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Involvement of mGluR5/Homer crosstalk disruption in the pathophysiology of Fragile X Syndrome

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PhD Thesis

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ABSTRACT

Fragile X Syndrome (FXS) is the most common inherited form of intellectual disability and autism. FXS is caused by a mutation in the fragile X mental retardation 1 (*Fmr1*) gene which leads to the lack of the encoded FMRP protein. FMRP is an RNA binding protein involved in protein synthesis regulation at synapses. Many evidences suggest a central role of the Group-I metabotropic glutamate receptor subtype 5 (mGluR5) in the FXS pathophysiology. In particular, an exaggerated signaling response following mGluR5 activation may underlie synaptic dysfunction in this disorder. Although much work has focused on the dysregulation of synaptic protein synthesis as a consequence of this enhanced mGluR5 signaling, it becomes clear that in FXS there is also an altered balance of mGluR5 association with Homer scaffolding proteins, which are postsynaptic density (PSD) partners of mGluR5. Although an extensive literature describes the mGluR5/Homer association, very little is known about the consequences of the disruption of this interaction in the FXS context. Therefore, the goal of my thesis was to study the consequences of mGluR5/Homer crosstalk disruption in the *Fmr1* knockout (KO) mouse model of FXS in term of properties and functions of mGluR5, such as expression during development, surface expression and axonal/dendritic targeting, agonist-induced internalization, surface dynamics and mGluR5-mediated modulation of NMDA receptor (NMDAR) currents.

In a first set of experiments we investigated the mGluR5 surface expression in cultured hippocampal neurons from WT and *Fmr1* KO mice by using immunofluorescence techniques and biotinylation assay. We found that mGluR5 was more expressed on the neuronal surface and was differently distributed in dendrites and axons of *Fmr1* KO cultured neurons. We then hypothesized that these alterations were a direct consequence of the mGluR5/Homer crosstalk disruption. We demonstrated that these altered expression and targeting of mGluR5 were critically dependent on mGluR5/Homer crosstalk disruption. We also observed that mGluR5 did not undergo internalization upon sustained mGluR5 activation with DHPG in *Fmr1* KO neurons. This latter phenotype, however, was not dependent on the disruption of the mGluR5/Homer crosstalk. Altogether, these results demonstrate that mGluR5/Homer crosstalk disruption contributes to the pathophysiology of FXS altering expression and targeting of mGluR5 on the surface of *Fmr1* KO neurons.

In the second part of my study we investigated the consequences of the disrupted mGluR5/Homer crosstalk for the mGluR5 surface dynamics, and consequently for

NMDAR function in *Fmr1* KO neurons. Using a combination of live-cell imaging and single-molecule tracking, we found that mGluR5/Homer crosstalk disruption specifically increased the mGluR5 lateral diffusion at the synapse of cultured *Fmr1* KO hippocampal neurons. The higher mGluR5 mobility resulted in an increased probability of transient physical interaction with NMDAR in the PSD of *Fmr1* KO. This interaction altered the mGluR5-mediated modulation of NMDAR currents as evidenced by the two following changes. First, using patch-clamp recordings from CA1 pyramidal neurons, we found that NMDAR-mediated excitatory postsynaptic currents (NMDAR-EPSCs) evoked by *Schaffer* collateral stimulation showed lower amplitudes in *Fmr1* KO neurons. Second, the postsynaptic expression of mGluR5 mediated long term depression (LTD) of NMDAR-EPSCs was reduced in *Fmr1* KO neurons. Finally, we demonstrated that these defects in NMDA currents were strongly dependent on the mGluR5/Homer crosstalk disruption and altered mGluR5 dynamics.

Altogether, our results show that mGluR5/Homer disruption contributes to the mGluR5 dysregulation in *Fmr1* KO neurons. This study might have implication for the treatment of mGluR5 synaptic dysfunctions in FXS by targeting mGluR5/Homer interaction and provide new suggestions to correct the defective signaling underlying cognitive impairment and autism.

RÉSUMÉ

Le Syndrome de l'X Fragile (FXS) est la forme héréditaire majoritaire de déficience intellectuelle et la cause monogénique de l'autisme. Le FXS est causé par une mutation du gène *Fragile X Mental Retardation 1* (*Fmr1*), qui entraîne son inactivation et l'absence d'expression de la protéine codée: Fragile X Mental Retardation Protein (FMRP). FMRP est une protéine de liaison à l'ARN, impliquée dans la régulation de la synthèse protéiques à la synapse. Un rôle central est attribué au sous-type 5 des récepteurs métabotropiques au glutamate du groupe I (mGluR5) dans la physiopathologie du FXS. En effet, une réponse exagérée suite à l'activation de mGluR5 pourrait expliquer le dysfonctionnement synaptique dans ce syndrôme. Bien que de nombreux travaux aient mis l'accent sur la dérégulation de la synthèse des protéines synaptiques comme une conséquence de cette signalisation accrue du mGluR5, il y a aussi un équilibre altéré dans l'association de mGluR5 avec les différentes isoformes des protéines Homer, partenaires de densité post-synaptique (PSD) du mGluR5. Bien qu'une abondante littérature décrit l'association mGluR5/Homer, les conséquences de la perturbation de cette interaction dans le contexte du FXS sont peu connues. Par conséquent, l'objectif de ma thèse était d'étudier les conséquences de la perturbation de l'interaction mGluR5/Homer au niveau des propriétés et des fonctions de mGluR5, telles que l'expression durant le développement, l'expression de surface et le ciblage axonal/dendritique, l'internalisation déclenchée par l'agoniste, les dynamiques de surface, et la modulation des courants NMDAR induite par mGluR5.

Dans un premier temps, nous avons étudié l'expression de surface de mGluR5 dans des neurones hippocampiques *in vitro* issus de souris sauvages et *Fmr1* KO, par des techniques d'immunofluorescence et de biotinylation. Nous avons constaté que mGluR5 est plus exprimé à la surface neuronale et est différemment distribué dans les dendrites et les axones des neurones *Fmr1 KO*. Puis, nous avons démontré que cette altération d'expression et de ciblage est une conséquence directe de l'altération de l'interaction mGluR5/Homer. Nous avons aussi observé que mGluR5, indépendamment de l'altération de l'interaction mGluR5/Homer, ne subit pas d'internalisation suite son activation soutenue par DHPG dans les neurones *Fmr1 KO*.

Dans la seconde partie de mon étude, nous avons étudié les conséquences de la perturbation de l'interaction mGluR5/Homer dans les dynamiques de surface de mGluR5 et par conséquent pour la fonction du NMDAR dans les neurones *Fmr1* KO. Par des techniques d'imagerie et de pistage moléculaire, nous avons constaté que l'altération du

complexe mGluR5/Homer augmente spécifiquement la diffusion latérale à la synapse des neurones hippocampiques *Fmr1 KO in vitro*.

La mobilité élevée du mGluR5 conduit à une probabilité accrue d'une interaction physique transitoire avec NMDAR dans la PSD du *Fmr1 KO*.

Cette interaction altère la modulation, induite par mGluR5, des courants NMDAR. En effet, en utilisant des enregistrements en patch-clamp de neurones pyramidaux de CA1 sur tranches couplés à la stimulation des fibres collatérales de *Schaffer,* nous avons constaté que les courants excitateurs post-synaptiques induits par NMDAR (NMDAR-EPSCs) présentent des amplitudes plus faibles dans les neurones *Fmr1* KO. De plus, l'expression post-synaptique de mGluR5, induite par la dépression à long-terme de NMDAR-EPSCs est réduite dans les neurones *Fmr1* KO. Finalement, nous avons démontré que ces défauts des courants NMDAR sont dépendants de la perturbation de l'interaction mGluR5/Homer et altèrent les dynamiques de mGluR5.

Cette étude pourrait avoir des conséquences dans le traitement des dysfonctionnements synaptiques du mGluR5 dans le FXS, en ciblant l'interaction mGluR5/Homer, et offre de nouvelles suggestions pour corriger la signalisation défectueuse sous-jacente aux troubles du spectre autistique.

