granules by mCherry-FXR2P (figure 3.5d).

mRNA localisation of mutant *FMR1* mRNA

One of the mRNA targets of FMRP is *FMR1* mRNA itself (Sung et al., 2000, Schaeffer et al., 2001, Dolzhanskaya et al., 2003). Therefore, to study the transport of *FMR1* mRNA by different FMRP variants, we examined the mRNA localisation of both wildtype and *FMR1* mRNA variants in *Fmr1* KO neurons using *in situ* hybridisation.

FMR1 mRNA in *Fmr1* KO neurons transfected with *EGFP-FMR1* is transported into the dendrite and co-localises with FMRP in dendritic granules (figure 3.6a). Most FMRP-positive RNA-granules also contained *FMR1* mRNA. The pathogenic mutation in the second KH-domain of EGFP-FMRP_I304N may affect the mRNA binding capacity of the mutant protein. *EGFP-FMR1_I304N* transfected in *Fmr1* knockout neurons showed a co-localisation of *FMR1_I304N* mRNA with EGFP-FMRP_I304N and correspondingly showed a diffuse dendritic mRNA distribution compared to wildtype *FMR1* mRNA positive RNA-granules (figure 3.6b). *Fmr1* KO neurons transfected with *FMR1_Iso12* showed that *FMR1_Iso12* mRNA was mainly localised in the cytoplasm and only detected marginally in proximal dendritic RNA-granules (figure 3.6c). When co-transfected with FMRP_I304N the mRNAs of both variants were also incorporated in dendritic RNA-granules similar to the proteins (figure 3.6d), although we were unable to distinguish between the two mRNAs since both are recognized simultaneously by the same probe.

As shown above, FXR2P was able to recruit both FMRP variants in dendritic RNA-granules. Therefore, we were also interested to study whether the mRNAs of both

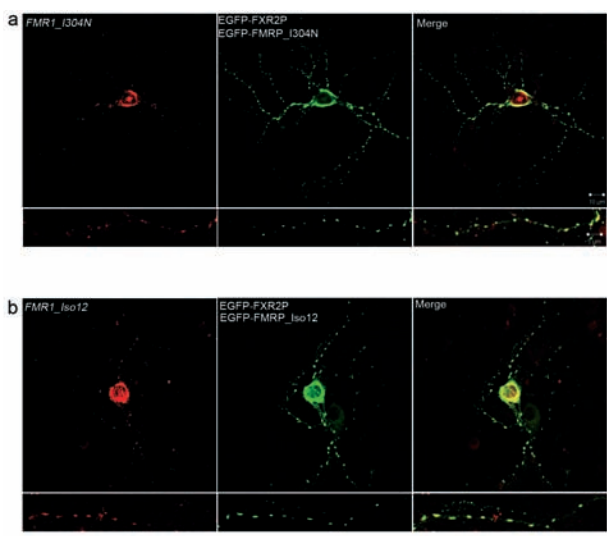

Figure 3.7. *In situ* hybridisation for *FMR1* mRNA after co-transfection of FMRP variants and FXR2P in *Fmr1* KO neurons. (a) Neurons co-transfected with *EGFP-FMR1_I304N* and *EGFP-FXR2* or (b) *EGFP-FMR1_Iso12* and *EGFP-FXR2* showed that *FMR1* mRNAs were incorporated in dendritic RNA-granules.

variants were also incorporated in the dendritic RNA-granules when the neurons were co-transfected with FXR2P combined with one of the FMRP variants. Therefore, *Fmr1* KO neurons were co-transfected either with *EGFP-FXR2* and *EGFP-FMR1_I304N* or with *EGFP-FXR2* and *EGFP-FMR1_Iso12* followed by an *in situ* hybridisation for *FMR1* mRNA. *FMR1_I304N* and *FMR1_Iso12* mRNAs were indeed incorporated in dendritic RNA-granules and co-localised with the EGFP fusion-proteins. (figure 3.7a and 3.7b).

In addition to wildtype *FMR1, FMR1_I304N* and *FMR1*_Iso12 constructs, we also examined a construct with a mutation that prohibits the formation of the G-quartet structure in *FMR1* mRNA without affecting the amino acid sequence of the protein (*EGFP-FMR1_ΔG1+2*) (Didiot et al., 2008). Since the amino acid sequence is not changed but only the G-quartet structure in the mRNA is affected, the protein shows the same granular pattern as wildtype Cherry-FMRP. Neurons transfected with *EGFP-FMR1*_ΔG1+2 showed the same mRNA distribution as wildtype *FMR1* mRNA in *EGFP-FMR1* transfected cells (figure 3.6e). The *FMR1_ΔG1+2* mRNA was incorporated in dendritic RNA-granules and co-localised with EGFP-FMRP.

3.4 DISCUSSION

It has been proposed that FMRP binds target mRNAs and mediates mRNA transport into the dendrite. During this dendritic mRNA transport, FMRP might play a role in silencing its target mRNAs. Upon arrival of the target mRNAs in spines, FMRP seems to play a role in local translation of these mRNAs (Brown et al., 2001, Weiler et al., 2004). Moreover, spine morphology in both FXS patients and in *Fmr1* KO mice is also affected, showing an immature morphology (Hinton et al., 1991, Comery et al., 1997, Irwin et al., 2001, McKinney et al., 2005). In 2004, the mGluR theory was proposed to explain the spine and electrophysiological characteristics seen in FXS (Bear et al., 2004). The spine phenotype in FXS is explained by exaggerated AMPA-receptor internalisation after mGluR5 stimulation and increased protein synthesis resulting in immature spines and enhanced LTD. Despite these findings, not much is known about the basic properties of FMRP-mRNA binding, target mRNAs and transport kinetics.

Our hippocampal neuron cultures of the *Fmr1* KO mice are the perfect tool to study RNA-granule formation and dendritic mRNA transport. Hippocampal neurons of the *Fmr1* KO mice allow us to solely study the effects of mutant forms of FMRP on FMRP-mRNA binding, target mRNAs and transport kinetics without endogenous Fmrp that might interfere with results.

Fmr1 KO neurons transfected with *FMR1_I304N* showed a diffuse pattern in the dendrite and a significantly lower number of EGFP-positive RNA-granules. This is in line with previous work showing that FMRP_I304N display a more diffuse pattern in cultured PC12 cells and hippocampal neurons (Schrier et al., 2004, Wang et al., 2008). However, the disadvantage of cells used in these studies is that there is endogenous

Fmrp present which interfere with the distribution of the FMRP mutants (figure 3.4). The significantly reduced number of FMRP_I304N-positive granules can be due to the inability of the mutant protein to bind to polyribosomes, leaving only single FMRP_I304N present in RNP particles bound to microtubules to be transported into the dendrite. Indeed, Wang et al. showed that GFP-FMRP_I304N could still be found in granules although these granules were much smaller than RNA-granules containing wildtype FMRP (Wang et al., 2008). Strikingly, if neurons were co-transfected with wildtype *Cherry-FMR1* and *EGFP-FMR1_I304N* constructs, both proteins co-localised in dendritic RNA-granules. Previously is was shown that *in vitro* that FMRP_I304N lost the function to dimerise with itself, but that FMRP_I304N can still dimerise with wildtype FMRP, FXR1P and FXR2P (Laggerbauer et al., 2001), although Feng et al. found no co-sedimentation in sucrose-gradient of FXR2P and FMRP_I304N using I304N-patient lymphoblasts (Feng et al., 1997a). In agreement with these results, we now show in primary hippocampal neurons that wildtype mCherry-FMRP can mediate, probably by dimerisation, the presence of EGFP-FMRP_I304N in RNA-granules.

EGFP-FMRP_Iso12 is an alternatively spliced variant of FMRP (Sittler et al., 1996). Several experiments have shown that FMRP without a NES (FMRP-NES) accumulates in the nucleus in different cell lines (Fridell et al., 1996, Willemsen et al., 1996, Tamanini et al., 1999a, Hu et al., 2005), although some studies also have shown the presence of small quantities of FMRP-NES in the cytoplasm (Kim et al., 2008). *Fmr1* KO neurons transfected with *EGFP-FMR1_Iso12* showed a predominantly nuclear localisation, while the dendrites did not show dendritic EGFP-FMRP_Iso12-positive RNA-granules. Since the FMRP_Iso12 construct lacks a NES signal, the fusion protein cannot be exported out of the nucleus and accumulates in the nucleus. Moreover, the lack of EGFP-FMRP_Iso12-positive RNA-granules indicates that FMRP_Iso12 cannot be incorporated direct after synthesis in the cytoplasm in RNA-granules before it is transported into the nucleus. In theory, the absence of EGFP-FMRP_Iso12-positive RNA-granules could also result from the lack of the RNA-binding RGG-box in this construct. However, earlier experiments by Sittler et al. using a FMRP-NES construct that lacks only exon 14 without a frameshift, showed a similar localisation pattern as the natural FMRP-Iso12 in COS cells (Sittler et al., 1996). Furthermore, it has been showed that the C-terminal part of FMRP is important for microtubule-dependent transport into the dendrites via kinesin (Dictenberg et al., 2008). Thus, FMRP_Iso12 might show a diffuse distribution pattern because it cannot bind to kinesin and consequently is not transported along microtubules. Moreover, the small amount of EGFP-FMRP_Iso12 found in the dendrite and cell soma might arise from a different nuclear export mechanism (Kim et al., 2008) or might reflect newly synthesized fusion proteins waiting for nuclear shuttling. Although there is some cytoplasmic FMRP_Iso12, our results suggest that FMRP requires to be first transported into the nucleus to pick up

target mRNAs or interact with other RNP proteins, before it can be incorporated in RNA-granules and subsequently transported into the dendrite.

Neurons co-expressing EGFP-FMRP_Iso12 and wildtype Cherry-FMRP show positive labelling of RNA-granules for both proteins. This indicates that EGFP-FMRP_Iso12 can be incorporated into dendritic RNA-granules in the presence of wildtype FMRP. It is already known that FMRP can dimerise with itself and FXR1P and FXR2P (Tamanini et al., 1999b, Laggerbauer et al., 2001). The observation that EGFP-FMRP_Iso12 could be incorporated into RNA-granules together with wildtype FMRP suggests that the variant EGFP-FMRP_Iso12 most likely dimerise with wildtype FMRP in the nucleus and in this way can escape accumulation in the nucleus. Surprisingly, when neurons were co-transfected with *Cherry-FMR1_I304N* and *EGFP-FMR1_Iso12*, RNA-granules were labelled positive for both proteins. This result suggests that EGFP-FMRP_Iso12 and mCherry-FMRP_I304N may dimerise using their coiled-coiled domains and ultimately be incorporated in RNA-granules using each others conserved domains, i.e. the NES of FMRP_I304N to exit the nucleus and the normal KH2 domain of FMRP_Iso12.

Finally, we were interested to see whether the FMRP-homologue FXR2P was also capable of binding the FMRP variants resulting in FMRP-positive dendritic mRNA-granules. FXR2P shares all the conserved domains of FMRP and therefore it is suggested that the homologues might be able to compensate to some extent for the lack of FMRP in *Fmr1* KO mice. Like FMRP, FXR2P is incorporated in dendritic mRNA-granules and is localised in a subpopulation of spines (figure 3.5a). Although endogenous Fxr2p levels in the neurons are most likely not sufficient to recruit both variants into RNA-granules, co-transfection of *EGFP-FXR2* together with one FMRP variants resulted RNA-granules containing both mutant FMRP and FXR2P. In addition, similar to FMRP, FXR2P was able to recruit mutant forms of FMRP in dendritic RNA-granules. Our results suggest that FXR2P and FMRP can functionally interact in neurons.

mRNA localisation

There are several studies showing that FMRP can bind its own mRNA (Sung et al., 2000, Schaeffer et al., 2001, Dolzhanskaya et al., 2003). However, we were interested whether mutant proteins still posses this function. Therefore, *Fmr1* KO neurons were transfected with several *FMR1* variants, followed by an *in situ* hybridisation to study the subcellular localisation of *FMR1* mRNAs. As expected, wildtype FMRP co-localised with its mRNA in RNA-granules as previously described (Antar et al., 2004). However, some granules were only positive for *FMR1* mRNA and not for FMRP. This suggests that *FMR1* mRNA can also bind to other mRNA-binding proteins to be incorporated in RNA-granules and transported into the dendrite.

Furthermore, we studied *FMR1* mRNA localisation of the mutant *FMR1_I304N*. The mutation is located in the second KH-domain, which is involved in binding of mRNAs with a sequence-specific element in a complex tertiary structure termed the

FMRP kissing complex (Darnell et al., 2005). *FMR1-I304N* mRNA was also diffusely localised in the dendrites, like FMRP_I304N. Wang et al. showed with propidium iodide, which stains all mRNAs, that mutant FMRP-I304N co-localises with mRNAs, suggesting that it still has the property to bind mRNAs (Wang et al., 2008). In contrast, Siomi et al. showed that FMRP_I304N has impaired mRNA-binding properties (Siomi et al., 1994). Our results suggest that FMRP_I304N can still bind its own mRNA but that the protein/RNA complex has lost the property to be incorporated in RNA-granules.

We also studied the *FMR1_Iso12* mRNA localisation. The protein lacks the NES in exon 14 and due to a frameshift mutation the protein also does not contain exon 15 to 17. However, this frameshift does not hamper the G-quartet structure in the mRNA. *FMR1_Iso12* mRNA is found in the cell soma of neurons, but was hardly detected in dendritic RNA-granules. This suggests that *FMR1_Iso12* mRNA incorporation in dendritic RNA-granules is very inefficient or delayed, probably due to accumulation of EGFP-FMRP_Iso12 in the nucleus that would otherwise transport the mRNA. Interestingly, when both variants were co-transfected in neurons, dendritic RNA-granules that are positive for both EGFP-FMRP_Iso12 and EGFP-FMRP_I304N also showed the incorporation of *FMR1* mRNA.

In a similar fashion, FXR2P was able to recruit not only the FMRP variants in dendritic RNA-granules, but also their corresponding mRNAs. Unfortunately, we could not discriminate whether FXR2P or the FMRP variant is able to bind to the *FMR1* mRNA. However, our results suggest that FXR2P interacts with *FMR1* mRNA in neurons.

Finally, the *FMR1_ΔG1+2 in situ* hybridisation experiments showed that *FMR1-ΔG1+2* mRNA co-localise with FMRP-positive RNA-granules and was still transported into the dendrite. Recently it has been reported that this mutant *FMR1_ΔG1+2* mRNA has a disrupted binding to FMRP, but that the mRNA is normally localised in HeLa cells (Didiot et al., 2008). Our results suggest that *FMR1-ΔG1+2* mRNA can still be transported into the dendrite and therefore the G-quartet structure is not essential for incorporation of *FMR1-ΔG1+2* mRNA in dendritic RNA-granules.

In conclusion, we show that the KH2-domain and the C-terminal part of FMRP are important for *FMR1* mRNA transport from the nucleus into dendritic RNA granules in primary hippocampal neurons. Moreover, mutations in these domains can be functionally overcome by co-expression of wildtype FMRP or FXR2P. This suggests that hetero-dimers of mutant and wildtype FMRP or FXR2P are functionally formed in these neurons. Finally, the G-quartet RNA structure was found not to be necessary for the dendritic transport of *FMR1* mRNA.

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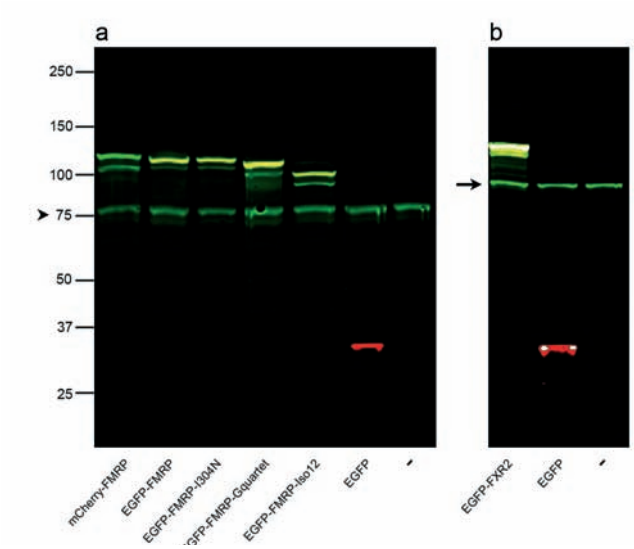
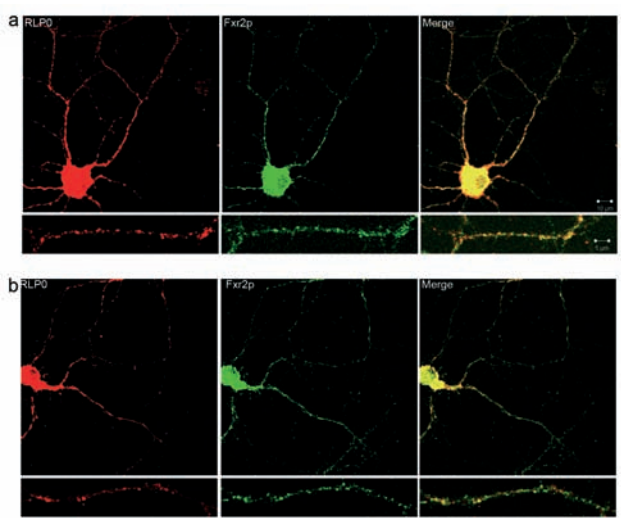


Supplemental figure S3.1. Western blot of HEK 293 cells transfected with fusion constructs was performed as described in Coevoets et al, 2009. HEK cells were transfected with following constructs: lane 1: *mCherry-FMR1*, lane 2: *EGFP-FMR1*, lane 3: *EGFP-FMR1_I304N*, lane 4: *EGFP-FMR1_Gquartet*, lane 5: *EGFP-FMR1_Iso12*, lane 6: *EGFP* alone, lane 7: non transfected, lane 8: EGFP-FXR2P, lane 9: EGFP alone, lane 10: non transfected. Blots were immunostained with (A) primary antibodies rb anti GFP (Abcam) and ms anti FMRP (T1A) and (B) primary antibodies mouse anti FXR2P (2G1) and rb anti GFP (Abcam). Following fluorescent secondary antibodies were used: goat anti rabbit 680nm and goat anti mouse 800nm secondary antibodies. All fusion construct do express the protein as expected and are not degraded.



Supplemental figure S3.2. Wildtype (a) and *Fmr1* KO (b) primary hippocampal neurons were stained for ribosomal RPLO and Fxr2p to visualize granules in the dendrite. Some granules contain both ribosomal subunits and Fxr2p, but there are also granules that are only positive for P0 or only positive for Fxr2p. There are no obvious differences in size or number of granules between wildtype and *Fmr1* KO hippocampal neurons. These results demonstrate that in the total absence of Fmrp, overall dendritic RNA-granule formation is normal.

4

RESCUE OF BEHAVIOURAL PHENOTYPE AND NEURONAL PROTRUSION MORPHOLOGY IN *FMR1* KO MICE

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ABSTRACT

Lack of fragile X mental retardation protein (FMRP) causes fragile X syndrome, the most common form of inherited mental retardation. FMRP is an RNA-binding protein and is a component of messenger ribonucleoprotein complexes, associated with brain polyribosomes, including dendritic polysomes. FMRP is therefore thought to be involved in translational control of specific mRNAs at synaptic sites. In mice lacking FMRP, protein synthesis-dependent synaptic plasticity is altered and structural malformations of dendritic protrusions occur. One hypothesised cause of the disease mechanism is based on exaggerated group I mGluR receptor activation. In this study, we examined the effect of the mGluR5 antagonist MPEP on fragile X related behaviour in *Fmr1* knockout mice. Our results demonstrate a clear defect in prepulse inhibition of startle in *Fmr1* knockout mice, that could be rescued by MPEP. Moreover, we show for the first time a structural rescue of fragile X related protrusion morphology with two independent mGluR5 antagonists.

Keywords: fragile X syndrome, spines, dendrite branching, MPEP, fenobam, prepulse inhibition of startle, metabotropic glutamate receptor, primary hippocampal neuron culture.

4.1 INTRODUCTION

Fragile X syndrome (FXS) is the most common heritable form of mental retardation. The syndrome is caused by a lack of expression of FMRP (fragile X mental retardation protein), which is the protein product of the *FMR1* gene. In most cases, the lack of expression is caused by expansion of a CGG repeat (>200 units) in the 5' UTR of the *FMR1* gene, leading to methylation of both the CGG repeat and the promoter region, accompanied by transcriptional silencing. FMRP is an RNA binding protein that associates with polyribosomes and is localised in neurons in the form of granules that move in a microtubule dependent manner with the speed of RNA transport (De Diego Otero et al., 2002; Antar et al., 2005; Wang et al., 2007). Moreover, FMRP has been shown to influence the translation efficacy of several of its target mRNAs (reviewed in (Bagni and Greenough, 2005; Bardoni et al., 2006; Zalfa et al., 2007), which also implicates local translation at synaptic sites (Weiler et al., 1997; Greenough et al., 2001; Weiler et al., 2004; Muddashetty et al., 2007). In most cases, FMRP acts as a translational repressor (Laggerbauer et al., 2001; Lu et al., 2004). Therefore, FMRP is thought to be involved in the transport and/or the regulation of local mRNA translation at synaptic sites (Weiler et al., 1997; Miyashiro et al., 2003; Weiler et al., 2004; Bagni and Greenough, 2005). The presumed loss of translational regulation at synaptic sites might underlie the cognitive impairment in FXS (Huber et al., 2000).

Over the last few years, the metabotropic glutamate receptor (mGluR) theory of FXS has gained much support (Bear et al., 2004). The mGluR theory states that AMPA receptor internalisation triggered by mGluR5 stimulation (Snyder et al., 2001), is exaggerated in *Fmr1* knockout (KO) mice, accounting for the enhanced hippocampal LTD found in KO mice (Huber et al., 2002; Bear et al., 2004). Recently it was shown that FMRP deficient dendrites indeed show aberrant AMPA receptor trafficking resulting in a significantly reduced number of AMPA receptors at the plasma membrane (Nakamoto et al., 2007). Moreover, *Fmr1* KO mice that are crossbred with mice that have a 50% reduction in mGluR5 expression were shown to be rescued in several phenotypic aspects (Dolen et al., 2007). It is hypothesised that FMRP normally is involved in the inhibition of the translation of several local mRNAs that are important for the mediation of AMPA receptor internalisation. Since the amount of AMPA receptors in the postsynaptic density is correlated with protrusion shape, this might also explain the immature protrusion morphology that has been found in different brain areas of both patients with FXS and *Fmr1* KO mice (Hinton et al., 1991; Comery et al., 1997; Nimchinsky et al., 2001; Galvez and Greenough, 2005; Koekkoek et al., 2005; Grossman et al., 2006). The mGluR theory has also boosted the search for therapeutic targets for FXS. An antagonist of mGluR5 receptors would theoretically counteract the increased amount of AMPA receptor internalisation in *Fmr1* KO neurons. Behavioural studies have shown that *Fmr1* KO mice treated with the mGluR5 antagonist MPEP

(2-methyl-6-(phenylethynyl)-pyridine hydrochloride) clearly display less sensitivity to audiogenic seizures and more wildtype-like behaviour in an open field test compared with untreated mice (Yan et al., 2005). Also in a *Drosophila* model based on loss of function of *dfmr1*, the single homolog of the *FXR* family of genes in the *Drosophila* genome, MPEP was able to rescue courtship behaviour and mushroom body defects (McBride et al., 2005). However, the molecular mechanisms behind the effects of MPEP have not been elucidated.

In the present study, we show a defect in prepulse inhibition of acoustic startle (PPI) in *Fmr1* KO mice compared to wildtype littermates and a rescue of this behavioral phenotype by the mGluR5 antagonist MPEP. In addition, we demonstrate an altered protrusion morphology in *Fmr1* KO primary hippocampal neurons that could be rescued using two different mGluR5 antagonists, MPEP and fenobam, rendering protrusion densities indistinguishable from wildtype neurons.

4.2 MATERIALS AND METHODS

Mouse models

Fmr1 KO mice were generated in our lab as described previously (Bakker et al., 1994; Mientjes et al., 2006) and were backcrossed to C57BL/6J mice at least seven times.

Prepulse inhibition of startle

Prepulse inhibition of startle (PPI) was measured by analysis of eye blink reactions of mice to acoustic stimuli, based on the magnetic distance measurement technique (MDMT) used for eye blink conditioning (Koekkoek et al., 2002; Koekkoek et al., 2005). Adult *Fmr1* KO mice (n=8) and wildtype littermates (n=9) were anesthetised with an oxygenated mixture of nitrous oxide and isoflurane. A dental acrylic pedestal was placed on the skull and animals were allowed to recover for three days. Prior to the experiment the mice were very briefly sedated using the isoflurane/nitrous oxide mixture. A sensorholder with an airchannel and a magnetsensor was attached to the pedestal. A small neobdimium iron borium magnet (0.8*1.6*0.2 mm) was glued to the lower eyelid with a minute drop of cyanoacryllate and a silicon body harness was put on to protect the mice from strain on the pedestal. Mice were placed inside their own cages within soundproof training chambers and allowed to recover until normal behaviour (grooming, eating) returned, usually this was within 15 minutes. To test and calibrate the MDMT system air puffs were given as a measure of full eyelid closure. A background noise level of 60 dB white noise was present. Subsequently, the mice were presented with a white noise startle stimulus of 90 dB, which in the prepulse inhibition condition was preceded by a 70 dB white noise prepulse, 50 ms before the startle stimulus.

Each mouse was subjected to seven blocks of trials consisting of one air puff and three repeated measures of a startle stimulus followed fifty seconds later by a prepulse/

startle stimulus with a fifty seconds intertrial interval. The next day the same mice were analysed again in the same way after MPEP treatment. MPEP treatment was administered by i.p. injection of 20 mg/kg MPEP dissolved in PBS, 30 minutes before the experiment. Percentages of PPI of startle were compared by non-parametric Mann-Whitney U test.

Primary hippocampal neuron culture

In short, E18 wildtype and *Fmr1* KO mice litters were planned on the same day. Embryos were decapitated after which hippocampi were removed and dissociated by trypsin and mechanical treatment. Neurons were plated on poly-L-lysine (100 µg/ml, Sigma) and laminin (50 µg/ml, Sigma) coated 30 mm glass coverslips. The neurons were attached to the substrate in a drop of Neurobasal medium (Gibco), containing penicillin/streptomycin (Gibco), Glutamax (Gibco) and B-27 (Gibco) supplements. After 2 hours, medium volume was adjusted to 2ml per coverslip in 6-well plates. After 20 days *in vitro* cells were transfected, using Lipofectamine 2000 (Invitrogen), with an mCherry construct under control of a chicken bactin promoter to ensure neuron-specific expression. One day after transfection, cells were treated for four hours with 200 mM MPEP (Sigma), 300 mM fenobam (Sigma), 100 mM D-AP-5 (Sigma) or left untreated in supplemented Neurobasal medium. After treatment, neurons were fixed in 4% formaldehyde in PBS, washed in PBS and mounted in Mowiol mounting solution (Mowiol 4-88, Hoechst).

Quantification of protrusion density and dendrite branching

Images of bactin-mCherry transfected neurons were acquired using a Zeiss LSM510 confocal microscope. Twenty to forty neurons from three independent experiments were imaged for each condition. For each neuron, a z-stack of 10 x 0,3 mm was made. The projected images were analysed for protrusions with Metamorph software (Molecular Devices, Sunnyvale, CA). Two distal dendritic segments of 70-100 mm were chosen per neuron for protrusion morphometric analysis. For each protrusion, the length and the width were measured. The length was defined as the distance from the base to the tip of the protrusion; width was defined as the maximum distance perpendicular to the long axis of the protrusion. In order to make an objective distinction between spines and filopodia, we calculated the ratio of the width and the length for each protrusion. Protrusions with a ratio above or equal to 0,5 were considered as spines and conversely, protrusions with a ratio below 0.5 were considered as filopodia (Okamura et al., 2004). Averages of protrusion densities of three independent experiments were compared with unpaired two-tailed Student's T-tests.

Dendrite branching of bactin-mCherry transfected neurons was quantified by performing Sholl analyses of stacked Zeiss confocal generated images (40x objective, stack of 20 x 0,2 mm). With Metamorph software, concentric equally spaced circles

(every 20 mm) were drawn around the cell soma of each neuron and subsequently, the amount of dendrite crossings were counted per circle. Averages of counts of three independent experiments were compared with unpaired two-tailed Student's T-tests.

4.3 RESULTS

MPEP rescues prepulse inhibition of startle defect in *Fmr1* KO mice

One of the most common clinical features of FXS is heightened sensitivity to sensory stimulation (Miller et al., 1999; Frankland et al., 2004). PPI is a widely used model to study basic sensorimotor processing and has shown to be related to mGluR signalling (Grauer and Marquis, 1999). In our mouse model, we examined PPI in wildtype and *Fmr1* KO mice. Mice were presented with a startling acoustic stimulus of 90 dB, which in the prepulse condition was preceded by a 70 dB pulse, 50 ms before the startle stimulus. In wildtype mice, the startle response after a prepulse was inhibited by 73% compared to the response after a startle stimulus alone (figure 4.1). This indicates good PPI in the wildtype mice. In the *Fmr1* KO mice however, the startle response was inhibited by only 30% when a prepulse preceded the startle stimulus, illustrating that PPI is defective in *Fmr1* KO mice. To study if MPEP can rescue a behavioural FXS phenotype in our mouse model, we examined PPI in wildtype and *Fmr1* KO mice with or without MPEP treatment. Treatment of *Fmr1* KO mice with 20 mg/kg MPEP thirty minutes prior to the experiment, rescued the PPI to a level of 70%, indistinguishable from the wildtype PPI response (figure 4.1). Interestingly, wildtype mice also responded to MPEP treatment with higher PPI levels. This effect was not further examined in this study.

Rescue of protrusion phenotype of *Fmr1* KO primary hippocampal neurons

Although MPEP has been shown to rescue several behavioural phenotypes in mice and *Drosophila* (McBride et al., 2005; Yan et al., 2005), the molecular mechanism

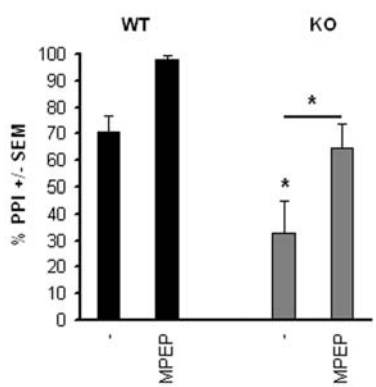

Figure 4.1. Rescue of prepulse inhibition of startle in *Fmr1* KO mice. Both wildtype and *Fmr1* KO mice were subjected to prepulse inhibition of startle procedures. *Fmr1* KO mice displayed a dramatic impairment of PPI on day 1 (baseline levels). This reduction was rescued to wildtype levels on day 2 by injection of 20 mg/kg MPEP 30 minutes prior to training. Interestingly, the wildtypes showed an equal improvement of PPI performance after injection of MPEP.

behind these rescue effects remains elusive. Therefore we decided to study if mGluR5 antagonists are also able to rescue FXS related altered protrusion morphology in an established *in vitro* model of primary hippocampal neurons.

In order to characterise our *in vitro* model, we first examined the basic neuronal properties of dendrite branching and protrusion morphology of our primary hippocampal cultures. Primary hippocampal neurons of wildtype and *Fmr1* KO mice were cultured in parallel. After twenty days *in vitro*, neurons were transfected with a bactin-mCherry construct in order to visualise the neurons, including dendritic protrusions. The next day, neurons were fixed, after which transfected neurons were imaged by confocal microscopy (figure 4.2). In order to quantify dendrite branching, we used Sholl analysis, which measures the number of dendrite crossings with equally spaced concentric circles around the cell soma. Quantification of three independent experiments comparing dendrite branching of *Fmr1* KO neurons and wildtype neurons did not reveal any significant difference (figure 4.3).

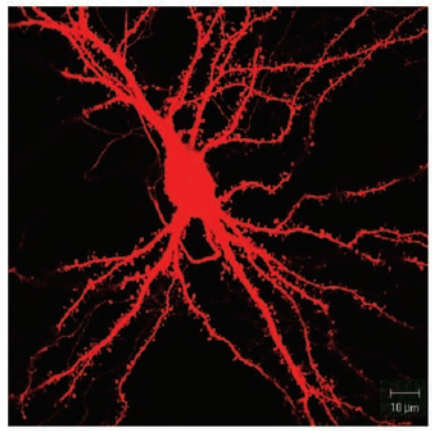

Figure 4.2. Representative image of a wildtype E18 hippocampal mouse neuron (DIV21), transfected with a βactin-mCherry construct.

Protrusions were quantified and measured for their length and width with Metamorph software. Based on these measurements, they were classified objectively as spines or filopodia (immature spines). Mature spines have a mushroom shaped appearance with a large spine head, while immature spines or filopodia have a long and thin appearance. Therefore, protrusions whose width was equal to or more than half the size of its length, were judged as standard mature mushroom spines. If this ratio was less than half the size of the length, protrusions were considered to be filopodia.

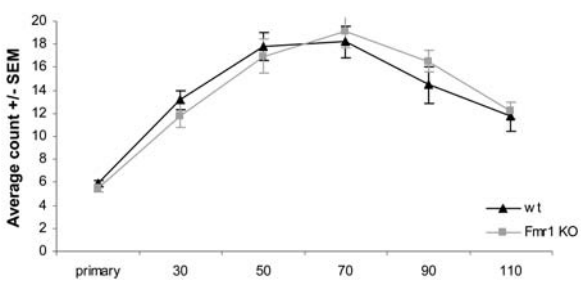

Figure 4.3. Dendrite branching is normal in *Fmr1* KO primary hippocampal neurons. Sholl analysis of wildtype and *Fmr1* KO primary hippocampal neurons cultured in parallel was performed with Metamorph software. Average of three independent experiments.

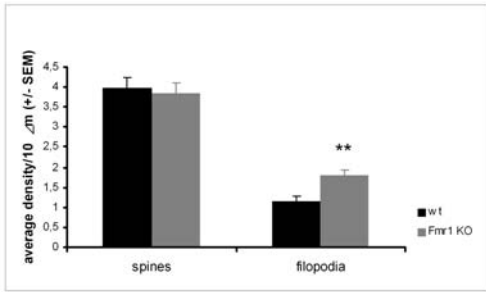

Figure 4.4. *Fmr1* KO primary hippocampal neurons have an immature protrusion phenotype. Protrusion densities of wildtype and *Fmr1* KO primary hippocampal neurons cultured in parallel were counted with Metamorph software. *Fmr1* KO neurons had significantly more filopodia than wildtype neurons ($p<0,001$), corresponding to an immature phenotype. Averages of 3 independent experiments, compared with Student's *T*-tests. Neurons were treated for four hours with 200 µM MPEP or 300 µM fenobam. The distinction between spines and filopodia was made objectively by using a threshold ratio of 0,5 for the width/length ratio of protrusions.

Using the Metamorph software we compared the protrusions of wildtype and *Fmr1* KO neurons. Based on the above described criteria, *Fmr1* KO neurons had an excess of filopodia when compared to wildtype neurons (figure 4.4).

After characterisation of our *in vitro* model and establishing an *Fmr1* KO phenotype in protrusion morphology, we continued to study the effect of MPEP on protrusion morphology. In addition, we also studied the effects of the more specific mGluR5 antagonist fenobam. Fenobam was originally discovered as an anxiolytic agent with unknown molecular target, that later was discovered to be a potent mGluR5 antagonist with an allosteric modularity site shared by MPEP, but different in structure (Porter et al., 2005). In parallel, wildtype and *Fmr1* KO neurons were subjected to treatment with the mGluR5 antagonists. Treatment of *Fmr1* KO neurons with 200 mM MPEP or 300 mM fenobam for four hours, rescued the protrusion phenotype (figure 4.5). The number of filopodia in treated *Fmr1* KO neurons was significantly lower than that in untreated *Fmr1* KO neurons, and indistinguishable from wildtype neurons (figure 4.5c). Protrusion numbers of wildtype neurons were not significantly altered by MPEP or fenobam treatment.

The total protrusion density did not differ significantly between wildtype and *Fmr1* KO neurons with or without treatments (figure 4.5a). Although the average number of protrusions classified as spines in *Fmr1* KO neurons were not statistically different from wildtype neurons (figure 4.5b), the average percentage of spines compared to filopodia per neuron was significantly lower in *Fmr1* KO neurons and was also rescued by either MPEP or fenobam treatment (figure 4.6). In other words, mGluR5 antagonist treatment restored the spine/filopodia ratio of *Fmr1* KO neurons to wildtype levels.

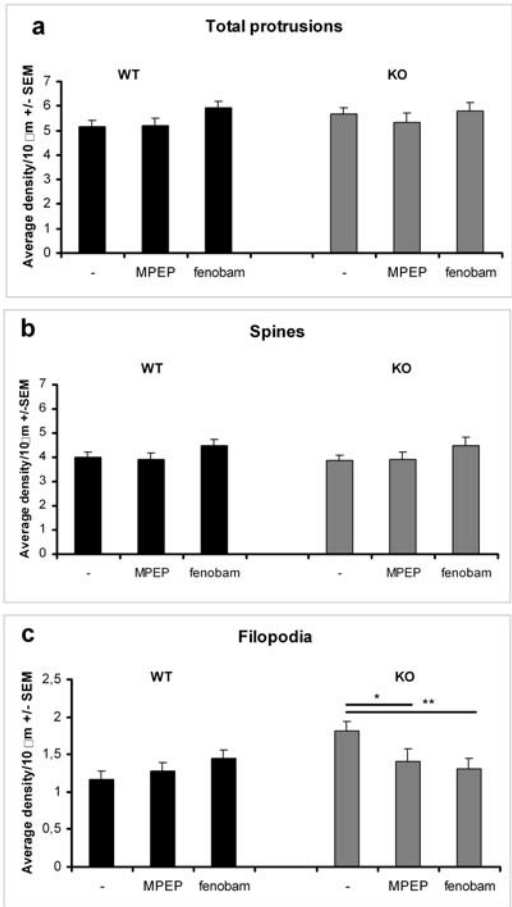

Figure 4.5. Rescue of protrusion morphology in *Fmr1* KO primary hippocampal neurons. *Fmr1* KO and wildtype neurons were treated for four hours with 200 μM MPEP or 300 μM fenobam. The total amount of protrusions (a) and the amount of mature spines (b) were unaffected by mGluR5 antagonist treatment. The *Fmr1* KO phenotype showing an increased number of filopodia was completely rescued by both mGluR5 antagonists (c). Averages of 3 independent experiments, compared with Student's *T*-tests (* =p<0,05, ** =p<0,01).