LIST OF ABBREVIATIONS

ABC	Aberrant Behavior Checklist
αCaMKII	Calcium/calmodulin-dependent protein Kinase II
ADHD	Attention-Deficit/Hyperactivity Disorder
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor glutamate receptor
Arc	Activity-regulated cytoskeletal protein
ASD	Autism Spectrum Disorders
CA	Cornu ammonis
CS	Conditioned stimulus
CTEP	2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine
DAG	Diacylglycerol
dFrm1	Drosophila Frm1 gene
DG	Dentate gyrus
DHPG	Dihydroxyphenylglycine
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
EC	Entorhinal cortex
EF1α	Elongation factor 1α
EPSC	Excitatory postsynaptic current
EPSP	Excitatory post-synaptic potentials
ERK	Extracellular signal-related Kinase
ERK1	Extracellular signal-related kinase 1
ERK2	Extracellular signal-related kinase 2
EVH	Enabled/vasodilator-stimulated phosphoprotein homology
EVH1	Enabled/vasodilator-stimulated phosphoprotein homology 1
Fmr1	Fragile X mental retardation 1 gene
FMRP	Fragile X mental retardation protein
FXPOI	Fragile X-related primary ovarian insufficiency
FXR1P	Fragile X-related protein 1
FXR2P	Fragile X-related protein 2
FXS	Fragile X Syndrome
FXTAS	Fragile-X-associated tremor/ataxia Syndrome
GABA	γ-aminobutyric acid
$GABA_AR$	GABA receptor type A
$GABA_BR$	GABA receptor type B
GABAR	γ-aminobutyric acid receptor
GluR	Glutamate receptors
GluR1	AMPAR subunit 1
GluR2	AMPAR subunit 2
ID	Intellectual disability

iGluRs	Ionotropic glutamate receptors
IP3	Inositol triphosphate
IP3R	Inositol triphosphate receptor
IPSP	Inhibitory post-synaptic potentials
IQ	Intelligence quotient
KAR	Kainate receptor
KO	Knockout
LTD	Long term depression
LTP	Long term potentiation
MAP1B	Microtubule-associated protein 1B
MAPK	Microtubuleassociated protein kinase
mGluR	Metabotropic glutamate receptor
mGluR1	Metabotropic glutamate receptor subtype 1
mGluR5	Metabotropic glutamate receptor subtype 5
miRNA	microRNA
MPEP	3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride
MTEP	3-((2-Methyl-4-thiazolyl)ethynyl)pyridine
mTOR	Mammalian target of rapamycin
NAM	Negative allosteric modulator
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NMDAR-EPSC	Excitatory postsynaptic current of NMDA receptor
PAM	Positive allosteric modulator
PI3K	Phoshpinositide-3 kinase
PIP2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PPI	Prepulse inhibition
PSD	Postsynaptic density
PSD-95	Postsynaptic density protein of 95 kDa size
SVN	Single-nucleotide variants
WT	Wild type

CHAPTER I

INTRODUCTION

Martin–Bell Syndrome, an X-linked intellectual disability, was first described in 1943 by James Purdon Martin and Julia Bell for several male members of the same family (J. P. Martin & Bell, 1943). Years later, in 1969, Herbert Lubs discovered the existence of a break on the X chromosome of affected males (Lubs, 1969), which was termed "fragile site" by Frederick Hecht in 1979 (Hecht & Kaiser-McCaw, 1979). This led to the name change from Martin–Bell Syndrome to Fragile X Syndrome (FXS). It was only in 1991 that the gene responsible for FXS was identified on the X chromosome at position q27.3, and named fragile X mental retardation 1 gene (*Fmr1*)(Verkerk et al., 1991).

In FXS, the *Fmr1* gene is silenced, and consequently its gene product, the fragile X mental retardation protein (FMRP), is strongly reduced in its expression or entirely absent. Meanwhile, FMRP has been shown to play a key role in regulating synaptic function and plasticity (Pfeiffer & Huber, 2009). FXS belongs to a larger group of psychiatric, neurological and childhood developmental disorders called "synaptopathies". It implies that disruptions in synaptic structure and function are potentially the major determinant of such brain diseases (Brose, O'Connor, & Skehel, 2010; Grant, 2012). The study of FXS has greatly enriched our understanding of the role of FMRP in physiology and pathophysiology of synaptic transmission. This knowledge holds the key to developing new therapies.

The monogenic nature of FXS makes it ideal for unraveling the underlying pathology mechanisms, and thus of intellectual disability disorders in general. The recent surge in interest for developing FXS therapies stems from significant progress in basic research initiatives aimed at unveiling the cellular and synaptic mechanisms of the disease. Several breakthrough discoveries have lead to the identification of a therapeutic target for FXS. The identification of the affected gene (Verkerk et al., 1991), the development of a mouse model (The Dutch-Belgian Fragile X Consortium, 1994), and the identification of the metabotropic glutamate receptor 5 (mGluR5)-dependent plasticity phenotype (Huber, Gallagher, Warren, & Bear, 2002), lead to the proposal of the "mGluR theory" (Bear, Huber, & Warren, 2004), validated by genetic rescue of FXS by mGluR5 knockdown (Dölen et al., 2007) as well as by pharmacologic blockade of mGluR5 (de Vrij et al., 2008; McBride et al., 2005; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005). So far, however, clinical trials employing novel mGluR5 antagonists for the treatment of FXS have failed to show improvement compared to placebo and were cancelled. An improved understanding of the cellular and subcellular nature of

mGluR5 dysfunction in FXS is thus needed for the identification of new therapeuthic targets.

1. Fragile X Syndrome

Fragile X Syndrome (FXS) is the most common inherited form of intellectual disability (ID) disorder, the second (after trisomy 21), and the best characterized cause for autism. FXS affects 1/4000 males and 1/7000 females and patients display a range of cognitive and behavioural deficits to varying degrees (Bassell & Warren, 2008; Garber et al., 2008). FXS results from transcriptional silencing of *Fmr1* gene and loss of the encoded protein, FMRP (fragile X mental retardation protein). In FXS patients, there is a large decrease or complete silencing of the expression of FMRP, indicating that its loss of function is responsible for the syndrome. FMRP is an mRNA-binding protein that functions as a translational regulator of target mRNAs in dendrites and in dendritic spines (A. E. Ashley & Sherman, 1995; Khandjian, Corbin, Woerly, & Rousseau, 1996; Laggerbauer, Ostareck, Keidel, Ostareck-Lederer, & Fischer, 2001). FMRP targets a large variety of mRNA molecules, with up to as many as 800 mRNAs as binding partners (V. Brown et al., 2001). This equals ~ 4% percent of all mRNA transcripts that occur in the mammalian brain (Bassell & Warren, 2008). A recent study identified that hundreds of targets of FMRP are mRNAs encoding part of the postsynaptic (>30% of FMRP targets) and presynaptic proteome (13% of FMRP targets) (Darnell et al., 2011). This large number of interactions explains why a single-gene deficit leads to such a complex sequence of events and makes it difficult to assess the full extent of the consequences of this cognitive disorder for synaptic functions.

1.1. Cause of the Fragile X Syndrome

FXS is caused by an expansion of the number of CGG repeats in the 5' untranslated region of the *Fmr1* gene. In the normal population, the CGG repeat is polymorphic and ranges from 5 to 55 CGGs with an average length of 30 CGG units (Fu et al., 1991). In Fragile X patients, however, the CGG repeat is found to be expanded beyond 200 repeats known as the full mutation, that are usually hypermethylated and the cytisine methylation extends to the adjacent promoter region of the *Fmr1* gene (Oberlé et al., 1991; Sutcliffe et al., 1992; Verkerk et al., 1991). As a consequence the gene is transcriptionally silenced and FMRP is absent (Verheij et al., 1993).

Unmethylated expansions of from 55 to 200 CGG units, called premutations, are unstable in meiosis and are found in both males and females and may expand to a full mutation only upon maternal transmission to the next generation. People with a premutation of the *Fmr1* gene do not have FXS but may have Fragile X-associated disorders. In these people, the *Fmr1* gene is in its normally unmethylated state and usually makes some FMRP. However in these conditions the *Fmr1* gene does not function normally. Premutations of the *Fmr1* gene may cause Fragile X-associated tremor/ataxia Syndrome (FXTAS) and Fragile X-associated primary ovarian insufficiency (FXPOI). FXTAS is a condition that causes balance, tremor and memory problems in some older male (and less commonly, female) carriers of the premutation. FXPOI is characterized by decreased ovarian function, which can lead to infertility and early menopause in some female carriers of the premutation.

FXS in an "X-linked" condition, which means that the gene is on the X chromosome. Since a women has two X chromosomes, a female carrier with a premutation or full mutation has a 50% chance of passing on the X with the mutation in each pregnancy, and a 50% chance of passing on her normal X. If she has a premutation, and it is passed on (to either males or females), it can remain a premutation or it can expand to a full mutation. If she has a full mutation and it is passed on (to either males or females), it will remain a full mutation. Because males have only one X chromosome, fathers who carry the premutation will pass it on to all their daughters and none of their sons (they pass their Y chromosome on to their sons). There have been no reports of premutations that are passed from a father to his daughter expanding to a full mutation. This appears to only occur when passed from a mother to her children.