4.4 DISCUSSION

In this study we have shown a clear defect in PPI in *Fmr1* KO mice measured by eye blink in response to loud sound. In support of the mGluR theory of FXS, this defect was rescued to wildtype levels after treatment of the mice with 20 mg/kg of the mGluR5 antagonist MPEP. The impaired PPI response in *Fmr1* KO mice is in line with sensorimotor gating deficits in patients with FXS (Frankland et al., 2004). However, in the Frankland study, PPI was found to be increased rather than decreased in *Fmr1* KO mice. One explanation could be that the measurement of startle eyelid responses with the magnetic distance measurement technique (MDMT) as performed in our study is more sensitive than standard whole-body startle measurements such as used in the Frankland study. Eyelid measurements of startle include the very first components of the startle response, whereas whole-body startle measurements require induction of very strong startle responses. Therefore, eyelid startle measurement allows for a

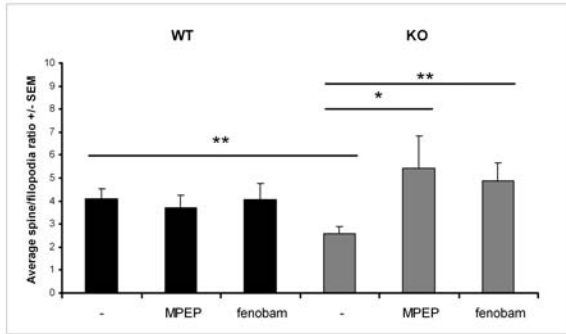

Figure 4.6. mGluR5 antagonist treatment changes the distribution of spines and filopodia in *Fmr1* KO neurons. The average percentage of spines also changes significantly in *Fmr1* KO primary hippocampal neurons after treatment with two independent mGluR5 antagonists. As total protrusion density is not different between wildtype and *Fmr1* KO neurons, we can conclude that the excess of filopodia in *Fmr1* KO neurons can successfully be changed into or replaced by spines.

better dissection of the more subtle differences in startle behaviour. In addition, our method allows us to reduce the sound pressure levels necessary for startle induction which is relevant since *Fmr1* KO mice react strongly to loud acoustic stimuli and are highly susceptible to audiogenic seizures (Musumeci et al., 2000). In another study, PPI in *Fmr1* KO mice was not significantly altered (Spencer et al., 2006). This could also be attributed to differences in the sensitivity of the methods used to measure PPI. Interestingly, we found that wildtype mice showed increased PPI after MPEP treatment which is in contrast with earlier studies in rats (Henry et al., 2002; Zou et al., 2007). The underlying molecular mechanisms of the increased PPI in MPEP-treated wildtype mice are unknown and beyond the scope of this study. Nevertheless, the rescue of PPI levels in the *Fmr1* KO mice underscores the therapeutic potential of MPEP (and/or other mGluR5 antagonists) for treatment of fragile X related behaviour. The PPI as measured in this study has therefore proven to be a valid behavioural test to study mGluR5 targeted therapeutic intervention in patients with FXS. In this study, an acute effect of MPEP was measured (thirty minutes after i.p. injection). However, in consideration of potential future therapeutic interventions in patients, it would be interesting to study these effects in a chronic model for MPEP treatment after long-term exposure (e.g. 2 months) of mice to MPEP. In addition, other mGluR5 antagonists that are more specific for the mGluR5 receptor and show less side effects are due to be tested in clinical trials in the future (see http://www.fraxa.org).

In an attempt to study the effect at the cellular level, we have shown altered protrusion morphology of *Fmr1* KO neurons in an established *in vitro* model. Primary hippocampal neurons of E18 wildtype and *Fmr1* KO mice were cultured for 21 days, a time at which dendritic spines have matured and form synaptic contacts characteristic

of those seen *in vivo* (Papa 1995). Protrusion morphology in *Fmr1* KO neurons was significantly different from wildtype neurons. *Fmr1* KO had more filopodia than wildtype neurons, corresponding to a more immature phenotype (figure 4.4). This is in accordance with literature for both patients with FXS and *Fmr1* KO mice (Hinton et al., 1991; Comery et al., 1997; Nimchinsky et al., 2001; Galvez and Greenough, 2005; Koekkoek et al., 2005; Grossman et al., 2006). In primary hippocampal neurons, reported quantities of protrusions tend to differ in literature. One study has even described fewer protrusions in hippocampal cultures of *Fmr1* KO mice (Braun 2000). Another more recent study showed increased density of filopodia-like spines in cultured *Fmr1* KO hippocampal neurons, but with many more protrusions per distance (3-5 filopodia/10 μm) than in our study (Antar et al., 2006). However, these cultures were not fully matured and different culture methods (such as use of glial cell feeder layers) might influence the protrusion number. In contrast, it was reported that specifically in hippocampal area CA1, *Fmr1* KO neurons have more stubby spines as opposed to filopodia (Grossman et al., 2006). In light of all these seemingly different findings, we analysed our own culture system extensively and used an objective measurement technique to distinguish mature mushroom-like protrusions from immature filopodia-like protrusions. With these criteria, *Fmr1* KO neurons in our culture system showed a decreased spine to filopodia ratio. Furthermore, we have shown rescue of this altered protrusion morphology in *Fmr1* KO primary hippocampal neurons by two independent mGluR5 antagonists, MPEP and fenobam. Since spine shape is correlated with the number of AMPA receptors in the postsynaptic density (Matsuzaki et al., 2001), these data correlate with the rescue effect of MPEP on AMPA receptor trafficking as shown by Nakamoto et al. (Nakamoto et al., 2007). In the latter study, the concentrations of MPEP used on primary neurons (10-50 mM) differed from our experiments due to different time courses of the experiments. In our study, a higher MPEP concentration was needed to visualise fast effects (within 4 hours) on protrusion morphology, whereas Nakamoto et al. studied MPEP effects after 16 hours and up to three days. In wildtype cerebellar Purkinje cells, daily treatment with 30 mM MPEP for ten days changes normal protrusion morphology into a more immature phenotype with more filopodia-like protrusions (Catania et al., 2001). In the present study, acute MPEP treatment had no significant effect on the protrusion morphology of wildtype hippocampal neurons (figure 4.4).

Others have shown that MPEP can target NMDA receptors at high concentrations (Popoli et al., 2004; Lea et al., 2005). However, it is unlikely that the rescue effect in this study is mediated by NMDA receptors, as we also see the rescue effect with the structurally different, more specific mGluR5 antagonist fenobam. Moreover, we also tested the effect of the NMDA specific antagonist D-AP-5 (100 mM) on protrusion morphology, which did not show rescue of the *Fmr1* KO protrusion phenotype (data not shown).

In conclusion, our *in vitro* model of primary hippocampal neurons and the *in vivo* measurement of PPI form excellent tools to further study the molecular mechanisms that underlie therapeutic intervention with mGluR5 antagonists in patients with FXS and have great potential for testing newly developed drugs.

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5

AFQ056, A NEW mGLUR5 ANTAGONIST FOR TREATMENT OF FRAGILE X SYNDROME

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ABSTRACT

Fragile X syndrome, the most common form of inherited intellectual disability, is caused by a lack of FMRP, which is the product of the *FMR1* gene. FMRP is an RNA-binding protein and a component of RNA-granules found in the dendrites of neurons. At the synapse, FMRP is involved in regulation of translation of specific target mRNAs upon stimulation of mGluR5 receptors.

In this study, we test the effects of a new mGluR5 antagonist (AFQ056) on the prepulse inhibition of startle response in mice. We show that *Fmr1* KO mice have a deficit in inhibition of the startle response after a prepulse and that AFQ056 can rescue this phenotype. We also studied the effect of AFQ056 on cultured *Fmr1* KO hippocampal neurons; untreated neurons showed elongated spines and treatment resulted in shortened spines. These results suggest that AFQ056 might be a potent mGluR5 antagonist to rescue various aspects of the fragile X phenotype.

Keywords: fragile X syndrome, protrusions, spines, AFQ056, prepulse inhibition of startle metabotropic glutamate receptor, primary hippocampal neuron culture.

5.1 INTRODUCTION

Fragile X syndrome (FXS) is the most common form of inherited mental retardation and is caused by expansion of the CGG repeat in the 5' UTR of the *FMR1* gene. In unaffected individuals the CGG repeat is less then 50, while in most patients with FXS the repeat has expanded to above 200. As a consequence, the CGG repeat and the promoter region are methylated, resulting in *FMR1* gene silencing and fragile X mental retardation protein (FMRP) deficiency. FMRP is predominately expressed in brain and testes. Microscopic analysis of post-mortem brain from patients with FXS and *Fmr1* knockout (KO) mice have shown no gross brain abnormalities except for subtle alterations in size and shape of dendritic spines (Rudelli et al., 1985; Hinton et al., 1991; Comery et al., 1997; Greenough et al., 2001; Nimchinsky et al., 2001; Koekkoek et al., 2005).

FMRP has three RNA-binding domains to bind specific target mRNAs. It is postulated that FMRP binds specific target mRNAs in the nucleus and is subsequently packed in to RNP particles. In the cell soma, FMRP is incorporated in RNA-granules that are transported into the dendrite and move in a microtubule-dependent manner (De Diego Otero et al., 2002; Antar et al., 2005; Wang et al., 2008). Although at present the function of FMRP is not fully understood, it has been suggested that FMRP is important for the transport of target mRNAs, and that it is essential to silence translation of the target mRNAs during transport (Miyashiro et al., 2003; Dictenberg et al., 2008). Moreover, at the synapse FMRP has shown to be important for translational regulation of target mRNAs in synaptosomes after mGluR5 stimulation (Weiler et al., 1997; Greenough et al., 2001; Weiler et al., 2004; Muddashetty et al., 2007).

In 2004, the mGluR theory was proposed as an explanation for the characteristic phenotype observed in FXS (Bear et al., 2004). Normally, upon group 1 metabotropic glutamate receptors (especially mGluR5) activation, mRNAs (including *FMR1* mRNA) are translated locally at the synapse and as a consequence, AMPA receptors are internalised (Nakamoto et al., 2007). During this process, FMRP acts as a translational silencer/repressor. Lack of FMRP results in elevated protein synthesis, leading to a net loss of AMPA receptors in the postsynaptic membrane. Loss of these receptors is proposed to be responsible for the changes in electrophysiology (enhanced LTD) and abnormal spine morphology.

Since the formulation of the mGluR theory, researchers have been searching for therapeutic intervention that can counteract the excessive activity of the group I mGluRs and the net loss of AMPA receptors. The most promising components tested thus far are mGluR5 antagonists, e.g. MPEP (2-methyl-6-phenylethynyl-pyridine hydrochloride). In *Fmr1* KO mice, MPEP has been shown to rescue audiogenic seizures (Yan et al., 2005), AMPA receptor internalisation (Nakamoto et al., 2007),

deficits in prepulse inhibition of startle (PPI), and immature spine morphology (De Vrij et al., 2008). Unfortunately however, MPEP is not mGluR5 specific and at high concentrations (> 30 mM) can inhibit the NMDA receptor (O'Leary et al., 2000; Lea et al., 2005). Therefore, it is important to develop specific mGluR5 antagonists that can reverse some symptoms in patients with FXS but have fewer adverse effects. Recently, a clinical phase II trial with AFQ056 has been conducted. We tested AFQ056 in *Fmr1* KO mice using PPI behavioural test. In addition, we studied the effects of AFQ056 on spine morphology in cultured hippocampal neurons of *Fmr1* KO mice.

5.2 MATERIAL AND METHODS

Animals used for PPI

The *Fmr1* KO mice were generated in our lab as described previously (Mientjes et al., 2006). The line was completely backcrossed in C57BL/6J mice. Both the *Fmr1* KO mice and the wildtype mice (only males were used in this study) were housed under standard conditions. All experiments were carried out with permission of the local ethical committee.

Prepulse inhibition of startle

Prepulse inhibition of startle (PPI) was measured as described before (De Vrij et al., 2008). Wildtype mice and *Fmr1* KO mice used for this experiment were littermates. Briefly, PPI was measured by analysis of eye blink reactions of mice to acoustic stimuli, based on the magnetic distance measurement technique (MDMT) used for eye blink conditioning (Koekkoek et al., 2002; Koekkoek et al., 2005). At twelve weeks of age, adult wildtype littermates (n=9) and *Fmr1* KO mice (n=10) were anesthetised with an oxygenated mixture of nitrous oxide and isoflurane. A dental acrylic pedestal was placed on the skull and animals were allowed to recover for three days. Prior to the experiment the mice were briefly sedated using the isoflurane/nitrous oxide mixture. A sensor holder with an air channel and a magnet sensor was attached to the pedestal. A small neodymium iron borium magnet (0.8*1.6*0.2 mm) was glued to the lower eyelid with a minute drop of cyanoacryllate. Mice were placed inside their own cages within soundproof training chambers and allowed to recover until normal behaviour (grooming, eating) returned, usually this was within 15 minutes.

Corneal air puffs were presented during the experiment which induced a reflexive full eyelid closure. These were used to test and calibrate the MDMT signal. A background noise level of 60 dB white noise was present. Subsequently, the mice were presented with a white noise startle stimulus of 95 dB, which in the prepulse inhibition condition was preceded by a 70 dB white noise prepulse, 50 ms before the startle stimulus. Each mouse was subjected to 20 startle trials and 20 prepulse trials all randomly applied. The experiment set up was randomised and the investigator was always blinded to the genotype of the mice.

Drug treatment

AFQ056 is a selective mGluR5 antagonist, with a non-competitive inhibitory mode of action. AFQ056 is currently in clinical development and undergoing a number of clinical trials such as in L-dopa induced dyskinesia in Parkinson's disease patients (clinicaltrial.gov; NCT01092065, NCT00986414), Huntington's disease (clinicaltrial.gov; NCT01019473) and in patients with FXS (clinicaltrial.gov; NCT00718341). PK experiments in mice showed that AFQ056 has short plasmatic and brain halflife (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)(personal communication). In a sub chronic study (5 days), AFQ056 did not show any accumulation in mice (personal communication).

The mice were intraperitoneally (i.p.) injected with the vehicle, methylcellulose, to measure basal PPI. Subsequently, on the same day, the mice were tested once more for PPI after an i.p. injection with AFQ056 (3 mg/kg) suspended in methylcellulose (injection 30 minutes prior to experiment). At least 7 days later, the same mice were tested again for PPI to study the effect of fenobam treatment. Fenobam (20 mg/kg) suspended in methylcellulose was i.p. injected 30 minutes prior to the PPI experiment. To make sure that AFQ056 was eliminated from the body, 2 mice that showed an effect after AFQ056 were tested before the fenobam injection to study their PPI levels (see figure 5.1 for overview time schedule). Percentages of PPI within genotypes were tested using the paired t-test (before and after treatment with AFQ056). The independent Student's *T*-test was used to test if there was a significant difference between wildtype mice and *Fmr1* KO mice before treatment.

Dissociated neuronal culture

Dissociated neuronal cultures were generated using E16 mouse hippocampal neurons. Briefly, mouse embryonic brains were obtained from E16 embryos of *Fmr1* KO mice; C57BL/6J (Jackson; stock 003025) or FVB/NJ (Jackson; stock 002700), and their respective control C57BL/6J (Jackson Lab; stock 000664) or FVB/J (Jackson Lab; stock 001800). The embryonic brains were dissected in Leibovitz's L15 medium (Gibco) containing penicillin/streptomycin at 1/100 dilution (Gibco). Following the isolation of hippocampus, the tissue was minced with scissors, transferred to 15 ml Falcon tube, and left for 5 minutes at room temperature for sedimentation. Extra medium was removed from the tube, and 0.25% trypsin/EDTA was added for digestion of tissues at 37°C for 20 minutes. Digested materials were neutralised by addition of "complete medium" that contains Dulbeco modified essential media (DMEM [Gibco]), 10% of fetal bovine serum, and penicillin/streptomycin at 1/100 dilution, and were triturated (approximately 20 times) completely with gentle force using Pasteur pipette. Triturated materials were spun down at 6,000 rpm for 6 minutes and the supernatant was removed. Cell pellet was washed with "complete medium". Washed materials were

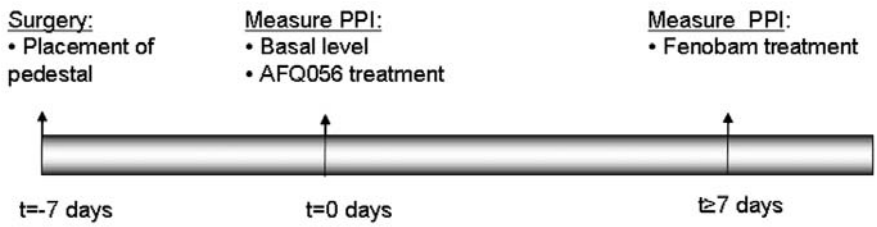


Figure 5.1. Schematic overview of PPI experiments. At least 7 days prior to the PPI experiments, the pedestal was placed on the skull of the mice. At day one, mice were tested for their basal PPI and the PPI after AFQ056 treatment. At least 7 days after the experiment, the mice were once more subjected to a PPI experiment to test the effect of fenobam on PPI. Two mice that showed an effect after AFQ056 treatment were tested for their PPI levels before fenobam treatment.

spun down at 9,000 rpm for 6 minutes and its supernatant was discarded. Precipitant was resuspended with "culture medium" that consists of Neurobasal media (Gibco), B27 supplement (Gibco) at 1/50 dilution, L-glutamine (Gibco) at 1/400 dilution, and Penicillin/Streptomycin (Gibco) at 1/100 dilution. The resulting suspension was passed through a cell strainer (BD Falcon) to remove clumped materials and plated at a density of 20,000 cells per well onto 8 well glass chamber slide (Lab-TeckII Chambered coverglass) that was pre-coated with poly-D-lysine (50 µg/ml in Borate buffer; 50mM Boric acid and 12.5 mM sodium borate) overnight and with Laminin (10 mg/ml in Hanks balanced salt solution) for 3-4 hours. Neuronal cultures were maintained by changing medium on the day after dissection, and then every three days. In order to sustain good culture conditions, only a half of the medium in the well was replaced with fresh media.

Transfection

Visualisation of postsynaptic structure was performed by transfection of a plasmid that expresses Enhanced Green Fluorescence Protein (EGFP) at DIV 5. Transfection was carried out using Lipofectamine 2000 (Invitrogen). As the presence of antibiotics during transfection reduces transfection efficiency, within 1 hour prior to transfection, 250 µl of medium from each well was removed (the removed medium was kept at 37°C and used later, see below) and was replaced with the same volume of fresh "culture media" that lacks penicillin / streptomycin. Cells in each well were transfected with 0.5 µg of DNA. A DNA complex was formed by mixture of two solutions; DNA 0.5 µg in 7.5 µl opti-MEM (Gibco) and 0.2 µl Lipofectamine 2000 in 7.5 µl opti-MEM per well. The mixed solution was left at room temperature for 20-30 minutes to form DNA complex, and 15 µl of the mixed solution were dropped into each well. One to two hours after transfection, the transfection mix was removed. Cells were washed with "culture medium" containing penicillin/streptomycin once, and then the original "culture medium" that had been removed from the well and kept at 37 °C was added back to the well.

Treatment and dendritic spine morphometric analysis

AFQ056 treatment was performed at DIV14 for 2 hours and treated neurons were fixed with 4% paraformaldehyde at 37 °C, washed 3 times in PBS and kept at 4°C in PBS until visualisation. Imaging caption was performed using Zeiss Axiovert 200M using the 40x objective. The dendritic spine measurement was done using AxioVision software. Measurements for AFQ056 utilised Cintiq21 (WACOM) as a measurement tool. Per group minimal 30 neurons (three independent neuronal cultures; approximately ten neurons from each culture for each treatment group) were imaged and analysed. Per neuron, from two secondary dendrites, at least 4-5 dendritic segments were measured for a length of 40-60 μm each. Protrusion from dendrite surface to the tip was measured. Protrusions with length less than 3 μm were selected for quantification. Spine width was measured at spine head 90 degree across the spine protrusion at the widest point of spine head.

Averages of all independent experiments between genotypes were compared with unpaired two-tailed Student's *T*-tests. An ANOVA test has been used to test the effect of different concentration of AFQ056. Following ANOVA, post hoc differences were resolved using the Tukey's multiple comparison test.

5.3 RESULTS

PPI is an operational measure of sensorimotor gating whereby a weaker prestimulus (the prepulse) inhibits the motor response to a strong, startling stimulus. One of the most prevalent symptoms of FXS is heightened sensitivity to sensory stimuli and therefore PPI forms a relevant behaviour test. To measure the prepulse inhibition of startle we used the eye-blink response. Recently, we showed with the same technique that the inhibition of the startle response after prepulse was defective in *Fmr1* KO mice and that the deficits could be rescued using MPEP treatment (De Vrij et al., 2008). In search for more specific and potent antagonists, we tested the new mGluR5 antagonists AFQ056.