FXS shows anticipation, which refers to the number of the trinucleotide repeats increasing from one generation to the next, meaning the risk of FXS increases in successive generations. These stages start with the normal gene and then proceed to the premutation and then the full mutation. A recent study has estimated that 1/151 females and 1/468 males are carrier of a FXS premutation (Seltzer et al., 2012).

1.2. Fragile X phenotype

FXS is characterized by moderate to severe ID in affected males, with intelligence quotient (IQ) typically in the range of 40 - 70 (Merenstein et al., 1996). On the other hand 60% of carrier females present with mild to moderate ID (IQ ~80). Female individuals typically are less affected than male individuals because their normal

X chromosome yields some FMRP. Only ~25% of female individuals with the full mutation have an IQ below 70, although the majority of female patients present themselves with learning and/or behavioral problems (R. J. Hagerman & Hagerman, 2001) (Table 1).

Typical features are not evident at birth in most males born with full mutation of FXS and developmental delays become only evident during early childhood years (around three years of age) (J. E. Roberts et al., 2009). Moreover, most children with FXS do not have any specific physical features of this syndrome until they reach puberty. These features include macrocephalus, long narrow face, prominent ears, soft skin, mild connective tissue dysplasia (hyperflexible joints), hypotonia, flat feet, prominent forehead, and also post-pubertal macroorchidism (Lachiewicz, Dawson, & Spiridigliozzi, 2000). In general, Fragile X patients typically have a normal life expectancy (Turk, 2011).

The behavioral phenotypes of FXS include poor eye contact, shyness, social anxiety, hand flapping, aggression, impulsivity, hyperarousal to sensory stimuli, seizures, features of Attention-Deficit/Hyperactivity Disorder (ADHD) combined with language and other learning deficits, and impaired fne and gross motor skills (reviewed by (Tranfaglia, 2011)). FXS patiens often display autistic-like features, and this leads to a primary diagnosis of FXS as Autism Spectrum Disorders (ASD) (around one year of age). According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) the core features of autism are impaired social interaction, impaired social communication and the presence of perseverative or repetitive behaviors or restrictive interests (www.dms5.org). A recent study using the Autism Diagnostic Observation Schedule (a protocol for diagnosing and assessing autism) found that 35.1% of young males with FXS scored in the autistic range (Hall et al., 2010). Thus, FXS is currently considered to be the most common monogenetic cause of autism (Muhle et al., 2004; Schaefer and Mendelsohn, 2008) (Table 1).

A characteristic feature of FXS is an abnormal increase in brain weight and head circumference when compared with the control group over the course of the first two years of life (McCary, Machlin, & Roberts, 2013). Quantitative magnetic resonance imaging (MRI) studies show anatomical alterations in several brain regions that are thought to be neuroanatomical substrates for the processing of cognitive, emotional and behavioural responses such as the cerebellar vermis, prefrontal gyri, hippocampus, amygdala and caudate nucleus of basal ganglia. Specifically, Reiss and his colleagues

have reported the increase of lateral ventricular volume in Fragile X patients (Reiss, Lee, & Freund, 1994). In both male and female patients, the posterior vermis of the cerebellum was decreased (Reiss et al., 1994) and the caudate nucleus was increased (Reiss et al., 1994). Reiss also found the hippocampus to be enlarged in Fragile X children and young adults (Reiss et al., 1994) but Jäkälä et al. (1997) did not reproduce this finding in adults (Jäkälä et al., 1997). Moreover, the volume of caudate and amigdala was lower in Fragile X patients (Reiss et al., 1994). Since the limbic system is involved in the reinforcement of new behaviors and appropriate behavior selection (Hessl, Rivera, & Reiss, 2004), the inability to properly establish and reorganize the underlying neuronal circuits in response to experience may lead to the common behavioral abnormalities seen in FXS.

Changes in gross brain architecture are mirrored by alterations in synaptic structure. The first study of post-mortem tissue was performed by Rudelli et al. (1985) on a 62-year-old male with FXS (Rudelli et al., 1985). Using the Golgi method, these authors found that dendritic spines on parieto-occipital cortex were long and tortuous with prominent terminal heads and compared to those of unaffected individuals. Long and thin spines are reminiscent of immature dendritic spine structure found during early development (Ethell & Pasquale, 2005; Papa, Bundman, Greenberger, & Segal, 1995; Ziv & Smith, 1996), and could reflect errors in synapse maturation, stabilization or elimination. Subsequent autopsy studies of Fragile X patients reported similar alterations in spine structure in temporal and visual cortex as well as increases in the density of these immature spines (Hinton, Brown, Wisniewski, & Rudelli, 1991; Irwin et al., 2001; Wisniewski, Segan, Miezejeski, Sersen, & Rudelli, 1991) (Table 1).

These studies have identified an anatomical hallmark of FXS, that, when combined with the core behavioural and cognitive phenotypes provide cellular and behavioural fingerprints on which modelling FXS experimentally has flourished.

In males with a premutation (50-200 repeats), there is an increased probability of the development of the FXTAS (R. J. Hagerman & Hagerman, 2001). FXTAS associated symptoms emerge at a later age (around 50 years of age), and the syndrome has a progressive course. The clinical picture comprises autonomic dysfunction, such as hypertension and impotence, psychiatric features including agitation, dysinhibition and anxiety, neuropathy in the lower extremities, intention tremor, frequent falling, Parkinsonian symptoms, and cognitive deficits including executive function and memory deficits (Cornish et al., 2008). Moreover, about 20% of women who are carriers for the

FXS premutation are affected by FXPOI, which is defined as menopause before the age of 40 (Cronister et al., 1991). The number of CGG repeats correlates with penetrance and age of onset (Tassone et al., 2007). However, it is interesting to note that premature menopause is more common in premutation carriers than in women with the full mutation, and for premutations with more than 100 repeats the risk of FXPOI begins to decrease (Sullivan et al., 2005; Wittenberger et al., 2007).

1.3. Fragile X mental retardation protein

FMRP is widely expressed in all mammalian tissues, with the highest expression levels in the brain and testes (Devys, Lutz, Rouyer, Bellocq, & Mandel, 1993). It is highly expressed in neurons throughout the brain, and is also expressed in astrocytes of the mouse brain (Feng, Gutekunst, et al., 1997b; Pacey & Doering, 2007). FMRP is mainly cytoplasmic, although it possesses nuclear localization and export signals that enable its transfer between the cytoplasm and the nucleus, presumably to bind its mRNA targets (Eberhart, Malter, Feng, & Warren, 1996).

Sequence analysis shows that FMRP is a selective RNA-binding protein that contains an arginine-glycine-glycine (RGG) box and two K homology domains for binding RNA (KH1 and KH2) (H. Siomi, Siomi, Nussbaum, & Dreyfuss, 1993). In fact, FMRP binds up to 4% of the mRNAs in the brain (C. T. Ashley, Wilkinson, Reines, & Warren, 1993) and regulates its transport, stability and translation (reviewed by (Maurin, Zongaro, & Bardoni, 2014)). Evidence for a functional role for FMRP in translation comes from findings that it co-sediments with ribonucleoprotein (RNP) particles and translating polyribosomes (Corbin et al., 1997; Eberhart et al., 1996; Feng, Absher, et al., 1997a). Several *in vitro* and *in vivo* analyses suggest that FMRP acts as a translational repressor. Further evidence that FMRP functions as a translational repressor comes from studies showing that FMRP associates with several mRNA transcripts whose translation is affected in FXS models, such as mRNAs encoding microtubule-associated protein 1B (MAP1B), calcium/calmodulin-dependent protein kinase II (αCamKII), postsynaptic density protein of 95 kDa size (PSD-95) and elongation factor 1α (EF1α) (Hou et al., 2006; K. Xu et al., 2004; Zalfa et al., 2003). However, other studies suggest that FMRP promotes translational initiation and/or translational elongation of target mRNAs, such as Trailer- Hitch and Sod1 transcripts (Bechara et al., 2009; Monzo et al., 2006). In addition, recent studies suggest that FMRP might also regulate transcript stability, such as that of microRNA-124a (miRNA-124a) and PSD-95 (X.-L. Xu, Li, Wang, & Gao,

2008; Zalfa et al., 2007). Thus, the translation and expression of FMRP targets can be either positively or negatively affected by FMRP expression, which indicates that the potential role of FMRP as a translational regulator is much more complex than was originally believed.