Prepulse inhibition of startle

First we tested the basic PPI in wildtype and *Fmr1* KO mice after injection with the vehicle methylcellulose. Wildtype mice showed an inhibition of the startle response of 47% after a prepulse. In contrast, *Fmr1* KO mice showed 25% of inhibition of the startle response after a prepulse. As we have shown before, this indicates that *Fmr1* KO mice have a deficit in the inhibition of the startle response ($P<0.05$) (figure 5.2).

On the same day, the same mice were tested once more to study if AFQ056 was able to rescue the PPI deficits in *Fmr1* KO mice. The mice were i.p. injected with AFQ056 30 minutes prior to the PPI experiment. As shown in figure 5.2, AFQ056 was able to rescue the PPI deficits in the *Fmr1* KO mice without any side effects in the wildtype mice. After AFQ056 injection, the *Fmr1* KO mice showed a 48% inhibition of the startle response after the prepulse, whereas wildtype mice showed 53% inhibition. For

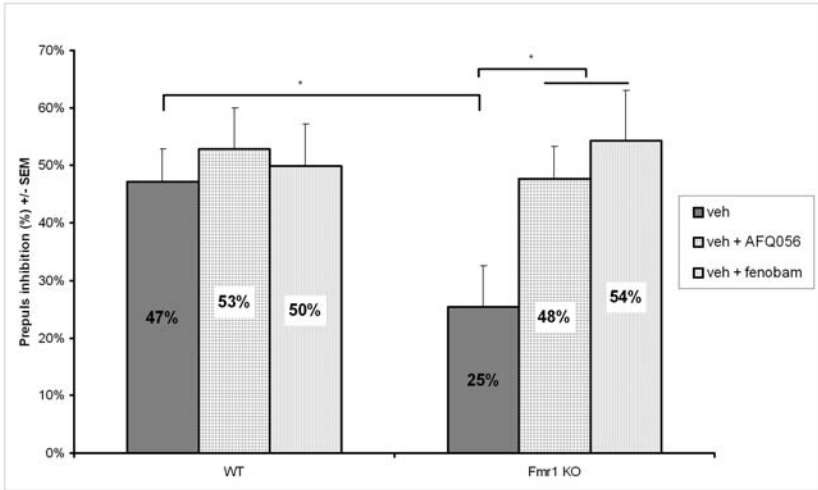


Figure 5.2. Rescue of PPI deficits in Fmr1 KO mice after treatment with AFQ056 or fenobam. *Fmr1* KO mice display a deficit in inhibition of the startle response after prepulse ($P < 0.05$). The PPI deficits in *Fmr1* KO were rescued after AFQ056 (3 mg/kg) or fenobam (20 mg/kg) treatment ($P < 0.05$) (paired Student´s *T*-test before and after treatment $P < 0.05$).

fenobam, the mice were i.p. injected 15 minutes prior to the PPI experiment. Like AFQ056, fenobam was also able to rescue the PPI deficits in the *Fmr1* KO mice (figure 5.2). After fenobam injection, *Fmr1* KO mice showed a 54% inhibition of the startle response after PPI, whereas WT mice showed 50% inhibition.

Differences in spine morphology

As we have shown before, hippocampal cultured neurons of *Fmr1* KO also had more immature protrusions, which could be rescued by MPEP and fenobam treatment (de Vrij, 2008). In order to investigate the genetic influence on dendritic spine morphology in dissociated neuronal cultures of *Fmr1* KO neurons, we have examined morphological differences of dendritic spines between wildtype and *Fmr1* KO mice at DIV 14 in B6 and in FVB background (figure 5.3a). *Fmr1* KO B6 neurons showed significantly longer mean dendritic spine length than wildtype B6 neurons (figures 5.3b and 5.3c). However, mean dendritic spine width and dendritic spine density of *Fmr1* KO B6 neurons did not differ from those of wildtype (figures 5.3b and 5.3c).

In contrast to the results from B6 background, no significant differences were observed in dendritic spine length, width, and density in hippocampus between wildtype and *Fmr1* KO mice in FVB background. In addition to hippocampal neurons, we also studied cortical neurons of *Fmr1* KO FVB mice and measured the spines at a later time-point, i.e. 20 DIV. In summary, no differences were present between wildtype and *Fmr1* KO neurons in the hippocampus or cortex in FVB mice at all DIVs tested

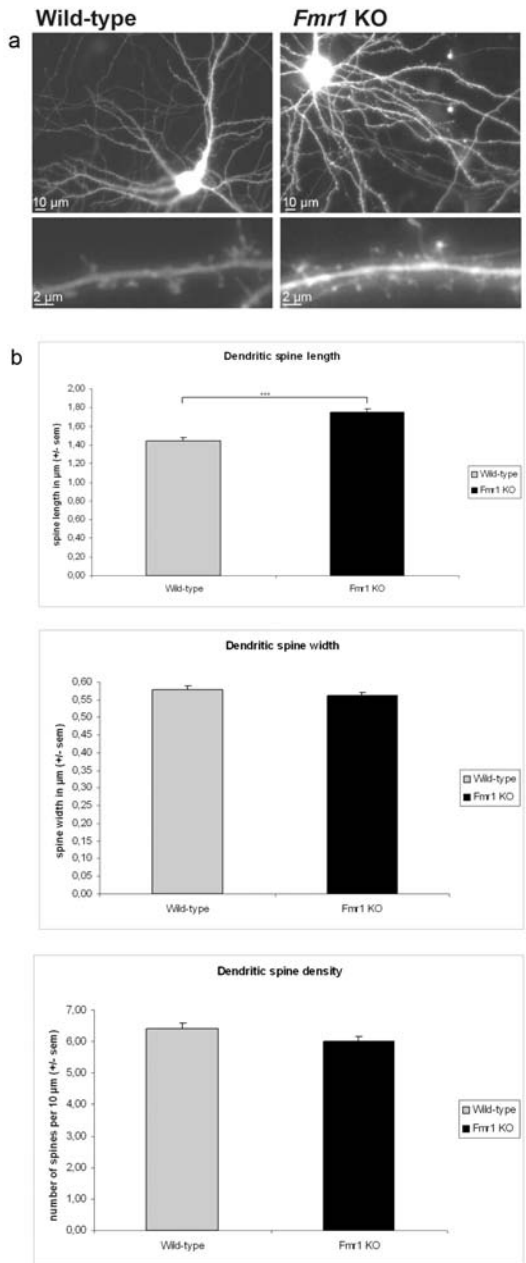


Figure 5.3. Hippocampal neurons from Fmr1 KO mice in B6 background showed significant increase in mean dendritic spine length. (a) Representative images of EGFP transfected neurons from each genotype and a higher magnification (lower panels). (b) Comparison of dendritic spine length, width, and density of hippocampal neurons from wildtype and *Fmr1* KO mice. Spine length of wildtype versus *Fmr1* KO hippocampal neurons (1.75 µm vs. 1.44 µm, $p<0.0001$). (c) Summary of mean values, std. deviation and std. error of dendritic spine length, width and density.

	Dendritic spine length		Dendritic spine width		Dendritic spine density	
Genotype	Wild-type	*Fmr1 KO*	Wild-type	*Fmr1 KO*	Wild-type	*Fmr1 KO*
Mean	1,44	1,75	0,58	0,56	6,41	6,00
Std. deviation	0,17	0,19	0,05	0,05	0,86	0,83
Std. error	0,034	0,035	0,010	0,009	0,175	0,152

Table 5.1. Summary of spine morphometric analysis of hippocampal and cortex neurons from wildtype and *Fmr1* KO mice in FVB background.

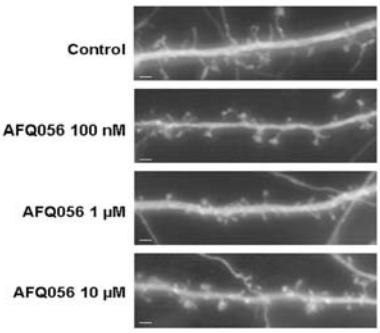
			DIV 14	DIV20
Spine length	cortex	WT	2,2 ± 0,07	1,6 ± 0,05
		Fmr1 KO	2,1 ± 0,05	1,7 ± 0,04
	hippocampus	WT	1,7 ± 0,04	1,5 ± 0,05
		Fmr1 KO	1,7 ± 0,05	1,5 ± 0,04
Spine width	cortex	WT	0,4 ± 0,01	0,6 ± 0,03
		Fmr1 KO	0,4 ± 0,01	0,6 ± 0,03
	hippocampus	WT	0,4 ± 0,01	0,6 ± 0,03
		Fmr1 KO	0,4 ± 0,01	0,6 ± 0,03
Spine density	cortex	WT	28,9 ± 0,7	35,7 ± 1,3
		Fmr1 KO	27,3 ± 0,9	32,0 ± 1,2
	hippocampus	WT	30,6 ± 1,0	32,3 ± 1,1
		Fmr1 KO	29,3 ± 1,1	34,8 ± 0,8

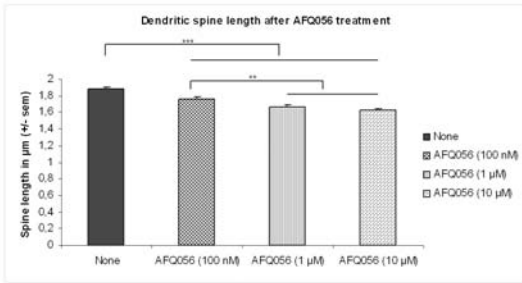
(14 and 20) with one exception; dendritic spine density of cortex neurons at DIV20 (table 5.1). Cortical neurons at 20 DIV from *Fmr1* KO FVB mice showed significantly less dendritic spines than wildtype FVB cortical neurons (35.7±1.3/50 µm in wildtype versus 32.0±1.2/50 µm in *Fmr1* KO ($P<0.04$)) (table 5.1). However, this difference was not consistent with what was observed in other experimental systems, which showed increased dendritic spine density in *Fmr1* KO neurons rather than a decrease. Therefore, we chose to do all further experiments in hippocampal *Fmr1* KO B6 neurons.

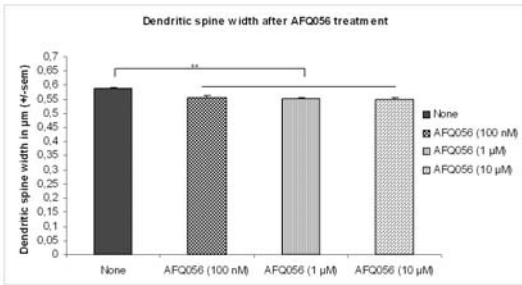
As we have demonstrated previously, MPEP resulted in a rescue of the dendritic spine phenotype in *Fmr1* KO hippocampal neurons (de Vrij et al, 2008). Here we examined the effect of the new mGluR5 antagonist AFQ056 (figure 5.4a). At all three concentrations tested (100 nM, 1 µM, and 10 µM), AFQ056 shortened dendritic spine length of hippocampal *Fmr1* KO B6 neurons in a concentration-dependent manner (figures 5.4b and 5.4c). Statistically significant differences were observed in all but one comparison, between 1 µM and 10 µM (figures 5.4b and 5.4c).

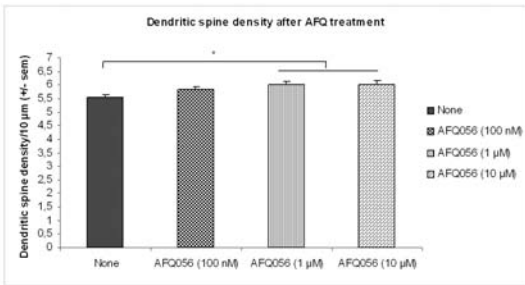
In addition, AFQ056 showed very marginal but statistically significant changes in both dendritic spine width and density of *Fmr1* KO hippocampal B6 neurons. Contrary to our expectations, AFQ056 significantly decreased dendritic spine width and increased spine density in dissociated hippocampal neurons from *Fmr1* KO mice. Although the degree of statistical significance in dendritic spine width was quite high, actual differences were less than 0.04 µm (figure 5.4b and 5.4c).

All together, these results showed that in dissociated hippocampal neurons of *Fmr1* KO B6 mice, AFQ056 changed dendritic spine morphology at the levels of spine length, and caused subtle changes in spine width and density.









Dendritic spine length

Treatment	None	AFQ056 (100 nM)	AFQ056 (1 µM)	AFQ056 (10 µM)
Mean	1,884	1,764	1,665	1,624
Std. deviation	0,1099	0,107	0,1101	0,1219
Std. Error	0,02007	0,0195	0,0201	0,0222

Dendritic spine width

Treatment	None	AFQ056 (100 nM)	AFQ056 (1 µM)	AFQ056 (10 µM)
Mean	0,5863	0,556	0,5511	0,5494
Std. deviation	0,0321	0,045	0,0314	0,0348
Std. Error	0,0058	0,0081	0,0057	0,0063

Dendritic spine density

Treatment	None	AFQ056 (100 nM)	AFQ056 (1 µM)	AFQ056 (10 µM)
Mean	5,552	5,843	6,009	6,01
Std. deviation	0,504	0,506	0,669	0,796
Std. Error	0,092	0,092	0,1222	0,1453

◁ **Figure 5.4. Change in dendritic spine morphology and organisation of *Fmr1* KO hippocampal neurons (C57B6 background) by mGluR5 antagonist AFQ056**. (a) Representative images of dendrites and dendritic spines with or without AFQ056 treatment (scale bar: 2 μm). (b) Comparison of dendritic spine morphology (length, width, and density) of cultured hippocampal neurons from *Fmr1* KO mice with or without AFQ056 treatment. AFQ056 treatment shortened dendritic spine length (control 1.88 μm, 100 nM AFQ056 1.76 μm, 1 μM AFQ 1.67 μm and 10 μM AFQ056 1.62 μm, all $p<0.001$). Marginal changes in spine width and density (spine width: control vs. 100 nM AFQ056 0.586 vs. 0.556 μm, $p < 0.05$, control vs. 1μM AFQ056 0.586 vs. 0.551 $p < 0.05$, control vs. 10μM AFQ056 0.586 vs. 0.549 $p < 0.001$) (spine density: control vs. 1 μM AFQ056 5.552 vs. 6.009 $p < 0.05$, control vs. 10 μM AFQ056 5.552 vs. 6.010 $p < 0.05$). (c) Summary of mean values, std. deviation and std. error of dendritic spine length, width and density.

5.4 DISCUSSION

The mGluR theory postulates a link between the neurological and the psychiatric fragile X phenotype by connecting the absence of FMRP to mGluR5 mediated activity (Bear et al., 2004). In both patients with FXS and *Fmr1* KO mice, the mGluR5 signalling seems to be disturbed resulting in elongated spines and enhanced mGluR5 mediated long-term depression (LTD). Further evidence that mGluR5 receptors play a major role was shown by an elegant study in which *Fmr1* KO mice were crossed with heterozygous mGluR5 KO mice, reducing mGluR5 expression by 50%. Reduced mGluR5 expression resulted in a rescue of the *Fmr1* KO phenotype in electrophysiology, spine morphology and behaviour (Dolen et al., 2007). Our results show that a new mGluR5 antagonist, AFQ056, and a well studied mGluR5 antagonist, fenobam, both were able to rescue the PPI deficits in *Fmr1* KO mice. In addition, AFQ056 showed a rescue effect on spine length in cultured hippocampal neurons of the *Fmr1* KO mice. In addition, AFQ056 showed a rescue effect on spine length in cultured hippocampal neurons of the *Fmr1* KO mice. Contrary to our expectations marginal effects on spine width and density were observed. However, the concentration dependent effect on spine length is more pronounced and complements the rescue effect on PPI.

PPI is a behavioural test used in several neuropsychiatric disorders, including schizophrenia, Huntington's disease and FXS. PPI is thought to reflect sensorimotor-gating mechanisms that are considered as a fundamental component of information processing in the brain. PPI is mediated by brain-stem circuits, but modulated by forebrain circuits (Bast and Feldon, 2003; Li et al., 2009). In rodents, the startle response and PPI are usually measured as a response of the whole body. In our study we used the magnetic distance measurement technique (MDMT) to measure the eye blink response after a startle pulse with or without a preceding prepulse. Using this technique, we are able to use a lower sound burst to measure the startle response or PPI with high sensitivity. Since this technique is relatively new to test PPI in *Fmr1* KO mice, this might explain our differences in results compared to whole body responses described in literature (discussed in (De Vrij et al., 2008)).

The mGluR5 receptor is expressed in forebrain areas that are important for PPI (Swerdlow et al., 2001; Brody et al., 2004). Additionally, mGluR5 has been described to play a functional role in sensorimotor gating, and mGluR5 KO mice show deficits in PPI and startle response (Kinney et al., 2003; Brody et al., 2004). *Fmr1* KO mice that were treated with mGluR5 antagonists AFQ056 or fenobam, showed a rescue in the inhibition of the startle response after a prepulse, while the wildtype mice did not show any increase of the PPI. Of note, AFQ056 and fenobam appear to be more specific than the mGluR5 antagonists MPEP, which in earlier experiments also showed a mild increase of PPI in wildtype mice (De Vrij et al., 2008). This indicates that both mGluR5 antogonists rescues the PPI phenotype in *Fmr1* KO mice by restoring the mGluR5 signalling.

In addition to the PPI, we also studied the effects of AFQ056 on spine morphology using dissociated hippocampal neurons. Previous studies demonstrated an increase in dendritic spine length and dendritic spine density in *Fmr1* KO neurons in brain slices, adult mouse brain and dissociated hippocampal cultures (Hinton et al., 1991; Comery et al., 1997; Nimchinsky et al., 2001; Antar et al., 2006; Grossman et al., 2006; Hayashi et al., 2007; De Vrij et al., 2008). In line with these studies, we found a clear difference in dendritic spine length between *Fmr1* KO and wildtype primary hippocampal neurons. Strikingly, differences in hippocampal spine morphology could only be observed in B6 mice, while *Fmr1* KO mice in a FVB background did not show a hippocampal spine phenotype and a reversed spine phenotype in cortical neurons. Behavioural studies and spine morphology studies have shown that the genetic background of mice can influence the phenotype (Kooy, 2003; McKinney et al., 2005; Errijgers et al., 2008). In literature, dissociated neuron cultures show highly diversified *Fmr1* KO phenotypes (discussed in (De Vrij et al., 2008)). In this study the significant difference in dendritic spine length between *Fmr1* KO and wildtype neurons was used as an evaluation criterion of the effect of compounds.

AFQ056 had a significant and concentration-dependent rescue effect on the spine length of *Fmr1* KO neurons. AFQ056 also caused a slight increase in spine width and a decrease in spine density. The latter two are counterintuitive since an *in vivo* study of reduction of mGluR5 activity in *Fmr1* KO mice showed opposite effects on spine width and density (Dolen et al., 2007). Despite subtle changes in spine width and density, striking dendritic shortening by AFQ056 suggested overall recovery of dendritic spine morphology. In order to properly address the extent of functional rescue of *Fmr1* KO neurons by AFQ056 further functional validation might be beneficial using different experimental systems, including morphological analysis in brain and changes in electrophysiological properties after AFQ056 treatment.

In previous studies, the mGluR5 antagonist MPEP has demonstrated that inhibition of the mGluR5 does not influence normal spine development in cultures from wildtype

animals (De Vrij et al., 2008). In contrast, the absence of FMRP has been shown to alter spine development and only in this situation inhibition of mGluR5 has demonstrated an effect on spine development. Thus we limited our investigations in cultures from *Fmr1* KO mice to assess the potential effect of this molecule in a disease state. Our studies do not allow to draw conclusions on the mechanism of action underlying the effects seen on the spine morphology or the restoration of the PPI. We can only state that the inhibition of the mGlu5 receptor is implicated in the correction of two typical dysfunctions due to the absence of FMRP.

In conclusion, we have shown that a new mGluR5 antagonists, AFQ056 and fenobam were able to rescue the PPI deficit in *Fmr1* KO mice. Moreover, AFQ056 treatment caused a significant and concentration dependent rescue of the spine length phenotype of *Fmr1* KO hippocampal neurons. We therefore emphasise that PPI and *in vitro* measurements of spine morphology are useful tools to study newly developed drugs for FXS. Our results suggest that AFQ056 might be an effective for treatment of several symptoms of FXS.

ACKNOWLEDGEMENTS

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6

EFFECTS OF SHORT-TERM AND LONG-TERM TREATMENT WITH MGLUR5 ANTAGONIST AFQ056 ON HIPPOCAMPAL PROTRUSION MORPHOLOGY IN ADULT *FMR1* KO MICE

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Manuscript in preparation

ABSTRACT

Fragile X syndrome (FXS) is the most common inherited form of mental retardation and is caused by the lack of fragile X mental retardation protein (FMRP). In the brain, spine abnormalities have been reported in both patients with FXS and *Fmr1* knokout mice. This altered spine morphology has been linked to disturbed synaptic transmission related to altered signalling in the excitatory metabotropic glutamate receptor 5 (mGluR5) pathway. To date, therapeutic approaches mainly target this signalling pathway to rescue FXS-related behaviour and spine abnormalities. An important question to these approaches is whether patients might benefit from therapy in adulthood, rather than only in development. Therefore, we investigated for the first time hippocampal protrusion morphology in adult *Fmr1* knockout mice. Our results show a hippocampal CA1-specific altered protrusion phenotype, which could not be rescued by long-term treatment with a mGluR5 antagonist, AFQ056. This suggests that long-term treatment with an mGluR5 antagonist can not rescue the abnormal protrusion morphology in *Fmr1* knockout mice.

Keywords: fragile X syndrome, *Fmr1* knockout mice, protrusions, mGluR5 antagonist, AFQ056, chronic treatment, hippocampus CA1, hippocampus CA3

6.1 INTRODUCTION

Excitatory synapses on pyramidal neurons in the cerebral cortex and hippocampus terminate at spines, which are short protrusions joined to the dendrite. Their morphology and density are abnormal in several mental retardation syndromes, including fragile X syndrome (FXS) (Purpura, 1974; Kaufmann and Moser, 2000; Fiala et al., 2002). FXS is the most common inherited form of intellectual disability and is caused by lack of fragile X mental retardation protein (FMRP). Lack of FMRP is a consequence of a trinucleotide (CGG) repeat expansion in the 5' UTR of the *FMR1* gene. In unaffected individuals, the CGG repeat in the *FMR1* gene ranges between 5-55, while in patients with FXS the repeat exceeds the 200 units, which results in hypermethylation of the promoter region. This process leads to silencing of the *FMR1* gene, resulting in lack of FMRP.

FMRP is an RNA-binding protein and plays a crucial role in controlling mRNA translation after specific stimulation. It has been shown that FMRP is synthesised after stimulation of group I metabotropic glutamate receptor (mGluR) in synaptosomes (Weiler et al., 1997), and that it acts as a translational repressor in rabbit reticulocyte lysate (Laggerbauer et al., 2001). Accordingly, lack of FMRP results in excessive translation of different target mRNAs, including Map1b, SAPAP3 and PIKE-S, which are involved in synaptic plasticity (Lu et al., 2004; Narayanan et al., 2008; Sharma et al., 2010). *Fmr1* knockout (KO) mice indeed exhibit enhanced group I mGluR LTD (mGluR1 and mGluR5). It was shown that group I mGluR LTD is protein-synthesis dependent in wildtype hippocampus, while in *Fmr1* KO hippocampus mGluR LTD could persist in the presence of protein-synthesis inhibitors (Huber et al., 2002). This suggested that due to the absence of FMRP, proteins that are important for the maintenance of mGluR LTD are abundantly present in *Fmr1* KO hippocampal slices.

Lack of FMRP has an effect on the protrusion morphology. Neurons of patients with FXS and *Fmr1* KO mice show an increased density of dendritic protrusions, which are on average are also longer and thinner, which are referred to as immature protrusions (Rudelli et al., 1985; Hinton et al., 1991; Comery et al., 1997; Irwin et al., 2001; Irwin et al., 2002; McKinney et al., 2005). It has been suggested that this phenotype results in excessive group I mGluR signalling, resulting in weakening of the synapse through increased levels of AMPA receptor internalisation at the postsynaptic membrane (Nakamoto et al., 2007). Pharmacological studies on wildtype neurons showed that prolonged group I mGluR stimulation also results in immature protrusions, resembling the protrusion phenotype in FXS (Vanderklish and Edelman, 2002; Abu-Elneel et al., 2008).

All evidence together implicates that FMRP plays an important role in repression of translation after group I mGluR stimulation. This observation led to the mGluR theory (Bear et al., 2004), which explains many clinical features found in patients with FXS

and *Fmr1* KO mice, and opened the door for therapeutic interventions. Many research groups are studying therapeutic intervention strategies targeting the mGluR5 pathway, although drugs targeting other signalling pathways involved in FXS are also under investigation (chapter 2).

For new potential FXS therapy, it is crucial to find out if adult patients will benefit from these drugs or if the drugs can only exert their action in development. We recently showed that AFQ056 is an effective mGluR5 antagonist, able to rescue the prepulse inhibition of startle response (PPI) deficit in *Fmr1* KO mice and abnormal spine morphology in cultured *Fmr1* KO hippocampal neurons (chapter 5). Therefore, we investigated the possibility of rescuing the abnormal protrusion morphology in adult *Fmr1* KO mice. In the present study, we show for the first time CA1-specific abnormal protrusion morphology in the hippocampus of adult *Fmr1* KO mice. Subsequently, we used this abnormal protrusion phenotype to study the effect of short-term and long-term AFQ056 treatment on hippocampal protrusion morphology in adult *Fmr1* KO mice.

6.2 MATERIALS AND METHODS

Animals

Fmr1 KO mice are generated in our lab as described previously (Mientjes et al., 2006). The line was completely backcrossed in C57BL/6J mice. Both the *Fmr1* KO mice and the wildtype mice were housed under standard conditions. All experiments were carried out with permission of the local ethical committee. For all experiments, we used *Fmr1* KO and wildtype littermates.

Treatment

Short-term treatment: one hour prior to perfusion, 23-28 week old wildtype and *Fmr1* KO mice were intraperitonealy (i.p.) injected with AFQ056 (3 mg/kg) dissolved in 0.5% methylcellulose. Control animals were injected only with the vehicle methylcellulose. *Long-term treatment:* 19 week old wildtype and *Fmr1* KO mice were fed for six weeks with food pellets containing AFQ056 (150 mg/kg). Mice eat approximately three to four grams a day, resulting in a dose of ~18 mg/kg/day. Three mice, fed for four days with AFQ056-food pellets, were sacrificed to measure the blood/brain ratio of AFQ056. AFQ056 concentration was determined using LC-MS (Liquid chromatography separation followed by mass spectrometry). Control mice were subjected to normal food.

Perfusion and tissue preparation

Brains were isolated as described in Shen et al. (Shen et al., 2008). Briefly, mice were deeply anesthetised with ketamine HCl (100mg/kg,) and xylazine (20mg/kg) i.p.

injected and perfused transcardially with 0.1M PBS (10ml), followed by 15 ml 1.5% paraformaldehyde (PFA) in 0.1 M PBS. It has been shown that 1.5% fixation of PFA is important for the dye to fill up small protrusions (Kim et al., 2007). Brains were removed and post-fixed overnight in 1.5% PFA. The next day, brains were coronally (150 µm thick) sectioned using a vibratome at room temperature. Sections were collected in PBS and on the same day diOlistically labelled.

DiOlistic labelling

Tungsten particles (1.3 µm diameter, Biorad) were coated with 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Sigma) as previously described (Wu et al., 2004). From each mouse, 6-10 hippocampi slices were isolated. The tungsten particles were delivered into the hippocampi using a Helios Gene Gun system (Biorad) at 180 psi. Prior to delivery, a polycarbonate filter with a 3 µm pore size (Falcon 3092; BD Biosciences, Franklin Lakes, NJ) was placed on top of the tissue to filter large clusters of particles. The hippocampi slices were stored at 4 °C for 48 hours to allow the dye to diffuse throughout the dendrites and axons. Subsequently, the slices were post-fixed with 4% PFA for 1 hour and briefly washed with PBS containing Hoechst. Finally, the slices were mounted onto slides with Mowiol (Mowiol 4-88, Hoechst).

Confocal imaging

To image the labelled pyramidal neurons in the hippocampal slices, we used a Leica confocal microscope (Leica TCS SP5 Confocal). The fluorescent DiI was visualised using a 543 nm laser line. Images of neurons were acquired using a 63x oil immersion objective, and the image size was 248 um * 248 um. The localisation of CA1 and CA3 regions was visualised by Hoechst staining (figure 6.1a). The neurons were scanned with an interval of 0.5 µm along the Z-axis. The investigator was always blind to the genotype of the mice.

Protrusion quantification and data analysis

To study the spine morphology of pyramidal neurons in the hippocampus, we used the Z-stacks of the neurons and zoomed in two times (figures 6.1b and 6.1c). Only the secondary or tertiary dendrites were analysed. For quantification 6 to 10 cells per genotype were identified (number of mice: WT (13); *Fmr1* KO (15); WT + AFQ for one hour (3); *Fmr1* KO + AFQ for one hour (6); WT + AFQ for six weeks (6); *Fmr1* KO + AFQ for six weeks (7)) and protrusions were measured as described previously (Okamura et al., 2004; De Vrij et al., 2008). The length was defined as the distance from the base to the tip of the protrusion; width was defined as the maximum distance perpendicular to the long axis of the protrusion. To discriminate between mature or immature protrusion morphology, we calculated the ratio of width/length. When the ratio is above or equal to 0.5 it is defined as a spine (mature) and if the ratio is

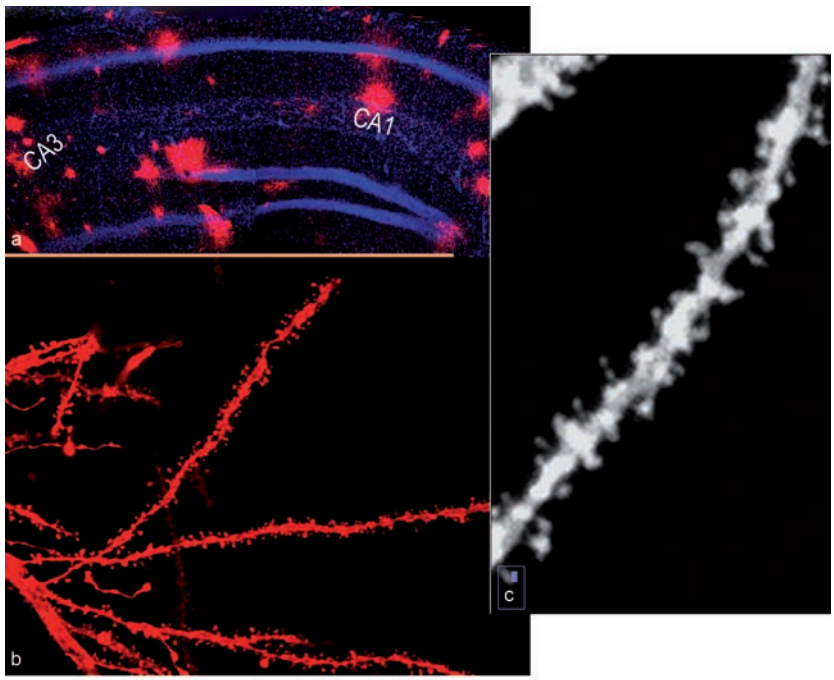

Figure 6.1. Representative pictures of DiOlistic labelled hippocampal slices. (a) Low magnification of a DiOlistic labelled hippocampal slice. (b) DiOlisitic labelled neuron shows clear dendritic branches with protrusions. (c) Overview of a dendritic branch (zoomed in). Morphology of protrusions is clearly visible, varying between long immature protrusions and mushroom-like spines.

smaller than 0.5, it is defined as a filopodia (immature). Morphometric analysis and quantification were performed using MetaMorph software (Universal Imaging Corporation, West Chester, PA) by the investigator who was blind to genotype and experimental manipulation. The independent Student's *T*-test has been used to test if there is a significant difference between wildtype mice and *Fmr1* KO mice at basal level. An ANOVA with a Bonferroni correction has been used to test for significant differences between the four different groups (i.e. wildtype, wildtype + AFQ056, *Fmr1* KO and *Fmr1* KO + AFQ056).

6.3 RESULTS

Altered protrusion morphology in CA1 hippocampal region

For a long time, it has been thought that the adult nervous system remained in a fixed state throughout adulthood. However, currently it is understood that synaptic plasticity in the adult brain is important for long-term memory formation (Holtmaat and Svoboda, 2009). For a new FXS therapy to be successful, it is not only crucial to assess

the benefit on behaviour in adult patients, but it is also important to study the effect on synaptic plasticity. Therefore, at a cellular level we investigated if the protrusion morphology can change after treatment with an mGluR5 antagonist. In this study, we described for the first time the protrusion phenotype in adult *Fmr1* KO mice (~ 25 week old mice). Patients with FXS show learning and memory deficits, and therefore we have chosen to study the protrusion morphology in the hippocampus, specifically in the CA1 and the CA3 region.

Protrusion quantification of *Fmr1* KO pyramidal CA1 neurons revealed that dendrites have significantly more filopodia (immature) compared to wildtype CA1 neurons (figure 6.2a). The density of protrusions was also altered in the *Fmr1* KO neurons, showing significantly more protrusions per dendritic distance. Surprisingly, the protrusion morphology and density of pyramidal neurons in the CA3 region were not significantly different in *Fmr1* KO neurons compared to wildtype (figure 6.2b).

Because only the pyramidal neurons in the CA1 region showed an increased protrusion density and abnormal protrusion morphology, we focused on this region to perform rescue studies using AFQ056.

Short-term treatment with AFQ056

AFQ056 is a selective mGluR5 antagonist, with a non-competitive, inhibitory mode of action. Recently, we have shown that i.p. injection of AFQ056 (3 mg/kg) 30 minutes prior to a PPI experiment rescued the PPI deficit found in *Fmr1* KO mice (chapter 5).

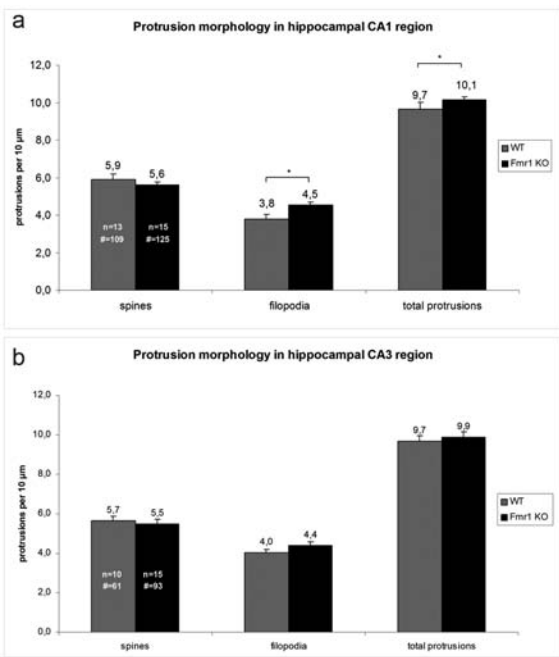

Figure 6.2. Protrusion morphology in wildtype and *Fmr1* KO in the CA1 and CA3 region of the hippocampus. (a) At age 25 weeks, pyramidal neurons in the hippocampal CA1 region of the *Fmr1* KO have significantly more filopodia compared to wildtype neurons ($P<0.05$). The dendrites of *Fmr1* KO CA1-neurons show an increased number of protrusions per 10 µm ($P<0.05$). (b) In the CA3 region, there are no significant differences observed between wildtype and *Fmr1* KO neurons.

Additionally, treatment for 2 hours with AFQ056 resulted in a rescue in the immature spine phenotype of E18 cultured *Fmr1* KO hippocampal neurons. This prompted us to study the rescue of the abnormal protrusion morphology in the CA1 region from adult *Fmr1* KO mice after short-term treatment with AFQ056 (i.p. injection of 3 mg/kg one hour prior to perfusion). Short-term treatment with AFQ056 did not rescue the abnormal protrusion phenotype (i.e. increased number of filopodia) nor did it rescue the increased density of protrusions found in *Fmr1* KO neurons in the CA1 hippocampal region (figure 6.3a). However, after short-term AFQ056 treatment there was a significantly reduced number of spines in *Fmr1* KO neurons compared to wildtype neurons.

Long-term treatment with AFQ056

Protrusions are highly dynamic structures and protrusion changes can occur in hippocampal slices within minutes after LTP induction (Matsuzaki et al., 2004). Since short-term treatment with AFQ056 did not result in changes of protrusion morphology we have chosen to treat wildtype and *Fmr1* KO mice for a long-term period (six weeks) with AFQ056 mixed in food pellets. To show that this is an effective delivery of the drug, we first determined the AFQ056 concentration in brain tissue and blood-plasma after four days of treatment with AFQ056 mixed in food pellets. The results showed that the ratio of brain/blood concentration of AFQ056 was on average 2.7, meaning that AFQ056 is effectively reaching its target receptor in the brain.

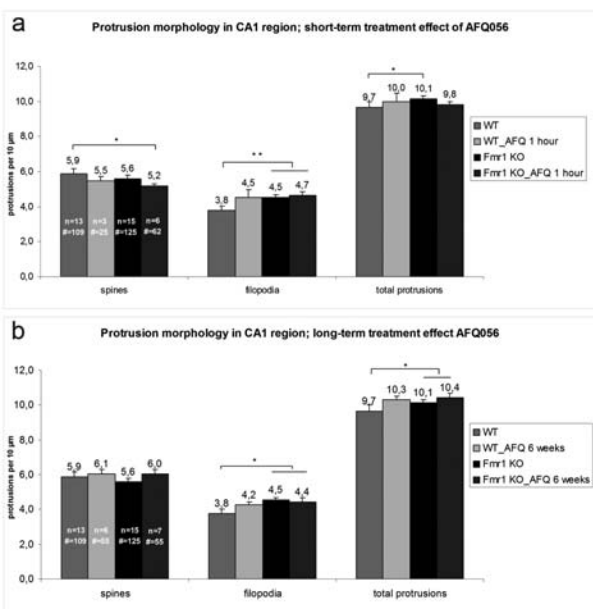

Figure 6.3. Protrusion morphology in wildtype and *Fmr1* KO in the hippocampus after short-term and long-term AFQ056 treatment. (a) Short-term AFQ056 treatment did not result in a rescue of the increased number of dendritic filopodia found in the CA1 region in *Fmr1* KO mice, although, there is an effect on the number of spines. AFQ056 does result in reduced number of spines in the *Fmr1* KO mice compared the wildtype animals ($P<0.05$). (b) Long-term AFQ056 treatment did not result in a rescue of the increased number of dendritic filopodia found in the CA1 region in *Fmr1* KO mice nor on the increased number of protrusions

After six weeks of treatment, no adverse effects on general health of the mice were found after AFQ056 treatment. In addition, no significant rescue effect on protrusion morphology was found in the CA1 region of *Fmr1* KO mice: CA1 pyramidal neurons in the *Fmr1* KO mice still had significantly more filopodia compared to wildtype CA1 pyramidal neurons (figure 6.3b). Additionally, the protrusion density was also still significantly different after AFQ056 treatment.