To complicate further the theory that FMRP acts at the level of translation, biochemical purification of FMRP-bound complexes suggests that FMRP interacts with multiple pathways that also regulate gene expression. For example, FMRP has been found to interact with components of the RNA interference (RNAi) pathway (Bardoni et al., 2003), a gene-silencing mechanism triggered by the presence of double-stranded RNA (dsRNA). Moreover, FMRP associates with miRNAs and components of the miRNA pathway (Caudy, Myers, Hannon, & Hammond, 2002; Edbauer et al., 2010; Jin et al., 2004). The FMRP associated miRNAs have been shown to regulate dendritic branching, which is rescued by genetically reducing FMRP expression and function (Edbauer et al., 2010). However, although these studies support the theory that FMRP acts as a regulator of gene expression, the molecular mechanisms remain to be elucidated.

FMRP is also involved in a number of protein-protein interactions, which may act to modify its affinity for certain target mRNAs and/or its function. Many of these protein binding partners are also RNA-binding proteins or cytoskeleton-associated proteins (Bardoni, Davidovic, Bensaid, & Khandjian, 2006). Specifically, NUFIP1 (nuclear FMRP interacting protein 1) and 82-FIP (82 kDa FMRP-interacting protein) are all RNA-binding proteins which interact with FMRP through its N-terminal domain (Bardoni et al., 2006). Cytoplasmic FMRP interacting protein-1 (CYFIP1) and CYFIP2, which also interact with FMRP via its N-terminus, may act to link FMRP to the Rho GTPase signaling pathway and actin cytoskeleton remodeling (Schenck, Bardoni, Moro, Bagni, & Mandel, 2001). Finally, FMRP's C-terminal domain is involved in interactions with microspheruleprotein 58 (MSP- 58), KifC3, Ran, BPM, and SMN (survival of motor neuron) (Bardoni et al., 2006; Davidovic et al., 2007; Menon, Gibson, & Pastore, 2004; Piazzon et al., 2008).

Along with FMRP there are two human homologous, Fragile X-Related Protein 1 (FXR1P) and Fragile X-related protein 2 (FXR2P). There is a high sequence similarity between FMRP and FXR1P and FXR2P especially in their functional domains and overlap in tissue distribution (Bontekoe et al., 2002; Mientjes et al., 2004; Tamanini et al., 1999). *In vitro* and *in vivo* evidence indicates that these proteins can form homo- and

heteromers with each other, suggesting potential interdependency among these proteins for their function (Tamanini et al., 1999). Despite this, FXR1P and FXR2P do not seem to be able to compensate for the lack of FMRP in FXS, suggesting that these proteins may have different functions (Coffee, Tessier, Woodruff, & Broadie, 2010).

1.3.1. FMRP and synaptic functions

Dendritic spines are basic units of neuronal information processing. Spines are sensitive to their environment and change density and morphology to a number of stimuli (Yuste & Bonhoeffer, 2001), and spine abnormalities have long been associated with mental retardation of unknown etiology (Purpura, 1974), as well as with Down's and Rett Syndromes (Kaufmann & Moser, 2000). The cognitive deficits that are characteristic of FXS correlate with the abnormal spine morphology seen in FXS patients and *Frm1* knock-out (KO) mouse (the animal model of the disease). The spine abnormatilities of FXS might be linked to the altered regulation of translation at the synapse, especially given that FMRP regulates the translation of proteins important for synaptic function and plasticity (Bagni & Greenough, 2005). Synaptic plasticity - a long-term change in synaptic strength after stimulation - is considered to be the mechanism of information storage in learning and memory (Steward & Schuman, 2001). As spines are thought to play a pivotal role in synaptic plasticity, the role of FMRP at the synapse has been a central question.

Accumulating evidence suggesting that FMRP regulates protein synthesis locally at synapses and in response to glutamate provides answers to this question. Dendrites and their associated synapses contain the necessary machinery to synthesize proteins. This dendritic protein synthesis is required for activity dependent long-term synaptic plasticity (Sutton & Schuman, 2005). A number of studies implicate FMRP in the translational regulation of dendritic mRNAs by activity and, more specifically, in response to the activation of Group-I mGluRs, mGluR1 and mGluR5 (Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004). *Fmr1* mRNA itself is expressed in dendrites and is bound by FMRP (Brown et al., 2001). In addition to the RNA for FMRP, other dendritically localized mRNAs, such as those of microtubule-associated protein 1b MAP1B, postsynaptic density protein of PSD-95, and EF1α, are FMRP targets (reviewed by (Bagni & Oostra, 2013)). Moreover, all of these RNAs are translated in response to mGluRs activation (Hou et al., 2006; Todd, Mack, & Malter, 2003; Zalfa et al., 2007).

These findings suggest that FMRP and mGluRs regulate the translation at synapses in a functionally opposing manner. mGluR activation initiates protein synthesis and FMRP suppresses it acting as a negative feedback regulator. In the absence of FMRP mGluR-dependent protein synthesis proceeds unbalanced, leading to a excess in protein synthesis-dependent plasticity (Dölen & Bear, 2008). Exaggerated protein synthesis is believed to be pathogenic in FXS and possibly in other disorders associated with autism (Kelleher & Bear, 2008) and the question of how synaptic activity can trigger FMRP regulation of mRNA translation is of particular interest.

Whilst there is a wealth of studies addressing the putative roles of FMRP at the postsynaptic site, recent studies have highlighted a presynaptic role for FMRP during neuronal development. FMRP localises to discrete granules (Fragile X Granules, FXGs) in neuronal axons and their terminals in a range of brain areas including frontal cortex, hippocampus, cerebellum and olfactory bulb glomeruli (Christie, Akins, Schwob, & Fallon, 2009). Overall, these findings suggest that presynaptic functional deficits associated with loss of FMRP might play an important role in the pathophysiologi of FXS.

1.4. Animal models of Fragile X Syndrome

The knowledge about behavioral, cellular and molecular mechanisms that underlie FXS has been greatly advanced by the generation and analysis of animal models. The first FXS animal model developed was the *Frm1* KO mouse (The Dutch-Belgian Fragile X Consortium, 1994), followed by Drosophila FXS models with a deleted or mutated d*Frm1* gene (Morales et al., 2002), and zebrafish FXS models. *Frm1* expression was knocked down with antisense morpholinos or the *Frm1* gene was deleted by genetic KO (Broeder et al., 2009; Tucker, Richards, & Lardelli, 2006). These transgenic or KO mice are distinct from the human patient in that they do not carry the trinucleotide expansion, but they are nonnetheless characterized by the absence of FMRP. These mouse models have helped tremendously to discover the molecular mechanisms underlying the FXS pathophysiology.

They recapitulate several symptoms observed in human patients with defects in neuronal development, dendritic spine morphology, synaptic plasticity, and behavior. FXS animal models have been proven helpful to identify, develop, and test potential therapeutic strategies to treat FXS.

1.4.1. Fragile X mouse model

The identification of *Fmr1* gene led to the development of FXS animal models such as the *Fmr1* KO mouse. The *Fmr1* mouse gene is 95% homologous to its human counterpart, and FMRP has similar patterns of expression in terms of developmental time course and tissue specificity in both species (O'Donnell & Warren, 2002; Oostra & Hoogeveen, 1997).

The first generation of *Fmr1* KO mice was generated by interrupting exon 5 with a neomycin cassette (Kooy, 2003). As mentioned above the model is not identical to the human model, because the *Fmr1* gene is not silenced by hypermethylation of an expanded CGG repeat. Although *Fmr1* KO mice do not express FMRP, the *Fmr1* promoter in those mice is intact and residual *Fmr1* transcription was found in these mice. To create a KO model completely deficient in *Fmr1* transcription, a second generation *Fmr1* KO mouse model was created lacking the first exon that includes the promoter region (Mientjes et al., 2006). This strategy resulted in a complete loss of *Fmr1* mRNA transcription and therefore total lack of FMRP expression.

These *Fmr1* KO mouse models have been extensively studied and were demonstrated to be a useful animal model to study FXS (Table 1). The *Fmr1* KO mice show increased testicular weight similar to male FXS patient's (Kooy et al., 1996; Mientjes et al., 2006). With respect to behavioral and cognitive phenotypes, *Fmr1* KO mice demonstrate subtly impaired cognitive function and aberrant behavior. Use of the Morris water-maze task to study spatial learning revealed *Fmr1* KO mice exhibit subtle spatial-learning phenotypes that depend on their genetic background (Kooy et al., 1996; Mientjes et al., 2006). More robust cognitive deficits have been identified in studies of extinction of memory that include inhibitory avoidance paradigms, trace fear conditioning and leverpress escape/avoidance tasks (Brennan, Albeck, & Paylor, 2006; Dölen et al., 2007; Eadie et al., 2009; Hayashi et al., 2007; Zhao et al., 2005) (Table 1).

One of the most robust and reproducible phenotypes observed in the mouse FXS model is its susceptibility to age-dependent audiogenic seizures, which is consistent with the symptoms of human patients (Dölen et al., 2007; Musumeci et al., 2000; 2007) (Table 1). Clinical and behavioral studies in patients have demonstrated that sensory hypersensitivity is a predominant feature of FXS. *Fmr1* KO mice display also altered sensory system development that may underlie the altered sensory hypersensitivities (Dölen et al., 2007; J. R. Gibson, Bartley, Hays, & Huber, 2008; Harlow et al., 2010; Till et al., 2012). *Fmr1* KO mice show a defect of prepulse inhibition of acoustic startle

response (PPI) and increased locomotor activity (Bakker & Oostra, 2003; de Vrij et al., 2008; Kooy, 2003). In a very recent study Zhang et al., (2014) described a detailed cellular mechanism that correlates neocortical hyperexcitability to tactile somatosensorial stimuli in *Fmr1* KO mice (Zhang et al., 2014). These findings suggest that *Fmr1* KO mouse is a suitable model for studying sensory hypersensitivity in FXS (Table 1).

Anxiety is also a main symptom of FXS patients, but this phenotype is controversial in *Fmr1* KO mice. Some studies suggest that *Fmr1* KO mice are less anxious in the open field test and in the elevated plus maze (Bilousova et al., 2009; Hébert et al., 2014; Kooy et al., 1996; Restivo et al., 2005), while others suggest that they are more anxious in a social context (Mineur, Sluyter, de Wit, Oostra, & Crusio, 2002). *Fmr1* KO mice have been found to exhibit a longer latency to enter a dark box (Michalon et al., 2012), an impairment in the acquisition of a visuospatial discrimination task (Krueger, Osterweil, Chen, Tye, & Bear, 2011) and a reduced freezing behavior to training context and sound (Guo et al., 2011) (Table 1) .

*Fmr*1 KO mice display autistc-like phenotypes. Repetitive behavior is one of the core features for autism as defined by the DSM-5 (www.dsm5.org). This phenotype was also found in *Fmr1* KO mice, either as an increased marble burying (Spencer et al., 2011) or as an increased grooming behavior (Pietropaolo, Guilleminot, Martin, D'Amato, & Crusio, 2011). Moreover, different social behavior phenotypes have been observed ranging from increased social preference in *Fmr1* KO (Gantois et al., 2013; Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005) to no difference (C. H. McNaughton et al., 2008), to decreased social interest (Mineur et al., 2002). These differences are most likely due to the protocols used or differences in the strains used, but at some extend they do fit the heterogeneity found in social behavior of FXS patients (Table 1).

Fmr1 KO mice exhibit abnormal spine morphology and altered spine density in different brain areas similar to what is found in FXS patients. Although it is recognized that there is some alteration in dendritic spine morphology in FXS and *Fmr1* KO mice, the literature is not in agreement on the specific alterations. Some studies found a higher dendritic spine density in cortex or hippocampus of *Fmr1* KO adult mice (Galvez & Greenough, 2005; McKinney, Grossman, Elisseou, & Greenough, 2005), or a decreased one (Braun and Segal, 2000), while others could not confirm that phenotype (Grossman, Elisseou, McKinney, & Greenough, 2006; Irwin et al., 2001). But most of the studies

report an immature dendritic spine phenotype either in the cortex or the hippocampus of *Fmr1* KO mice, compared with the wild-type (WT) littermate controls (Bilousova et al., 2009; Cruz-Martín, Crespo, & Portera-Cailliau, 2010; Galvez & Greenough, 2005; Grossman et al., 2006; Irwin et al., 2002; Levenga et al., 2011; McKinney et al., 2005; Nimchinsky, Oberlander, & Svoboda, 2001; Pop et al., 2014). A recent study examined the spine morphology at the nanoscale by using stimulated emission depletion microscopy (STED) (Wijetunge, Angibaud, Frick, Kind, & Nägerl, 2014). This super-resolution imaging approaches have shown that the developmental trajectory of spine morphogenesis was largely intact in *Fmr1* KO neurons, with only subtle differences that are dependent on age and brain region. Moreover, accordingly to previous studies (Cruz-Martín et al., 2010; Nimchinsky et al., 2001), no differences have been observed in *Fmr1* KO mice during the second and third postnatal weeks. All together these findings suggest that a direct comparison between studies is difficult due to differences in methodology, age (Galvez and Greenough, 2005), brain region (Comery et al., 1997), and statistical analyses (Nimchinsky et al., 2001). Nonetheless, effects of FMRP loss on spine morphology cannot be generalized between ages, brain regions or cell types (Table 1).

In the *Frm1* KO mouse, Till et al. found a delay in somatosensory map formation and alterations in the morphology profile of dendrites and spines of layer 4 neurons. They also found a decrease in the synaptic levels of proteins involved in mGluR signaling at times corresponding to the highest levels of FMRP expression. These results suggest that inaccurate timing of developmental processes caused by the loss of FMRP may lead to alterations in neural circuitry that underlie behavioral and cognitive dysfunctions associated with FXS (Till et al., 2012).

This animal model has given the opportunity to study defects in synaptic plasticity in various brain regions. Huber et al. showed that a form of long-term depression (LTD) dependent on mGluR5 is altered in the CA1 reagion of the hippocampus of *Fmr1* KO mouse (Huber et al. 2002). This form of LTD is normally protein synthesis-dependent, but in the case of *Fmr1* KO mice it occurs independently since the costitutive level of protein synthesis is occluded (Nosyreva & Huber, 2006; Ronesi & Huber, 2008). On the other hand, long-term potentiation (LTP) in the same region was not affected. Other studies, however, have shown deficits in LTP in the cortex and the lateral amygdala (Huber et al., 2002; Larson, Jessen, Kim, Fine, & Hoffmann, 2005; Volk, Pfeiffer, Gibson, & Huber, 2007; B. M. Wilson & Cox, 2007;

Zhao et al., 2005). These alterations in synaptic plasticity, which are widely accepted to underlie experience-dependent modification of brain function, support the idea that learning and memory deficits might be the result of aberrant synaptic plasticity.

	Fragile X patients		*Fmr1*KO mouse	
	Phenotypes	**References**	**Phenotypes**	**References**
Cognitive and behavioral features	Intellectual disability (IQ <70); Language deficits; Working and short-term memory problems; Deficits in executive function; Mathematical and visuospatial abilities;	Wright-Talamante et al., 1996; Hagerman, 2002; Moore et al., 2004; Koldewyn et al., 2008;	Mild learning and memory deficits (e.g. in spatial memory in Morris water maze); Impaired eyelid conditioning (motor skill learning); Decreased initial performance on Rotarod (motor skill learning); Object-recognition memory impairment;	Bakker et al., 1994; Paradee et al., 1999; Zhao et al., 2005; Brennan et al., 2006; Dolen et al., 2007; Hayashi et al., 2007; Eadie et al., 2009;
	Autistic features; Hand flapping; Biting; Irritability; Social deficits; Attention deficits;	Hagerman, 2002; Farzin et al., 2006; Clifford et al., 2007; Hessl et al., 2008;	Repetitive, perseverative digging; Impaired social behaviour; Increased marble burying (repetitive behavior);	Bakker et al., 1994;Spencer et al., 2011; Pietropaolo et al., 2011;
	Hyperactivity	Hagerman, 2002; Farzin et al., 2006;	Increased locomotor activity; Increased open-field activity; Increased center field behavior; Hyper-locomotion and anxiety-like behavior;	Bakker et al., 1994;
	Anxiety and sleep problems	Hagerman, 2002;	Decreased non-social anxiety and increased social anxiety; Anxiety-like behavior in the elevated plus-maze;	Bakker et al., 1994; Restivo et al., 2005; Hebert et al., 2014;
	Epileptic seizures	Musumeci et al. 1999; Sabaratnam et al., 2001; Berry-Kravis et al., 2010;	Increased susceptibility to audiogenic seizures; Prolonged epileptiform discharges in hippocampus; Increased persistent activity states in neocortex;	Musumeci et al., 2000, 2007; Kooy, 2003; Bakker &Oostra, 2003; Dolen et al., 2007; De Vrij et al., 2008;
	Deficit in sensorimotor gating	Cohen, 1995; Miller et al., 1999; Frankland et al., 2004; Hessl et al., 2008;	Altered sensorimotor gating (acoustic startle response and prepulse inhibition); Delayed myelination in the cerebellum;	Frankland et al., 2004; Chen & Toth, 2001; Nielsen et al., 2002; Pacey et al., 2013; Zhang et al., 2014;
Neuro-anatomical features	Anatomical alterations in several brain regions; Increased density and immature morphology of dendritic spines	Reiss et al., 1991, 1994, 1995; Hinton et al., 1991; Wisniewski et al., 1991; Kates et al. 1997; Mostofsky et al., 1998; Irwin et al., 1999; Eliez et al., 2001;	Age- and brain-region-dependent spine abnormalities; Altered spine morphology; Altered brain perfusion; Reorganization of GABAergic interneurons in neocortex;	Comery et al., 1997; Nimchinsky et al., 2001; Irwin et al., 2002; McKinney et al., 2005; Grossman et al., 2006; Pop et al., 2014;