6.4 DISCUSSION

Spines are highly dynamic structures that show fast activity-dependent changes in shape and strength (Kasai et al., 2010). An abnormal protrusion phenotype has been demonstrated in different brain regions from both patients with FXS and *Fmr1* KO mice (Hinton et al., 1991; Irwin et al., 2001; Nimchinsky et al., 2001; Irwin et al., 2002; McKinney et al., 2005). Although most studies investigated the protrusion morphology in post mortem brain material from patients with FXS or *Fmr1* KO mice (from 1-12 weeks old), it has never been studied in older *Fmr1* KO mice (25 wks) as described in this report. Due to the synaptic plasticity of the developing brain, early treatment would probably be most beneficial. For adult patients however, it is unclear if synaptic plasticity can still be normalised and, more importantly, if such changes would result in any favourable effects for patients with FXS.

In literature, any debate is concentrated at which developmental time-point the abnormal protrusion morphology starts in *Fmr1* KO mice and in which brain areas. It has been demonstrated that the protrusions of neurons in the somatosensory cortex of *Fmr1* KO showed both an immature phenotype (expressed in increased length) and an increased number of protrusions at 2 weeks of age, which disappeared around the age of 4 weeks (Nimchinsky et al., 2001). This suggests a transient nature of these protrusion abnormalities. Galvez et al. also studied the somatosensory protrusion morphology and indeed found no protrusion phenotype in *Fmr1* KO mice at 4 weeks old mice, nevertheless, the protrusion phenotype reappeared at 10 weeks of age (Galvez and Greenough, 2005). Altogether, these results suggest that during development the protrusion phenotype is variable.

The protrusion morphology in the hippocampus also seems to be variable. Grossman et al. demonstrated that in the CA1 region of young adult (10-12 weeks) *Fmr1* KO mouse, apical dendritic protrusions of pyramidal neurons are significant longer compared to wildtype neurons (Grossman et al., 2006). However, the density of protrusions in the CA1 region of *Fmr1* KO mouse did not differ from wildtypes. More recently, hippocampal organotypic slices of 7 days old *Fmr1* KO mice showed significant differences in protrusion morphology, including increased protrusion length and reduced spine head area (Bilousova et al., 2008).

To our knowledge, our study is the first attempt to show a FXS-related protrusion morphology in relative old-adult hippocampus of *Fmr1* KO mice *ex vivo*. The hippocampus consists of different regions and therefore, we decided to discriminate between the CA1 and CA3 region of the hippocampus. Unexpectedly, only the CA1 pyramidal neurons showed a clear protrusion phenotype in *Fmr1* KO mice, while there are no significant differences in the CA3 region. Thus, the abnormal protrusion morphology found in *Fmr1* KO mice seems not only to be age-specific, but also specific for brain subregions. Different studies have shown that the CA1 and CA3 hippocampal region have different functions in learning and memory (Guzowski et al., 2004). Consequently, the molecular blueprint of gene expression is also different between subregions of the hippocampus. Interestingly, recently is has been demonstrated in a common marmoset (a small monkey) that *Fmr1* mRNA is significantly less expressed in the CA3 region compared to the CA1 region (Datson et al., 2009). This agrees with our findings of altered protrusion morphology in the CA1 region, suggesting that lack of FMRP has more consequences for the protrusion morphology in this region, than for neurons in the CA3 region. Another explanation for this subregion difference could be the relation between FMRP and mGluR5 pathway. Defects in synaptic plasticity in the brain of patients with FXS and *Fmr1* KO mice have been linked to altered mGluR5 signalling (Bear et al., 2004). mGluR5 KO mice show impaired learning related to a clear phenotype in synaptic plasticity (reduced LTP) but only in the CA1 region of the hippocampus and not in the CA3 region (Lu et al., 1997). This may explain why altered mGluR5 signalling in *Fmr1* KO mice only results in a protrusion phenotype in the CA1 region and not in the CA3 region. Furthermore, the distribution of the mGluR5 is also different between hippocampal CA1 and CA3 region: immunolabelling of mGluR5 revealed less intensive staining in the CA3 compared to the CA1 region (Lujan et al., 1996).

The protrusion phenotype we found in adult hippocampal CA1 neurons was used to study the effect on the protrusion morphology after short-term and long-term treatment with the mGluR5 antagonist AFQ056. While short-term treatment with AFQ056 can rescue the PPI deficits in *Fmr1* KO mice (chapter 5), short-term treatment did not rescue the abnormal protrusion phenotype in the hippocampus of the *Fmr1* KO mice. This was surprising, since one would expect the abnormal protrusion morphology to be linked to the behavioural deficits of *Fmr1* KO mice. Apparently, it is not be mandatory to rescue the protrusion abnormalities to observe a rescue effect in the behavioural test, although is it not known at which exact time-point protrusions changes in adult mice are expected after inhibition of mGluR5 signalling. Long-term treatment did not rescue the abnormal protrusion morphology as well, however, in that study rescue experiments for the PPI test have not been performed thus we are unable to link the failure to rescue protrusion phenotype with behavioural deficits. Therefore, it is important to determine if long-term treatment with AFQ056 results in a rescue of the PPI deficits. In the *Drosophila Melanogaster* FXS model it has been demonstrated

that an effect of treatment on behaviour is not necessary linked to a rescue in neuronal morphology. In the *dFmr1* mutant fly, it has been shown that constant treatment with MPEP, another mGluR5 antagonist, could rescue the behavioural phenotype (measured in aberrant courtship behaviour) as well as the mushroom body defects (neuronal phenotype in *dFmr1* mutant fly). Alternatively, treatment with MPEP during adulthood did not rescue the mushroom body defects, but did rescue the abnormal behavioural phenotype.

Another reason why the spine morphology did not normalise might be that it is necessary to trigger the rescue of protrusion morphology. In our experimental set-up, mice were only i.p. injected or fed with AFQ056 and were not subjected to a behavioural task. Although we have shown that short-term treatment with AFQ056 can result in rescue of the PPI deficits (chapter 5), it might be important to trigger the rescue of protrusion morphology with a more (challenging) behavioural test, such as an enriched environment, morris water maze or open field test (Restivo et al., 2005; Meredith et al., 2007). However, it is still possible that mGluR5 antagonist will only dampen down the excessive mGluR5 signalling, resulting in improvement of behaviour but not in changes in protrusion morphology.

Besides AFQ056, different specific mGluR5 antagonists and other therapeutic approaches are also under investigation to rescue behaviour deficits and aberrant protrusion morphology (chapter 2). Concerning the altered protrusion morphology, our results show that treatment with an mGluR5 antagonist during adulthood is not likely to induce changes in the protrusion morphology phenotype in the CA1 region of the hippocampus from *Fmr1* KO mice. If protrusion morphology is indeed linked to behaviour, it is possible that adult patients with FXS show a reduced treatment-response because the protrusion morphology is less plastic in adulthood compared to the juvenile period. On the other hand, it might not be mandatory to change the protrusion morphology to induce a behavioural rescue by mGluR5 antagonists.

In conclusion, for future therapeutic interventions in FXS, it is important to further investigate the influence of age and protrusion phenotype and the relation of these two factors on therapeutic possibilities. Finally, it will be fascinating to investigate if treatment with mGluR5 antagonists from a young age on results in a rescue of the protrusion morphology in adult *Fmr1* KO mice.

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7

GENERAL DISCUSSION

Mental retardation (MR) is a developmental disability, characterised by an overall deficiency in cognitive abilities and functional skills, and is usually diagnosed during childhood. MR can be defined by an intelligence quotient (IQ) less then 70 and approximately 2-3% of the world population is affected (Chiurazzi and Oostra, 2000). MR can be associated with congenital abnormalities to form part of a clinical syndrome, or can be nonsyndromic. If nonsyndromic, MR is the only detectable sign. MR can be highly variable in severity and is heterogeneous with regards to the known cause, including environmental and genetic factors (Chiurazzi and Oostra, 2000). Environmental factors include maternal malnutrition, fetal alcohol exposure and anoxia. Chromosomal abnormalities, single-gene mutation and polygenic predisposition are genetic causes of MR. The most common genetic cause of MR is trisomy of chromosome 21 causing Down's syndrome, while FXS is one of the most frequent single gene defects responsible for MR.

7.1 FMRP FUNCTION

The symptoms of FXS can range from learning disabilities to severe cognitive and intellectual disability. FXS is caused by a lack of FMRP expression. Since the identification of the gene responsible for FXS including lack of the gene product in patients with FXS, many researchers have been studying the molecular mechanisms underlying the FXS phenotype. The mGluR theory suggests that the misregulation of translation of specific proteins ultimately results in increased AMPA receptor internalisation, leading to weakening of the synapse (Bear et al., 2004). The mGluR theory is a hypothesis that explains many clinical features found in patients with FXS, however many question marks remain. For example:

- Which key proteins are misregulated in the absence of FMRP?
- What is the exact mechanism by which FMRP represses translation and what is its role in transport of specific mRNAs?
- What is the role of the two autosomal paralogous proteins, FXR1P and FXR1P?
- What role does FMRP play in the microRNA pathway?
- What role does the GABA receptor signalling pathway play in development of the FXS phenotype?

The research topic of this thesis is focused on the cellular function of FMRP in neurons and to investigate the feasibility of therapeutic interventions for FXS using both an *in vitro* and *in vivo* approach. In this chapter I will discuss the results of my research and review recent developments in the FXS research field.

FMRP and functional domains

Cultured hippocampal neurons were used to study the cellular function of FMRP and the effect of potential therapies. This allowed us to image single neurons and study morphological and functional changes. FMRP can be alternatively spliced in 12 potential isoforms (Ashley et al., 1993b; Verkerk et al., 1993; Sittler et al., 1996). For example, exon 15 can be alternatively spliced resulting in three different isoforms of exon 15. Most *Fmr1* mRNAs in the brain contain the complete exon 15 (Xie et al., 2009). On the other hand, the extended form of the second KH domain of a result of the inclusion of exon 12, is absent in the majority of *FMR1* transcripts found in the cell (Xie et al., 2009). The distribution of isoforms appears to be developmentally regulated and can alter during differentiation. Furthermore, these FMRP isoforms have different mRNA binding capacities, and the different mRNA isoforms also seem to have a specific cellular localisation (Denman and Sung, 2002; Xie et al., 2009). In chapter 3, we studied the contribution of several functional domains of FMRP in mRNA-granule formation and dendritic transport of one target mRNA, *FMR1* itself, using transfection studies in cultured hippocampal neurons. To study the different functional domains, we used one isoform of FMRP and the pathogenic FMRP mutant. We found that a specific isoform of FMRP, FMRP_Iso12, and the pathogenic mutant, FMRP_I304N show different subcellular localisation compared to wild type FMRP in transfected *Fmr1* knockout (KO) neurons. FMRP_Iso12 is missing the C-terminus of the protein, and lacks the Nuclear Export Signal (NES) and the RGG-box, an RNA-binding domain. Probably due to the lack of the NES, the protein is trapped in the nucleus and is not present in dendritic RNA-granules. This observation clearly suggests that the nuclear export signal is important for FMRP to be incorporated in dendritic RNA-granules. The *FMR1_Iso12* mRNA is also barely present in the dendrites suggesting that incorporation in dendritic RNA-granules is very inefficient or delayed, most likely due to accumulation of the FMRP_Iso12 in the nucleus that would otherwise transport the mRNA into the dendrite. Furthermore, we showed that FMRP_I304N (a pathogenic FMRP variant that results in severe mental retardation) is no longer incorporated into dendritic mRNA-granules. Other research groups had already shown that FMRP_I304N can no longer associate with polyribosomes and can no longer bind to the kissing complex sequence, the specific artificial RNA-sequence that can bind to the second KH domain (Feng et al., 1997; Darnell et al., 2005b).

Interestingly, we found that both wild type FMRP and one of its homologues, FXR2P, could dimerise with these FMRP forms, FMRP_Iso12 and FMRP_I304N, resulting in normal dendritic RNA-granule formation and *FMR1* mRNA localisation. These results show that FXR2P and FMRP variants can functionally interact in neurons. It has been postulated that both autosomal homologous proteins, FXR1P and FXR2P, can compensate for the absence of FMRP in patients with FXS due to a similar cellular

function. However, there is still debate if FXR1P and FXR2P share similar functions with FMRP or if there is a clear difference. For example, in contrast to the *Fmr1* KO and *Fxr2* KO mouse models, the *Fxr1* KO mouse model is lethal, probably due to its function in striated muscle tissue suggesting a specific function of FXR1P (Bakker et al., 1994; Bontekoe et al., 2002; Mientjes et al., 2004). Bechara et al. also showed that the longest isoform (isoform e) of FXR1P, which is only expressed in muscle tissue, can bind with a lower affinity to G-quartet mRNAs compared to FMRP, while two other FXR1P isoforms (isoform a and e) negatively regulate the affinity of FMRP for G-quartet mRNA (Bechara et al., 2007). More recently, it has been shown that dFmrp has an evolutionary conserved neuronal function which is not shared with FXR1P and FXR2P (Coffee et al., 2010). The *Drosophila* genome encodes for a single *dFmr1* gene with close similarity to all three human FXR genes. The lack of *dFmr1* recapitulates the FXS-phenotype, including elevated protein levels and altered neuromuscular junction. It was shown that only *hFMR1* fully rescue molecular and cellular defects in *Drosophila* neurons, while *hFXR1* and *hFXR2* provide no rescue. This suggests that *FMR1* has developed a specific neuronal function in evolution that cannot be compensated by either *FXR1* or *FXR2*. When reviewing all the evidence, it can be assumed that there is a significant difference in cellular function and this would be a fascinating area of research.

It has been proposed that two G-quartet structures, a structure organised in stacks of planar layers of guanine tetrad units, in the coding sequence of *FMR1* mRNA are important for binding to FMRP (Didiot et al., 2008). Mutation of the G-quartet, without affecting the amino acid sequence of FMRP, has been shown to alter *in vitro* binding of *FMR1_ΔG1+2* to FMRP, but does not change the localisation of the mRNA in HeLa cells (Didiot et al., 2008). Additionally, the G-quartet structure in *FMR1* mRNA appears to be a potent exonic splicing enhancer (Didiot et al., 2008). This suggests that the G-quartet might be a control element of *FMR1* alternative splicing, especially around exon 15, and that FMRP might be involved in splicing regulation of other genes containing a G-quartet structure in their protein coding sequence. In chapter 3, we show that the G-quartet structure is not essential for the incorporation of *FMR1* mRNA in RNA-granules and that *FMR1_ΔG1+2* mRNA (both G-quartet mutated) can still be transported into the dendrite in RNA-granules.

Our results suggest that transport to the nucleus is important for the function of FMRP, as also shown by Kim et al. who demonstrated that FMRP binds mRNA in the nucleus (Kim et al., 2009). Despite our efforts to investigate the precise cellular function of FMRP in the nucleus, it remains unclear whether the nuclear transport of FMRP is important for the binding of mRNA or microRNAs, or for its incorporation into ribonucleoprotein (RNP) particles.

Validation of FMRP mRNA targets

Understanding FMRP function relies fundamentally on the identification of its RNA targets, and this is the most likely way of understanding the specific processes that go awry in the neurons of patients with FXS. Quickly following the discovery of the *FMR1* gene, the first reports were published showing that FMRP can bind mRNAs with U-rich sequences, G-quartet structures or without specific structures (Ashley et al., 1993a; Brown et al., 2001; Chen et al., 2003; Dolzhanskaya et al., 2003; Miyashiro et al., 2003; Darnell et al., 2004; Darnell et al., 2005b). Although each research group revealed their list of "specific" mRNA targets, not many of these overlapped. This raised the question whether the techniques used were sensitive and accurate enough to identify the mRNA targets of FMRP. First, some studies relied on polysomal fractions and immunoprecipitation of FMRP-positive RNP particles, and therefore it is difficult to know if the identified mRNAs are specific FMRP targets rather than mRNA targets of other RNA-binding proteins in these complexes. Second, it is difficult to compare results, because different arrays were used with weak overlap (Schaeffer et al., 2003). Finally, in retrospect, Brown et al. used conditions (high concentration of EDTA that binds Mg^{2+}) during co-immunoprecipitation that abrogates binding of target mRNAs specific to the FMRP KH2 domain (Darnell et al., 2005a).

More recently, probably the most specific method, the HITS-CLIP (high throughput sequencing-crosslinking immunoprecipitation) technique, has been used to identify specific mRNA targets of FMRP (personal communication Darnell (Ule et al., 2003)). The advantage of this technique is that FMRP and its mRNA targets are crosslinked before lysis. After lysis the wash steps are very stringent, losing most protein-protein interactions and by immunoprecipitation of FMRP, only the mRNAs that are specifically bound could be identified. It is important to identify the specific target mRNAs of FMRP, not just the mRNAs that are in the same complex as FMRP. Identifying the specific mRNAs will facilitate the exact role of FMRP in misregulation of specific proteins, especially the proteins that are important for synaptic plasticity.

Many approaches to identifying the mRNA targets of FMRP use a high throughput screening method and several mRNA targets have been validated as FMRP targets, including *Psd95*, *CaMKII*, *Arc*, *Map1B* and *Fmr1* itself (Ashley et al., 1993a; Schaeffer et al., 2001; Zalfa et al., 2003; Lu et al., 2004). However, what are the consequences for these target mRNAs if FMRP is absent? In the early 90's, when FMRP was found to be an RNA-binding protein and was localised in dendrites of neurons, it was thought that FMRP was important for transport of specific mRNAs to the synapse. However, Steward et al. reported that two target mRNA, *CaMKII* and *Arc*, were normally distributed at basal state and 2 h after stimulation in the *Fmr1* KO mice using *in situ* hybridisation (Steward et al., 1998). In contrast, it has been demonstrated that in absence of FMRP several mRNA targets show altered subcellular distribution and abundance, and that FMRP might be

important for activity-dependent mRNA transport after mGluR stimulation (Miyashiro et al., 2003; Dictenberg et al., 2008). These results suggest that the absence of FMRP leads to mislocalisation and misregulation of mRNA targets in the brain.

A greater body of evidence has shown that FMRP seems to play an important role in the translational control of target mRNAs. First of all, it has been proven that binding of FMRP to actively translating polyribosomes is very important for its function, since abolishment of this function results in severe mental retardation (Feng et al., 1997). Second, FMRP is an translational repressor *in vitro,* important for translational control of target mRNAs (Laggerbauer et al., 2001; Li et al., 2001). These observations were followed by many studies demonstrating that in absence of FMRP, different target mRNAs, such as *Map1B*, *Arc*, *CaMKII* and *SAPAP3*, show elevated protein expression levels (Zalfa et al., 2003; Lu et al., 2004; Schuett et al., 2009). FMRP is therefore important to repress translation of several target mRNAs, although recently it has been shown that FMRP might also play a role in translational activation (Bechara et al., 2009).

However, what are the consequences of dysregulation in expression of several synaptic proteins? Communication between neurons is a highly sensitive process with a low threshold for errors. Other studies related to MR and psychiatric disorders have shown that altered protein expression during embryonic development or postnatal development is linked to schizophrenia, autism and multiple sclerosis. Of interest, balanced protein expression has shown to be important for behaviour of individuals and mice. For example, *CaMKII* is one of the target mRNAs of FMRP and might be misregulated in FXS. A transgenic mouse over-expressing CaMKIIα in the forebrain of mice results in increased anxiety-related behaviour and offensive-aggression (Hasegawa et al., 2009). Also FMRP over-expression in transgenic mice results in a phenotype, which is distinct from FXS (Peier et al., 2000).