Table 1. Comparison of the most prominent features of Fragile X patients and *Fmr1* KO mouse.

2. Information processing in the brain

The human brain is an extremely complex organ that functions as the information-processing unit of the central nervous system. The major building blocks of the brain are neurons and glial cells. Neurons are specialized for the transmission of electrical signals and possess a soma, multiple dendrites, and an axonal arbor. In contrast, glial cell are not capable of electrical signaling. Neurons make contact with each other and communicate via chemical and electrical synapses. At electrical synapses or gap junctions, the cytoplasm of the connecting cells is continuous via channels, allowing direct and fast electrical or chemical signaling (Bennett & Zukin, 2004). At chemical synapses, on the other hand there is no cytoplasmic continuity since pre- and postsynaptic compartments are separated by a synaptic cleft. Chemical synapses are the basis for the major mode of information transfer between neurons. Through these synapses, neurons form networks in the brain to relay information in the form of electro-chemical signals, which control all conscious and unconscious behavior.

2.1. Chemical synaptic transmission

Chemical synaptic transmission is initiated by the invasion of an action potential in the presynaptic bouton, leading to opening of voltage gated Ca^{2+} channels and influx of Ca^{2+} ions into the presynaptic terminal. This transient elevation of Ca^{2+} ion concentration in the presynaptic terminal facilitates binding of Ca^{2+} to specific Ca^{2+} sensors, leading to the fusion of neurotransmitter-containing synaptic vesicles with the plasma membrane (Sudhof, 2004). The neurotransmitter released from the pre-synaptic site diffuses through the synaptic cleft to bind neurotransmitter receptors present at the postsynaptic membrane. Specialized receptors for each type of neurotransmitter used at a synapse, as well as additional ion channels, reside in the postsynaptic membrane. These receptors are embedded in a scaffold that anchors them at appropriate locations (Iasevoli, Tomasetti, & de Bartolomeis, 2013). In the postsynaptic side, neurotransmitters induce excitatory or inhibitory postsynaptic potentials (EPSPs or IPSPs, respectively). Therefore, neurotransmitter are classified into two main groups: those that lead to EPSPs are termed as excitatory NT, and those that lead to IPSPs are termed as inhibitory neurotransmitter. In the brain, the most common excitatory neurotransmitter is glutamate, and the most important inhibitory neurotransmitter is γ–aminobutyric acid (GABA). EPSPs and IPSPs are then summed up (integrated) in a spatio-temporal

manner by the postsynaptic neuron. The action of the neurotransmitter on the postsynaptic compartment are stopped by the enzymatic degradation in the synaptic cleft, by uptake of the neurotransmitter back into the cell or by diffusion out of the synaptic cleft (where the neurotransmitter can, for example, be taken up by astrocytes).

2.2. The postsynaptic site of excitatory synapse

The postsynaptic membrane of excitatory synapses contains a highly organized structure called the postsynaptic density (PSD), a protein-rich subdomain lining the inner surface of the postsynaptic membrane located in front of neurotransmitter release sites. The PSD is composed of glutamate receptors (GluRs), associated signaling proteins, scaffolding proteins, and cytoskeletal elements (Sheng, 2001). There are two major classes of GluRs: ionotropic (iGluRs) and mGluRs (Table 2). In the synapse, GluRs interact with several auxiliary proteins that regulate their trafficking, lateral mobility, clustering turnover, subcellular localization, synaptic stabilization, and signal transduction. These two families of receptors influence the postsynaptic potential with very different time courses, producing postsynaptic responses that range from less then a millisecond (for iGluRs) to minutes or hours (in the case of mGluRs).

iGluR are non-selective cation channels. Based on pharmacological and electrophysiological properties, iGluR have been classified into three major subtypes: α-amino-3-hydroxy-5-methyl-4-isozaxole propionic acid (AMPAR), kainate (KAR), and N-methyl-D-aspartate (NMDAR) receptors (Mayer, 2004; Smart & Paoletti., 2012) (Table 2). All three types of iGluRs are integral membrane proteins composed of four large subunits that form a central ion channel pore (Mayer, 2004; Smart & Paoletti., 2012). The tetramers are composed of different subunits: AMPARs are formed by co-assembly of GluA1-4 subunits, KARs by coassembly of GluK1-5, and NMDARs by coassembly of GluN1 with GluN2A-D and GluN3A-B. The iGluR subunits are modular structures that contain four discrete semiautonomous domains: the extracellular amino-terminal domain, the extracellular ligand-binding domain, the transmembrane domain, and an intracellular carboxyl-terminal domain (Traynelis et al., 2010).

In contrast to ionotropic iGluRs, mGluRs are G-protein-coupled receptors (GPCRs). mGluRs compose a family of eight receptors that can be subdivided into three groups (I, II, and III) based on sequence homology, pharmacology, and signal transduction pathways (Gregory, Noetzel, & Niswender, 2013) (Table 2). Group-I includes mGluRs 1 and 5 that are canonically linked to the Gαq/11 heterotrimeric G-

proteins. These receptors are predominantly postsynaptic and act primarily through activation of phospholipase C (PLC), leading to the activation of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) pathways which mobilize receptor-mediated release of intracellular stores of Ca^{2+} (reviewed by (Niswender & Conn, 2010)). In contrast with Group-I, Group-II and III mGluRs are coupled predominantly to Gi/o proteins that classically lead to adenylate cyclase (AC) inhibition and/or direct regulation of ion channels (activation of K^+ channels and inhibition of Ca^{2+} channels) via liberation of Gβγ subunits. Group-II, which includes mGluRs 2 and 3, can be pre- or postsynaptic, whereas the Group-III includes mGluRs 4, 7 and 8, which are localized mainly presynaptically (reviewed by (Niswender & Conn, 2010)). Although single mGluRs can bind G proteins, a dimeric organization of mGluRs is required for signaling induced by agonists (reviewed by (Niswender & Conn, 2010)). All mGluRs have seven α-helical transmembrane domains contain a large NH_2 extracellular portion containing a Venus fly trap (VFT) glutamate binding domain and a cysteine rich domain, and an intracellular COOH terminal portion, which is the site of interaction with several scaffolding and regulatory proteins (reviewed by (Niswender & Conn, 2010)).

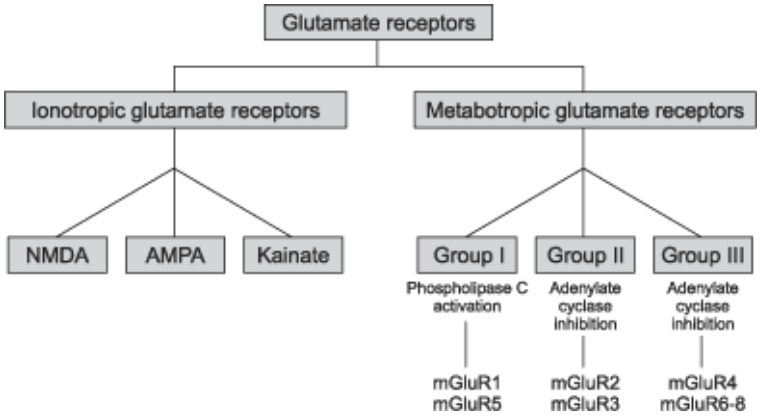

Table 2. Classification of glutamate receptors

2.3. Synaptic plasticity

Synaptic transmission is the main mean of communication between neurons and allow the transfer of information in the central nervous system. The magnitude of synaptic transmission is a reflection of the synaptic connection strength, which can be altered by many means including activity patterns, neuromodulation and neurotrophic

factors (reviewed by (Malenka & Bear, 2004)) Synaptic plasticity specifically refers to the modification of the strength or efficacy of synaptic transmission but also to structural plasticity, including synapse formation and elimination (reviewed by (Citri & Malenka, 2008; Holtmaat & Svoboda, 2009)). Some form of a plastic has been proposed to play a central role in the capacity of the brain to incorporate transient experiences into persistent memory traces. This idea was put forward over 100 years ago by the Spanish Nobel laureate Santiago Ramon y Cajal, and was further advanced in the late 1940s by Donald Hebb, who proposed that associative memories are formed in the brain by a process of synaptic modification that strengthens connections when presynaptic activity precedes with postsynaptic firing, so that the presynaptic neuron contributes to the firing of the postsynaptic neuron leading to strengthening (reviewed by (Cooper, 2005)).

Experimental support for the very existence of such long-lasting, activity-dependent changes in synaptic strength was lacking until the early 1970s when Bliss and colleagues (Bliss & Collingridge, 1993; Bliss & Lomo, 1973) reported that repetitive activation of excitatory synapses in the hippocampus caused a potentiation of synaptic strength that could last for hours or even days. Over the last three decades, this phenomenon, eventually termed LTP, has been the object of intense investigation because it is widely believed that it provides an important key to understanding some of the cellular and molecular mechanisms by which memories are formed (S. J. Martin, Grimwood, & Morris, 2000; Pastalkova et al., 2006; Whitlock, Heynen, Shuler, & Bear, 2006). Importantly, it is well established that most synapses that exhibit LTP also express one or more forms of LTD, correlated with a reduction of the synaptic strength (reviewed by (Malenka & Bear, 2004)). Thus, a key concept is that synaptic strength at excitatory synapses is bidirectionally modifiable by different protocols. Furthermore, it is now clear that the terms “LTP” and “LTD” describe a class of phenomenon, the underlying mechanisms depending on the circuit’s function.

The regulation of glutamate-mediated excitatory neurotransmission has been shown to play a critical role in many forms of synaptic plasticity. The phosphorylation of glutamate receptors (GluRs) has been demonstrated to alter their function and trafficking, suggesting that they may be targets of various kinases and phosphatases during the induction and maintenance of synaptic plasticity (reviewed by (Roche, Tingley, & Huganir, 1994)). Moreover, the dynamic movement of synaptic components has emerged in the last decades as the main mechanism to dynamically organize the synaptic membrane and as a key feature of synaptic transmission and plasticity (Bredt &

Nicoll, 2003; Collingridge, Isaac, & Wang, 2004; Luthi et al., 2001; Malenka & Nicoll, 1999; Mammen, Huganir, & O'Brien, 1997; Triller & Choquet, 2005; 2008).

For some years, endocytosis and exocytosis were thought to be the only routes for exit and entry of receptors from and to postsynaptic sites, respectively. In the early 2000s, it has been established that lateral diffusion of receptors in the plane of the plasma membrane is a key step for modifying receptor numbers at synapses (Borgdorff & Choquet, 2002; Tardin, Cognet, Bats, Lounis, & Choquet, 2003). Indeed, receptors constantly switch between mobile and immobile states on the neuronal surface, driven by thermal agitation (i.e. brownian motion) and reversible binding to stable elements, such as scaffolding proteins, cytoskeletal anchoring slots, or extracellular anchors (review by (Choquet & Triller, 2013)). The local enrichment of GluRs at postsynaptic densities (PSD) is thought to result from receptor immobilization by stable elements interacting in a biochemical and structural network.

LTD and LTP at pre-existing excitatory synapses involvea modification of synaptic molecules, in term of properties and/or numbers. Our understanding of the implicated molecular mechanisms has evolved in the last two decades from a model dominated by post-translational modifications of stable molecules leading to changes in their biophysical properties to a refined one in which the same modifications induce primarily a change in the trafficking rates of stable molecules, leading to changes in their type/number at synapses.

2.4 Neuronal transmission in the hippocampus

The hippocampus is part of the limbic system of the mammalian brain and is essential for memory formation as well as spatial navigation (Bliss & Collingridge, 1993; Malenka & Bear, 2004; C. H. McNaughton et al., 2008; M. A. Wilson & McNaughton, 1993). The clearly laminated structures and highly organised excitatory and inhibitory input-output networks have made the hippocampus one of the most widely investigated brain region. It is therefore not surprising that activity-dependent long-lasting potentiation of synaptic transmission, postulated as a mechanism of memory formation (Hebb, 1949), was first observed in the hippocampus (LTP (Bliss & Lomo, 1973)). In the same period, place cells, neurons with space selective activity, were discovered in the hippocampus (O'Keefe & Dostrovsky, 1971) and suggested to form a neural substrate of “cognitive maps” (O'Keefe, Nadel, & Willner, 1979). Indeed, damage to the hippocampus disrupts the navigation that requires memory for spatial

relationships, but not the navigation to a visible cue in a water maze (Morris, Garrud, Rawlins, & O'Keefe, 1982). These findings suggested that the hippocampus plays a specific role in spatial memory. Note that in humans, damage to the hippocampus results in severe deficits in declarative memory (reviewed by (Squire, 2004)). More recently, it was found that the hippocampus is also necessary for context-dependent memory (Biedenkapp & Rudy, 2007; J. J. Kim & Fanselow, 1992; Wiltgen & Silva, 2007), consistent with a critical role of the hippocampus in context-rich episodic memory (Vargha-Khadem et al., 1997; Winocur, Moscovitch, & Sekeres, 2007).

In human, the hippocampus is shaped like a sea horse and seated within the medial temporal lobe of the mammal's brain. It has a head (posterior to the amygdala), a body, and a tail (which follows the upwardly curving lateral ventricle). The majority of its output is via the alveus into the fimbria, which breaks free to the hippocampus to form the fornix. The term "hippocampal formation" generally applies to the *dentate gyrus* (DG), CA1, CA2 and CA3 fields (CA, *cornu ammonis*), and the subiculum (parahippocampal gyrus). CA4, is frequently called "the hilus" and considered part of the DG. The proper hippocampus is made of CA1, CA2 and CA3 fields. Information flow through the hippocampus proceeds from DG to CA3, then to CA1, and then to the subiculum, with additional input and ouput information. CA2 represents only a very small portion of the hippocampus and its presence is often ignored in accounts of hippocampal function, though it is notable that this small region seems unusually resistant to conditions that usually cause large amounts of cellular damage, such as epilepsy.

In a simplified view, the excitatory circuits in the hippocampus are characterised as conveying unidirectional flow of information through a well-characterised "trisynaptic" pathway (Bliss & Gardner-Medwin, 1973; Malenka & Nicoll, 1999) (Figure 1). Much of the neocortical input reaches the hippocampus through the enthorinal cortex (EC), which can be considered as the starting point of the intrinsic hippocampal circuit. The EC receives most of the sensory information via the adjacent perirhinal and postrhinal cortices (also termed parahippocampal cortex). Subsequently, the pyramidal cells in the EC layer II send axon projections into the DG (also called the perforant pathway). This pathway serves as the major input of the hippocampus. From the DG granule cells, the mossy fibre delivers information into the CA3 area of the hippocampus. The pyramidal cells in the CA3 then innervate the CA1 area of the hippocampus through long axon projections called *Schaffer* collaterals. Finally, the CA1

pyramidal cells send axons to the subiculum, which directs projections back to the EC. As a relay, the information flow starts in the superficial layers and finishes in the deep layers of the EC, and this is summarized as the EC-DG-CA3- CA1-EC pathway.

It is widely accepted that each of these regions has a unique functional role in the information processing of the hippocampus, but to date the specific contribution of each region is poorly understood.