7.2 PROTRUSION ABNORMALITIES

Several studies have demonstrated an altered protrusion morphology and often an increased protrusion density in the different brain areas of patients with FXS and *Fmr1* KO mice compared to controls (Hinton et al., 1991; Comery et al., 1997; Irwin et al., 2001; Irwin et al., 2002; McKinney et al., 2005; Grossman et al., 2006). In order to study the protrusion morphology in *Fmr1* KO neurons we used primary cultured hippocampal neurons. In chapters 4 and 5, we demonstrated that *Fmr1* KO hippocampal neurons cultured for 21 days *in vitro* (21 DIV) have more protrusions with an immature appearance (named filopodia) compared to wild type neurons. Antar et al. also studied the protrusions in cultured *Fmr1* KO hippocampal neurons and observed significantly more protrusions in the *Fmr1* KO neurons than in the wild type neurons (Antar et al., 2006). Because these neurons were only cultured for 14 DIV, it was difficult to distinguish between mature and immature protrusions. The protrusion

phenotype in our hippocampal cultures is in accordance with the descriptions of post mortem brain material from patients with FXS and *Fmr1* KO mice, in which neurons of the cortex showed abnormally long, thin and immature filopodia-like protrusions (Hinton et al., 1991; Irwin et al., 2001). The abnormal protrusion morphology in our cultured hippocampal neurons allowed us to perform rescue studies using specific drug treatments which will be discussed in paragraph 7.3.

It is also important to study effects of potential drugs *in vivo* and therefore, we investigated the protrusion morphology in the hippocampus of adult *Fmr1* KO mice using DiOlistic approach. We have chosen to study the spine morphology in the hippocampus because it is an important structure for learning and memory, which is affected in patients with FXS. As described in chapter 6, only the pyramidal CA1 neurons of adult *Fmr1* KO mice displayed an altered protrusion morphology, while the protrusions of neurons in the CA3 region appeared normal. Although different research groups have studied the protrusion morphology in *Fmr1* KO mice, generally the pyramidal neurons of the cortex were studied and not the hippocampal protrusions in adult *Fmr1* KO mice. Only Grossman et al. studied the hippocampal CA1 neurons of 10 week old *Fmr1* KO mice, but not the CA3 pyramidal neurons (Grossman et al., 2006). Bilousova et al. did study the protrusion morphology in pyramidal CA1 and CA3 *Fmr1* KO neurons, however, this was in postnatal organotypical slices of 7 days old (Bilousova et al., 2008). At P7, protrusions in both CA1 and CA3 regions of the *Fmr1* KO mice showed an abnormal morphology. It is possible that during postnatal development, pyramidal neurons in both areas show altered protrusion morphology, which only in CA3 region disappears during adulthood.

Our results suggest that the protrusion morphology appears to be age and brain region specific, even subregion specific (CA1 versus CA3). Recently, it has become clear that the alterations in the brain due to lack of FMRP is not observed in all brain regions. For example, over-expression of specific proteins in the *Fmr1* KO brain can be brain region specific. Schütt et al. analyzed the abundance of synaptic proteins in wild type and *Fmr1* KO mice (Schuett et al., 2009). They observed for example that SAPAP 1 and Shank3 expression were only elevated in the neocortical synaptosomes of the *Fmr1* KO mice, while SAPAP2 and SAPAP3 expression was only elevated in the hippcampal synaptosomes. Consequently, the protrusion abnormalities can also be region specific. At basal level, there are differences in mRNA expression between subregions of the hippocampus. Recently, Datson et al. showed that *Fmr1* mRNA is significantly more abundant in the CA1 region than in the CA3 region in the marmoset, suggesting that FMRP might play a more prominent role in the CA1 region of the hippocampus compared to CA3 region (Datson et al., 2009).

Finally, another explanation for the CA1 and CA3 differences might be due to the presynaptic role of FMRP. During development, FMRP is present in presynaptic fragile-X-granules (FXGs) containing besides FMRP, also its homologues FXR2P and

sometimes FXR1P (Christie et al., 2009). FXGs are only present in the CA3 region, and not in the CA1 region. Although FXGs are only present during development, it is possible that the function of FMRP in the CA3 region is more presynaptic.

The molecular mechanisms underlying the altered protrusion phenotype in FXS is another area of focus for research. According to the mGluR theory, due to the excessive protein synthesis after group I mGluR stimulation, excessive numbers of AMPA receptors are internalised which results in the immature protrusion phenotype in FXS. Several studies have shown that prolonged treatment of wild type hippocampal neurons with DHPG, a group I mGluR agonist, results in increased number of long, thin dendritic protrusions (filopodia), resembling the protrusion phenotype found in FXS. However, evidence is missing identifying which proteins are responsible for the excessive AMPA receptor internalisation in FXS (Vanderklish and Edelman, 2002; Abu-Elneel et al., 2008). Activity-regulated cytoskeleton-associated protein (Arc) (also termed as Arg3.1) is an immediate-early gene (IEG) induced in response to sensory experience, learning, LTP, spatial exploration, and novelty (Gusev et al., 2005; Guzowski et al., 2006) and might be a good candidate for the excessive AMPA receptor internalisation. It has been suggested that *Arc* is an mRNA target of FMRP and that the protein levels of Arc are increased in absence of FMRP (Zalfa et al., 2003). Park et al. demonstrated that Arc synthesis is required for mGluR-LTD induction, and Waung et al. showed that Arc is important for mGluR-dependent LTD by increasing the AMPA receptor internalisation rate (Park et al., 2008; Waung et al., 2008). Therefore, Arc may play a major role in the excessive AMPA receptor internalisation and altered protrusion morphology. Striatal Enriched Phosphatase (STEP) is another interesting candidate in AMPA receptor internalisation (Zhang et al., 2008). STEP can phosphorylate AMPA and NMDA receptors to mediate internalisation of these receptors. STEP mRNA is present in the dendrites and is synthesised after group I mGluR stimulation. STEP expression might be elevated in *Fmr1* KO mice, and in this way induces increased AMPA receptor internalisation. Recently, a STEP/*Fmr1* double KO mouse model have been generated and preliminary data showed that this mouse model show a rescue in open field behaviour compared to *Fmr1* KO mice (personal communication Paul Lombroso). Whether STEP is indeed an mRNA target of FMRP is thus far only hypothesised, and needs to be confirmed.

Cytoskeleton

Excessive AMPA receptor internalisation alone can not induce the immature appearance of the protrusions in FXS, since the cytoskeletal structure and the proteins leading up to cytoskeleton changes are also important for the protrusion morphology. It has been reported that the cytoskeletal structure does not only play a role in FXS, but also in other forms of MR (Ramakers, 2002; Newey et al., 2005). Small GTPases, such

as Rho, Rac and Cdc42 form a large family of proteins characterised by their ability to bind and hydrolyze GTP. They act as molecular switches affecting various biological activities regulating growth and migration and their action is not restricted to neurons and synapses alone, although their role in synapses seems to be important for the pathophysiology of MR (Boda et al., 2010). RhoA, Rac1, and Cdc42 are best known for their characteristic effects on the actin cytoskeleton, but more recently has also been found to influence microtubule organisation (Gundersen et al., 2004). Because microtubules and actin filaments make up the structural framework of dendrites and protrusions, the function of these networks in dendritic development and protrusion morphogenesis has been extensively studied in a variety of model systems.

Several lines of evidence have linked FMRP to GTPase signalling. Firstly, *Fmr1* KO mice show a general impairment in LTP that can be rescued by activation of Ras/PI3K cascade (Hu et al., 2008). Secondly, work in *Drosophila* has shown that dFmrp affects dendritic development by regulating the actin cytoskeleton through a translational suppression of Rac1 and profilin (Reeve et al., 2005). Thirdly, FMRP appears to be a negative regulator of PP2Ac mRNA translation, and absence of FMRP results in increased expression of PP2Ac leading to alterations in actin remodelling in fibroblast cell lines (Castets et al., 2005). Finally, another downstream effector of Rac, p21-activated kinases (PAK), a family of serine-threonine kinases that consists of at least three members, PAK1, PAK2 and PAK3, has been associated with FXS. Inhibition of PAK in the *Fmr1* KO can rescue cellular and behavioural FXS phenotypes, including the abnormal protrusion morphology, locomotor activity and anxiety (Hayashi et al., 2007). Furthermore, FMRP seems to play an important role in microtubule stability. FMRP represses the translation of microtubule associated protein1b (*Map1b*) mRNA during active synaptogenesis in neonatal brain development. In the absence of FMRP, elevated Map1b protein expression leads to abnormally increased microtubule stability, thereby hindering normal development of dendritic protrusion (Lu et al., 2004).

Lack of FMRP expression leads to abnormal protrusion morphology in different brain areas and this abnormal protrusion morphology seems to be a hallmark of many other MR disorders, syndromic or nonsyndromic. However, whether this phenotype is a cause or a consequence of MR and if we can use this feature to investigate the therapeutic effects of pharmacological interventions are questions that will be addressed in the next section.

7.3 THERAPEUTIC INTERVENTION

In 2004, Bear et al. postulated the mGluR theory that tried to explain the clinical and behavioural phenotypes observed in patients with FXS and *Fmr1* KO mice (Bear et al., 2004). This theory opened the door for potential therapeutic strategies that might improve the quality of life of patients with FXS.

The mGluR5 is a potential drug target. By blocking this receptor using a specific antagonist, it might reduce the excessive protein synthesis and altered synaptic plasticity in FXS. This may result in a rescue of abnormal transmission signalling and leads to improved behaviour. For our research it was important to choose an accurate metric to evaluate any drug effect in *Fmr1* KO mice. Unfortunately, the *Fmr1* KO mice have no robust behavioural phenotype, mice show a mild learning deficit demonstrated by Morris water maze, but barely any phenotype in non-cognitive problems, such as anxiety (Kooy, 2003). The most robust phenotypes that are repeatedly demonstrated are audiogenic seizures (Chen and Toth, 2001; Yan et al., 2005). In this thesis we describe a novel behavioural test to demonstrate a robust behavioural phenotype in prepulse inhibition of acoustic startle (PPI). In PPI, conditioning and learning do not occur and therefore, the mice can be tested multiple times. We measure this PPI response by measuring the eye-lid response, while other research groups measure whole body-response. Eyelid measurements of startle include the very first components of the startle response, whereas whole body startle measurements require induction of very strong startle responses. Therefore, eyelid startle measurement can detect more subtle differences in startle behaviour. Using our PPI set-up, the *Fmr1* KO mice show a significantly reduced PPI response compared to wild type mice, and this was replicated in multiple independent experiments. However, our results are in contrast to other research groups, where it has been shown that the PPI response in *Fmr1* KO mice was increased (Frankland et al., 2004) or not altered (Spencer et al., 2006). This can be explained by our differing outcome measures or differences in genetic background strains of the mice.

The altered PPI response in *Fmr1* KO mice was used as a metric to study three different mGluR5 antagonists, i.e. MPEP, fenobam and AFQ056. All three antagonists could rescue the deficits in PPI response in *Fmr1* KO mice. Surprisingly, only MPEP had a significant effect on the PPI response in wild type mice, i.e. MPEP further increased the PPI response. It is unclear why MPEP had this effect, however MPEP may have a small effect on NMDA receptors (Popoli et al., 2004; Lea et al., 2005). The results found with these mGluR5 antagonists are in line with other studies. It has been shown that MPEP can rescue audiogenic seizures in *Fmr1* KO mice and aberrant courtship behaviour in the *dFmr1* null mutant flies (McBride et al., 2005; Yan et al., 2005). In patients with FXS, one single dose of fenobam demonstrated an improved effect on PPI response in 6 out of 12 patients with FXS as well. Inter-individual genetic or environmental differences could explain the differences in drug efficacy.

The next aim of our study was focused on the rescue of the altered protrusion phenotype found in FXS with specific mGluR5 antagonists. It has been reasoned that the altered protrusion phenotype is linked to the typical FXS behaviour. We tested the effect of the same three mGluR5 antagonists in cultured hippocampal neurons.

Interestingly, all three mGluR5 antagonists could rescue the immature appearance of protrusions of cultured *Fmr1* KO neurons (chapter 4 and 5), however, *in vivo* short-term (1 hour) and long-term administration (6 weeks) with AFQ056 did not rescue the altered protrusion morphology found in the CA1 region of the hippocampus of adult *Fmr1* KO mice (chapter 6). These results need further investigation to determine if the abnormal morphology can be rescued when treatment is started earlier and if the morphology is linked to the characteristic behavioural phenotype exhibited by patients with FXS.

As mentioned above, acute treatment (~ 30 min) with these mGluR5 antagonists can rescue the altered PPI response in adult *Fmr1* KO mice, however, apparently the altered protrusion morphology can not be rescued in adult *Fmr1* KO mice under the conditions that we used. It might be that blocking mGluR5 only dampen down the mGluR5 signal transmission, but has no effect on the protrusion morphology. Another explanation might be linked to the protrusion turnover rate. It has been shown that in wild type cortex, the protrusion formation during motor learning and sensory experience is high, and only a small percentage of these newly formed protrusions are preserved during life (Yang et al., 2009). Recently, it was found that during early development (postnatal days 10-12), the *Fmr1* KO mice have an overabundance of immature protrusion subtypes and an increased protrusion turnover rate compared to wild type mice (Cruz-Martin et al., 2010). These results show that FMRP is important for maturation and stabilisation of dendritic protrusions. Blocking mGluR5 by MPEP could not rescue this abnormally high turnover rate at this specific developmental period, nor could it rescue the overabundance of immature protrusion subtypes. The failure to rescue turnover rate might be due to the fact that blocking the mGluR5 can dampen down the mGluR5 signal cascade of stable synapses, but that the turnover rate is still increased during mGluR5 blockade in adult *Fmr1* KO mice.

When protrusion morphology is indeed linked to behaviour, it might be possible that adult patients with FXS show a reduced treatment-response because the protrusion morphology is less plastic in adulthood compared to the juvenile period. On the other hand, it might not be mandatory to change the protrusion morphology to induce a behavioural rescue by mGluR5 antagonists.

7.4 CONCLUDING REMARKS

The work described in this thesis contributes to what is known about functional domains of FMRP and future therapeutic interventions for FXS. The use of *Fmr1* KO mouse model provides us with the perfect tool to investigate therapeutic interventions that ultimately can be used in a clinical setting. Research in the FXS field is now focused on studying different therapeutic targets. Besides mGluR5s, GABA receptors have been shown to play a role in the FXS phenotype and therefore are potential therapeutic

targets (chapter 2). The pathology of FXS involves many receptors and a loss of a delicate balance in synaptic plasticity and therefore, treatment with drug combinations should be considered. Several clinical trials have been conducted to study the effect of different types of drugs in patients with FXS. However, we believe it is crucial to conduct a robust clinical trial. This clinical trial should: i) be randomised placebo-controlled, ii) be double-blind, iii) include an adequate number of patients, iv) be a homogenous group, and v) use reliable objective read-outs to determine the therapeutic efficacy. At present different psychological questionnaires are used to determine the therapeutic efficacy of new treatments. However, improvement of behaviour is subjective to caregivers and teachers. To guarantee objectivity, it is important to investigate other reliable and objective read-outs that will facilitate determining the therapeutic efficacy of new treatments. The PPI test may be useful in providing an objective outcome measure for drug trials. In addition to clinical trials, pre-testing in mouse models is important for basic research in understanding the pathology of FXS and as a precursor for clinical trials. Furthermore, for future therapeutic interventions in FXS, it is important to investigate the influence of age and protrusion phenotype and the relation of these two factors on therapeutic possibilities using the FXS mouse model. Finally, it will be worthwhile to investigate if treatment with mGluR5 antagonists starting at young age will result in a rescue of the protrusion morphology in adult *Fmr1* KO mice.

Despite all the research focused on FXS many questions regarding the pathophysiology of FXS remain unanswered. Further potential areas of research that should be pursued include; (i) which mRNA targets show elevated translation at the synapse in FXS and in which brain areas, (ii) what is the relation between the elevated protein expression and the AMPA receptor internalisation, (iii) what are the mechanisms used by FMRP to repress translation of its target mRNAs including the involvement of microRNAs. Unravelling these basal mechanisms is important to increase our knowledge about synaptic plasticity and its role in FXS, autism and other MR disorders.

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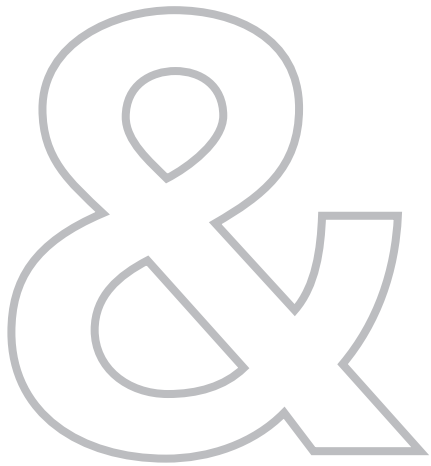

SUMMARY

Intellectual disability affects 2-3% of the population. Fragile X syndrome (FXS) is one of the most common inherited causes of intellectual disability. In 1991 was the fragile X mental retardation 1 (*FMR1)* gene, localised on the X chromosome, identified as the gene responsible for FXS. It was discovered that a CGG trinuclueotide repeat located in the 5' untranslated region of the *FMR1* gene was the cause of FXS. The CGG repeat in control individuals ranges from 5 to 55 CGG units, but has been shown to be unstable at higher repeat numbers. In patients with FXS it was found that this repeat comprised of over 200 CGG units. As a consequence of the CGG repeat expansion, the promoter region is hypermethylated. Typically, hypermethylation of the promoter results in silencing of the gene, resulting in the absence of *FMR1* transcription and its protein product, the fragile X mental retardation protein (FMRP).

FMRP is ubiquitously expressed in all cells of the body, but is predominantly present in specific cells of the brain and testes. In the brain, FMRP is abundantly present in the neurons. Microscopic analyses of brain material from both patients with FXS and *Fmr1* knockout (KO) mice reveal no gross morphological abnormalities. However, in specific brain areas long and thin dendritic protrusions have been observed, consistent with an immature protrusion phenotype. Patients with FXS have intellectual disabilities and show behavioural disturbances, due to the lack of FMRP. Efforts have been taken to study the cellular function of this protein and the consequences at molecular, electrophysiological and behavioural levels. To study FXS, several animal models have been generated, such as the *Fmr1* KO mouse model. These mice lack Fmrp expression, similar to patients with FXS.

Research in the past 20 years has finally led to a theory that attempts to explain the FXS phenotype. This theory is called the mGluR (metabotropic glutamate receptor) theory, which hypothesises that a specific signal transmission cascade is altered in patients with FXS. The first clinical trials, based on this theory, has been started to test the effect of drugs on behaviour and intellectual capacity. This thesis is divided in two parts: first, we investigated the function of the protein in neurons and second, we investigated the effects of different drugs on behaviour and spine morphology in *Fmr1* KO mice.

Chapter 1 is a general introduction and describes the *FMR1* gene and the important roles that FMRP plays in many cellular processes in neurons. FMRP harbours several domains that are important for its function in the cell. The protein can shuttle in and out the nucleus using its Nuclear Localisation Signal and Nuclear Export Signal. Furthermore, FMRP is an RNA-binding protein and harbours three RNA-binding domains: two KH domains and one RGG box. FMRP can bind to specific mRNAs using these domains, including to its own *FMR1* mRNA. The binding of specific mRNAs to FMRP is dependent on specific sequences and structures in the target mRNA. For example, an mRNA can contain a Gquartet structure that permits the mRNA to fold

in a specific manner to allow the mRNA to bind to FMRP. FMRP is mainly localised in the cell soma, but FMRP is also transported in RNA granules into the dendrite, in proximity of the synapse. It has been found that FMRP acts as a translational repressor, meaning that it is involved in repression of translation of specific mRNAs into protein upon synaptic activation. Therefore, in patients with FXS the translational repression of specific mRNAs is reduced, resulting in elevated protein synthesis at the synapse. These proteins seem to be important for AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptor internalisation, and therefore, lack of FMRP presumable results in increased AMPA receptor internalisation, consequently resulting in long and thin protrusions and reduced signal transmission.

Chapter 2 provides an in-depth discussion of the mechanism of learning and memory in the brain. Also, the function of two neurotransmitter systems that are involved in the FXS phenotype, the excitatory mGluR and the inhibitory GABA (γ-aminobutyric acid) systems, are explored. The alterations of these pathways in the FXS phenotype are explained in more detail together with the mGluR theory and GABA hypothesis. These theories have led to potential therapeutic strategies, which are extensively discussed. To date, many different compounds have been evaluated in FXS animal models or patients with FXS in clinical trials. However, many clinical trials do not always require the scientific standards to conclude if a medication has indeed any effect. Therefore, to establish definitive efficacy, it is crucial to set up clinical trials that: (i) are randomised and placebo-controlled; (ii) are double blind; (iii) include adequate number of patients; and (iv) utilise reliable objective readouts to determine therapeutic efficacy.

In chapter 3 we study several functional domains of FMRP by using different FMRP variants in transfection studies. We show that normal FMRP is incorporated into dendritic RNA-granules and that it co-localises with its own *FMR1* mRNA in the dendrite. A pathogenic FMRP variant, referred to as I304N, contains a mutation in the second KH domain and has been identified in a patient with a severe form of FXS. We find that this FMRP variant can not be incorporated in dendritic RNA-granules, but is still able to bind to its own mRNA. Another variant we study lacks the C-terminal part of the protein which contains the Nuclear Export Signal and the RGG box, another RNA-binding domain of FMRP. This variant, FMRP_Iso12, is trapped in the nucleus and is not incorporated in dendritic RNA-granules. In addition, almost no *FMR1_Iso12* mRNA localises in the dendrite, suggesting that FMRP plays an essential role in transport of its own mRNA. Another FMRP variant only contains silent point mutations that disrupt the Gquartet structure of *FMR1* mRNA, without affecting the amino acid sequence of FMRP. This Gquartet structure in the *FMR1* mRNA is important for the binding of the *FMR1* mRNA to FMRP. We show that the Gquartet structure is not essential for the incorporation of *FMR1_Gquartet* mRNA in dendritic RNA-granules and that it is still transported into the dendrite.

In chapter 4 we investigate the effect of an mGluR5 antagonist in *Fmr1* KO mice on behaviour and on protrusion morphology using cultured hippocampal neurons. The mGluR theory suggests that a reduction in the mGluR5 signal transmission might result in a rescue of the FXS phenotype. We introduce a new behavioural test to measure the prepulse inhibition of the startle (PPI) response. *Fmr1* KO mice show a significant reduction in PPI response compared to control mice. After treatment with MPEP (2-methyl-6-(phenylethynyl)-pyridine hydrochloride), an mGluR5 antagonist, is the PPI response of the *Fmr1* KO mice rescued and indistinguishable from control mice. Next, we examine cultured hippocampal neurons of *Fmr1* KO mice. As described in the literature, we find that cultured hippocampal neurons of *Fmr1* KO mice have an increased number of immature protrusions, referred as filopodia. Treatment of *Fmr1* KO neurons with MPEP for four hours results in a rescue of the altered protrusion morphology and is indistinguishable from control neurons.

In chapter 5, we describe a new mGluR5 antagonist, AFQ056, for its effects on the PPI response and protrusion morphology. Similar to MPEP, we show that also AFQ056 is able to rescue the reduced PPI response in *Fmr1* KO mice and the altered protrusion morphology of cultured *Fmr1* KO hippocampal neurons.

In chapter 6 we investigate the effect of treatment with AFQ056 on protrusion morphology *in vivo* using adult *Fmr1* KO mice (25 week old). For this, we have set up the DiOlistic labelling technique whereby a fluorescent dye fills up whole neurons to analyse the dendritic protrusion morphology in the hippocampus *in vivo*. We show that only neurons in the hippocampal CA1 region of the *Fmr1* KO mice have more filopodia compared to control mice, while neurons of the hippocampal CA3 region in *Fmr1* KO mice have a normal protrusion number and morphology. Subsequently, we investigate the effect of AFQ056 treatment on the altered protrusion morphology in the CA1 region of the adult *Fmr1* KO mice. Although short-term treatment with AFQ056 can rescue the reduced PPI response in *Fmr1* KO mice, short-term treatment can not rescue the abnormal protrusion morphology in the CA1 region of adult *Fmr1* KO mice. Long-term treatment for six weeks also has no effect on the altered protrusion morphology in the CA1 region of adult *Fmr1* KO mice.

In the final chapter are the results discussed in conjunction with other research. We show that different domains of FMRP are important for its incorporation in dendritic RNA-granules. Furthermore we discuss our results using mGluR5 antagonist as a potential treatment. The results on treatment with AFQ056 in adult *Fmr1* KO mice suggest that rescue of the altered protrusion morphology is not essential to improve specific behaviour abilities. Another explanation might be that the abnormal protrusion morphology in adult *Fmr1* KO mice are more difficult to correct with an mGluR5 antagonist. Hence, an earlier onset of treatment may be necessary. In conclusion, my research has gained knowledge on the normal cellular function of FMRP in neurons and the molecular mechanisms underlying the development of potential therapeutic strategies for FXS.

SAMENVATTING

Ongeveer 2-3% van de totale bevolking heeft een verstandelijke handicap. Het fragiele X syndroom (FXS) is één van de meest voorkomende erfelijke oorzaken van een verstandelijke handicap. In 1991 is het fragiele X mentale retardatie 1 (*FMR1*) gen, gelegen op het X chromosoom, geïdentificeerd als het gen dat verantwoordelijk is voor FXS. Een verlenging van een aaneenschakeling van CGG nuclueotiden in de 5' onvertaalde regio van het *FMR1* gen is de oorzaak van FXS. Een controle individu heeft een CGG herhaling variërend tussen de 5 en de 55 CGG units, terwijl de CGG herhaling in patiënten met FXS meer dan 200 CGG units is. Als gevolg van deze verlengde CGG herhaling is de promotor regio hypergemethyleerd. De hypermethylatie van de promotor resulteert in het uitschakelen van het *FMR1* gen waardoor patiënten geen *FMR1* mRNA maken en het bijbehorende eiwit, het fragiele X mentale retardatie eiwit (FMRP), missen.

FMRP is aanwezig in alle cellen van het lichaam, maar is in hogere mate aanwezig in specifieke cellen van de hersenen en de testikels. In de hersenen, is FMRP grotendeels te vinden in de zenuwcellen. Microscopische analyse van hersenmateriaal van zowel patiënten met FXS als *Fmr1* knockout (KO) muizen duiden niet op grote morfologische afwijkingen. Echter, in specifieke hersengebieden worden lange en dunne dendritische spines waargenomen, in overeenstemming met een onrijp spine fenotype. Door een gemis van FMRP zijn patiënten met FXS verstandelijk gehandicapt en hebben ze vaak ook gedragsstoornissen. Veel onderzoekers hebben onderzocht wat de precieze functie van dit eiwit is en wat de gevolgen zijn van het missen van dit eiwit op moleculaire, elektrofysiologische en gedragsmatige niveaus. Om onderzoek te doen naar FXS wordt veelal gebruik gemaakt van diermodellen, waaronder het *Fmr1* KO muismodel. Deze muizen hebben net als patiënten met FXS geen Fmrp expressie.

Onderzoek in de afgelopen 20 jaar heeft uiteindelijk geresulteerd in een eerste theorie die het FXS fenotype probeert te verklaren. Deze theorie heet de mGluR (metabotrope glutamaat receptor) theorie, die veronderstelt dat een specifiek mechanisme in signaaloverdracht tussen zenuwcellen is aangetast in patiënten met FXS. De eerste klinische trials, gebaseerd op deze theorie, zijn onlangs gestart waarbij het effect van medicijnen wordt getest op het gedrag en verstandelijke vermogens van patiënten met FXS. Dit proefschrift is onderverdeeld in twee delen: in het eerste deel hebben we de functie van het eiwit in zenuwcellen onderzocht en in het tweede deel hebben we het effect van verschillende medicijnen op gedrag and spine morfologie getest in de *Fmr1* KO muis.

Hoofdstuk 1 is een algemene introductie en beschrijft het *FMR1* gen en de belangrijke functies die FMRP heeft in veel cellulaire processen in neuronen. Verschillende functionele domeinen in het eiwit spelen een belangrijke rol in de functie van FMRP. Met behulp van de "Kern Lokalisatie Signaal" en "Kern Export Signaal" kan FMRP de

kern in en uit worden getransporteerd. Ook is FMRP een RNA-bindend eiwit en heeft het drie RNA-bindende domeinen: twee KH domeinen en één RGG box. Met deze domeinen kan FMRP aan specifieke mRNAs (boodschapper RNA moleculen) binden, waaronder zijn eigen *FMR1* mRNA. De binding van mRNA aan FMRP is afhankelijk van bepaalde sequenties en structuren in het mRNA. Een mRNA kan bijvoorbeeld een Gquartet structuur hebben wat ervoor zorgt dat het mRNA op een bepaalde manier wordt gevouwen en daardoor aan FMRP kan binden. FMRP is vooral gelokaliseerd in het cellichaam, maar het ook in RNA-partikels kan worden geïncorporeerd. Deze RNA-partikels kunnen vervolgens naar de dendriet worden getransporteerd, op weg naar de synapse waar FMRP een functie heeft als translationele repressor. Dit betekent dat FMRP de vertaling van specifieke mRNAs naar eiwit onderdrukt totdat de synapse geactiveerd wordt. In patiënten met FXS is er dus een verminderende repressie van vertaling van specifieke mRNAs naar eiwit en dit verklaart waarom het ontbreken van FMRP leidt tot verhoogde eiwitsynthese. De eiwitten die teveel worden aangemaakt, lijken belangrijk te zijn voor de AMPA receptor internalisatie. Het gebrek aan FMRP leidt daarom tot een verhoogde AMPA receptor internalisatie, met als gevolg dat de dendritische spines langer en dunner worden en de signaaloverdracht tussen de zenuwcellen verstoord is.

In hoofdstuk 2 wordt dieper ingegaan op het mechanisme van leren en geheugen. Verder worden de functie van twee neurotransmitters systemen besproken die betrokken zijn bij het FXS fenotype: de stimulerende glutamaat en de remmende γ-aminoboterzuur (GABA). De veranderingen in deze neurotransmitter-systemen in FXS worden nader uitgelegd samen met de mGluR theorie en GABA hypothese. Deze theorieën zijn de basis voor potentiële therapeutische strategieën, die we in detail bespreken. Tot op heden zijn verschillende medicijnen getest in dierlijke FXS modellen en in klinische studies met patiënten. Het is echter gebleken dat veel klinische studies niet helemaal voldoen aan de wetenschappelijke eisen om vast te kunnen stellen of een medicijn ook daadwerkelijk een effect heeft. Daarom is het van cruciaal belang dat de klinische trials: (i) gerandomiseerd en placebo gecontroleerd zijn, (ii) dubbelblind zijn, (iii) een adequaat aantal patiënten toelaten en (iv) gebruik maken van betrouwbare objectieve testen om de therapeutische werkzaamheid te bepalen.

In hoofdstuk 3 bestuderen we de functionele domeinen van FMRP en hiervoor maken we gebruik van verschillende FMRP varianten met behulp van transfectie studies. We laten zien dat normaal FMRP in dendritische RNA-partikels opgenomen is en dat het in de dendriet co-lokaliseert met zijn eigen *FMR1* mRNA. Een pathogene FMRP variant, genaamd I304N, bevat een mutatie in het tweede KH domein. Deze mutatie is geïdentificeerd in een patiënt met een ernstige vorm van FXS. Wij laten zien dat deze FMRP variant niet in dendritische RNA-partikels geïncorporeerd kan worden, maar nog wel steeds in staat is om aan zijn eigen mRNA te binden. Een andere FMRP variant die we hebben bestudeerd mist het C-terminale deel van het eiwit,

inclusief de "Kern Export Signaal" en de RGG box, een ander RNA-bindend domein van FMRP. Deze variant, FMRP_Iso12, zit gevangen in de kern en kan daarom niet worden opgenomen in dendritische RNA-partikels. Verder is er in de dendriet weinig *FMR1_Iso12* mRNA gelokaliseerd, wat suggereert dat FMRP een rol lijkt te spelen in het transport van het eigen mRNA. De andere variant die we hebben bestudeerd bevat alleen stille puntmutaties die de Gquartet structuur van het *FMR1* mRNA verstoren, zonder de aminozuur code van FMRP te veranderen. Deze Gquartet structuur in het *FMR1* mRNA is belangrijk voor de binding van het *FMR1* mRNA aan FMRP. Wij tonen aan dat de verstoring van de Gquartet structuur niet essentieel is voor de incorporatie van *FMR1_Gquartet* mRNA in dendritische RNA-partikels en dat het *FMR1_Gquartet* mRNA nog steeds naar de dendriet vervoerd kan worden.

In hoofdstuk 4 onderzoeken we het effect van een mGluR5 antagonist, een tegenhanger van het natuurlijk substraat, op het gedrag van *Fmr1* KO muizen en op de spine morfologie van gekweekte hippocampale neuronen. De mGluR theorie suggereert dat het dempen van mGluR5 signaaloverdracht kan leiden tot een verbetering van het FXS fenotype. Om dit te kunnen testen introduceren we een nieuwe techniek waarbij we de prepulse remming van de schrikreactie (PPI reactie) meten. *Fmr1* KO muizen hebben een significant verlaagde PPI reactie in vergelijking met controle muizen. Na behandeling met MPEP (2-methyl-6-(phenylethynyl)-pyridine hydrochoride), een mGluR5 antagonist, is de PPI reactie van *Fmr1* KO muizen niet meer te onderscheiden van controle muizen. Vervolgens hebben we gekweekte hippocampale neuronen onderzocht van de *Fmr1* KO muizen. Zoals eerder is beschreven, vinden we dat gekweekte neuronen van de hippocampus van *Fmr1* KO muizen een verhoogd aantal lange en dunne spines hebben, die we filopodia noemen. Een behandeling van de *Fmr1* KO neuronen met MPEP voor vier uur resulteert in een verandering van deze filopodia naar de normale spine morfologie.

In hoofdstuk 5 beschrijven we het effect van een nieuwe mGluR5 antagonist, AFQ056, op de PPI reactie en op de spine morfologie. Net als bij de behandeling met MPEP, tonen we aan dat AFQ056 de verminderde PPI reactie in *Fmr1* KO muizen kan herstellen en dat het de spine morfologie van gekweekte *Fmr1* KO hippocampale neuronen kan verbeteren.

In hoofdstuk 6 onderzoeken we het effect van behandeling met AFQ056 op spine morfologie in volwassen *Fmr1* KO muizen. Daarvoor hebben we een techniek gebruikt die neuronen opvult met een soort kleurstof (DiOlistische opvulling) om de spines van hippocampale neuronen zichtbaar te maken voor verdere analyse. We laten zien dat alleen de neuronen in de hippocampus CA1 regio van de *Fmr1* KO muizen meer filopodia hebben in vergelijking met controle muizen, terwijl de neuronen in de CA3 regio van de hippocampus in *Fmr1* KO muizen een normale spine morfologie hebben. Vervolgens hebben we het effect onderzocht van AFQ056 behandeling op de spine morfologie in de CA1 regio van de volwassen *Fmr1* KO muizen. Hoewel een

kortdurende behandeling met AFQ056 de verminderde PPI reactie verbetert in *Fmr1* KO muizen, kan een kortdurende behandeling de afwijkende spine morfologie in de CA1 regio van volwassen *Fmr1* KO muizen niet herstellen. Ook een langdurige behandeling van zes weken met AFQ056 heeft geen effect op de afwijkende spine morfologie in de CA1 regio van volwassen *Fmr1* KO muizen.

In het laatste hoofdstuk worden de resultaten die verzameld zijn tijdens mijn promotieonderzoek bediscussieerd, samenhangend met andere onderzoeksresultaten. We laten zien dat verschillende domeinen van FMRP belangrijk zijn voor de incorporatie in RNA-partikels. Daarnaast bediscussiëren we de resultaten van de potentiële behandeling met mGluR5 antagonisten. De resultaten van de behandeling met AFQ056 van volwassen *Fmr1* KO muizen suggereert dat een herstel van de spine morfologie niet essentieel hoeft te zijn om een verbetering te krijgen in specifieke gedragsafwijkingen (hoofdstuk 6). Een andere verklaring voor deze resultaten kan zijn dat de afwijkende spine morfologie in volwassen *Fmr1* KO muizen moeilijker te behandelen is met een mGluR5 antagonist en dus moet de behandeling wellicht eerder worden gestart. Tot slot heeft mijn onderzoek meer inzichten gegeven in de cellulaire functie van FMRP in neuronen en in de moleculaire mechanismen die ten grondslag liggen aan de ontwikkeling van potentiële therapeutische strategieën voor het FXS.

CURRICULUM VITAE

Geertruida Josien Levenga was born in Veendam, the Netherlands on April 19th 1982. After finishing the secondary education at the Ubbo Emmius College, Stadskanaal in 1999, she attended technical college at the Hanzehogeschool Groningen, the Netherlands. One year later in 2000, she started studying Biology at the University of Groningen, the Netherlands. Next to her major in Medical Biology, she took Integrative Neurobiology and Animal Physiology as minors. As a student she took part in research in differentiation of neural stem cells into oligodendrocytes for treatment of multiple sclerosis (Department of Medical Physiology, University of Groningen, Groningen, the Netherlands). During a second internship in Cambridge, England, she investigated the effects of inosine on axonal regeneration in organotypic brain slices (Brain Repair Centre, Cambridge University, England). Furthermore, during her Master's studies, Josien extended her knowledge with minors in "Science in Policy and Management" and "Communication and Presentation".

Upon her graduation in 2005, Josien worked as a PhD student in the department of Clinical Genetics of the ErasmusMC in Rotterdam, under supervision of Prof.dr. B.A. Oostra and dr. R. Willemsen. The results obtained from this PhD research were presented at several national and international conferences and have been published in international, peer-reviewed scientific journals. In January 2011, Josien will continue her scientific career in the group of dr. C. Hoeffer at the Joan and Joel Smilow Research Center, Department of Physiology and Neuroscience, New York, United States. Her work will focus on the relationship between Down's syndrome and Alzheimer's disease.

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PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student:	Geertruida Josien Levenga	PhD period:	January 15th, 2006 – October 20th, 2010
Erasmus MC Department:	Clinical Genetics		
Research School:	Medical Genetics Centre South-West Netherlands	Promotor:	Prof.dr. B.A. Oostra
		Supervisor:	dr. R. Willemsen

1. PhD training	Year	Workload (hours)
General academic skills		
» Biomedical English Writing and Communication	2008	80
» Classical methods for Data-analysis Methodology	2007	160
Specific courses (e.g. Research school, Medical Training)		
» Laboratory animal science	2006	120
» Reading and Discussing Literature in Molecular and Cell Biology	2006	56
» Safe Laboratory Techniques	2006	6
» Master course 'Molecular and Cell Biology'	2007	168
» Confocal Microscopy	2007	15
(Inter)national conferences		
» EndoNeuroPsycho (ENP) meeting, Doorwerth, The Netherlands	2006-2009	24
» Genetic Retreat, Rolduc, The Netherlands	2006-2008	90
» (Award for Best Presentation 2007)		
» Gordon Research Conference. Dendrites; Molecules Structure and Function, Ventura, USA	2007	24
» FRAXA Meeting, Durham, USA	2008, 2010	50
Seminars and workshops		
» MGC PhD workshops: Oxford, Maastricht and Heidelberg	2006-2008	40
» Gordon Research Conference: Dendrites Graduate Seminar, Ventura, USA	2007	16
» Donders Discussion, Nijmegen	2010	10

2. Teaching activities	Year	Workload
Lecturing		
» Developmental Neurobiology, Utrecht	2010	5 hours
Supervising		
» Bachelor thesis: ' Fragile X syndrome and the mTOR signalling pathway'. Zhara Alawi.	2009	4 months
» Bachelor thesis: 'Fragile X syndrome. Influence of the mTOR signal transduction on S6K and FMRP activity'. Marsha den Uijl.	2009	5 months

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Josien