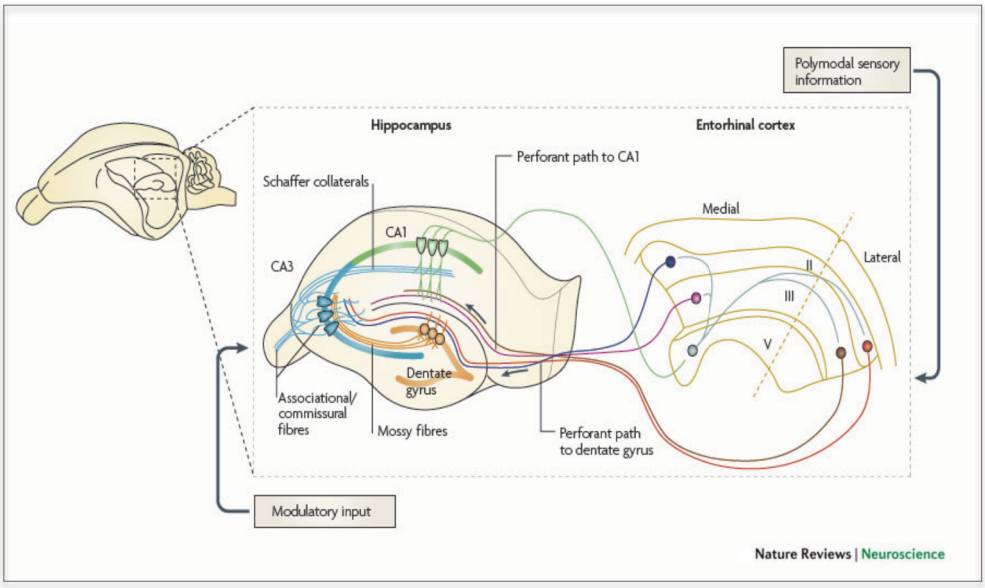

Figure 1. Wiring diagram of the hippocampus. The entorhinal cortex (EC) receives sensory information that is afterwards sent to the hippocampus trough several pathways. The EC projects directly to the *dentate gyrus* (DG) via the perforant path. Other layers of the EC project to the distal regions of CA1 cells. Granule cells of the DG region send their axons to the proximal regions of CA3 pyramidal cells. CA3 pyramidal cells form a densely interconnected, associative network. CA3 pyramidal cells also project to the proximal regions of CA1 cells. Finally CA1 works as the main output region of the hippocampus, sending fibers to the deeper layers of EC. Further modulatory input to hippocampal neurons is received by fibers from other brain areas such as the septum. (Modified from (Neves et al., 2008)).

The highly organized and laminar arrangement of synaptic pathways makes the hippocampus a convenient model for studying synaptic physiology and physiopathology *in vivo* and *in vitro* (Andersen, Bliss, & Skrede, 1971). The three principal excitatory synaptic connections of the hippocampus are all glutamatergic. In addition, the hippocampus also contains a great variety of GABA-ergic interneurons which modulate

neuronal transmission (Kasugai et al., 2010). Activation of CA3 pyramidal neurons leads to glutamate release from the axon terminals of the SCs in the *stratum radiatum* and *stratum lacunosum moleculare* of CA1 activating iGluRs (i.e. AMPARs and KARs) and mGluRs. AMPARs and KARs mediate the fast component of the EPSP (Karnup & Stelzer, 1999). NMDARs underlie the slow component of EPSPs and are thought to be responsible for certain forms of LTP (Kullmann, Erdemli, & Asztély, 1996). mGluR, which are located at both the presynaptic and postsynaptic side, act to modulate the release of neurotransmitter presynaptically (Baskys & Malenka, 1991), and to modify the responses postsynaptically by rapid redistribution of AMPAR and NMDAR (Snyder et al., 2001; M. Y. Xiao, Zhou, & Nicoll, 2001).

3. Group-I mGlu receptors

Group-I mGluR (i.e. mGluR1 and mGluR5) are expressed at many excitatory synapses and play an important role in several forms of synaptic plasticity (Bikbaev et al., 2008; Huber, Kayser, & Bear, 2000; Y. M. Lu et al., 1997) and learning behaviors (Balschun & Wetzel, 2002; Chiamulera et al., 2001; Y. M. Lu et al., 1997). mGluR1 and mGluR5 have complementary expression patterns during development (Catania et al., 1994). mGluR5 is more highly expressed in in forebrain regions during the first three postnatal weeks and declines afterwards, whereas mGluR1 is more highly expressed in the cerebellum and it increases with age and is maximal in adulthood (Catania et al., 1994). These expression studies suggest that Group-I mGluRs may have an important role in plastic changes occurring early during post-natal development (Catania et al., 2007).

In the hippocampus, mGluR1 and mGluR5 have a different pattern of expression. More specifically, mGluR1 is mostly found on cell bodies of granular layer of DG and pyramidal cells of CA3, whereas mGluR5 is mainly expressed in dendritic fields in most region of the hippocampus, predominantly in the CA1 pyramidal layer (Fotuhi, Standaert, Testa, Penney, & Young, 1994). This pattern supports the prominent role of mGluR5 in the modulatory postsynaptic action of glutamate in the CA1 area.

Both mGluR1 and mGluR5 are also found in astrocytes where they play key roles in glia-neuron interactions, regulation of glutamate reuptake, and the coupling of the neurovasculature to neuronal activity (D'Ascenzo et al., 2007; Shi et al., 2008; Vermeiren et al., 2005).

Group-I mGluR are mainly localized postsynaptically (Romano, van den Pol, & O'Malley, 1996; Shigemoto et al., 1997), although a presynaptic localization of these receptors has also been described (Gereau & Conn, 1995; L. S. Thomas, Jane, Harris, & Croucher, 2000) (Figure 2). In dendritic spines, mGluR1 and mGluR5 are typically localized in the perisynaptic region, and therefore they are generally recruited by the high levels of glutamate that are released during sustained synaptic transmission (Baude et al., 1993; Nusser, Mulvihill, Streit, & Somogyi, 1994; Vidnyanszky et al., 1996) (Figure 2). Interestingly, mGluR1 and mGluR5 are also found at extrasynaptic sites with a higher frequency of **m**GluR5 than mGluR1 (Luján, Roberts, Shigemoto, Ohishi, & Somogyi, 1997). Although Group-I mGluRs are canonically defined by their coupling to PLC transduction, more recent evidence suggests that these receptors activate at least

three distinct cascades (Figure 3).

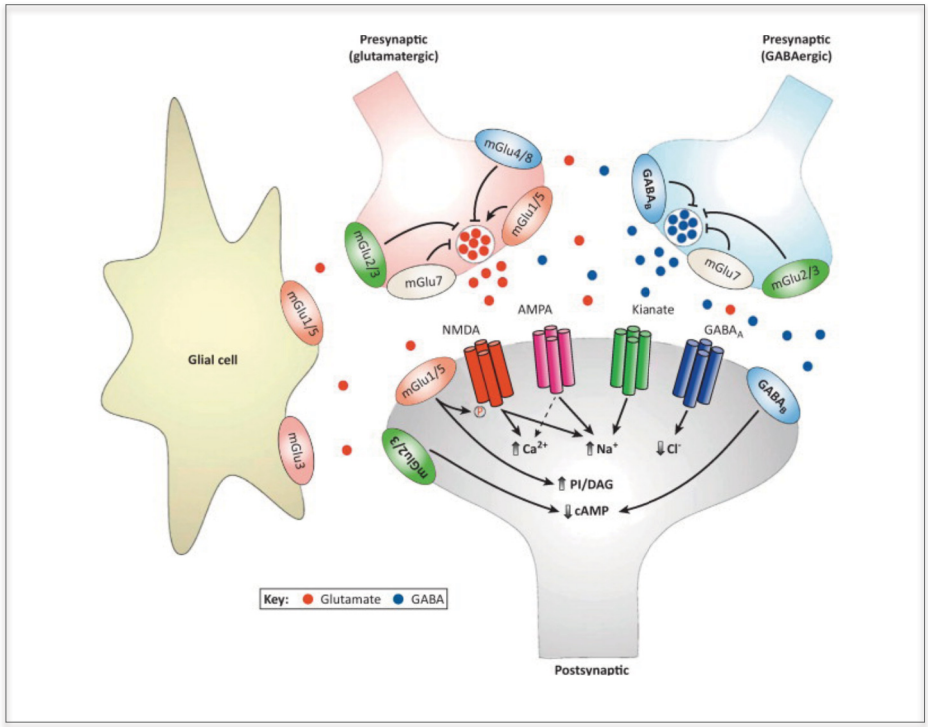

Figure 2. Localization of mGluRs in the synapse. (Modified from (Spooren, Lindemann, Ghosh, & Santarelli, 2012)).

(i) The Gq-dependent PLC cascade: phosphoinositol (PI) hydrolysis results in the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2) into two second messengers,namely DAG and IP3. DAG is an endogenous activator of protein kinase C (PKC), whereas IP3 mobilizes receptor-mediated release of intracellular stores of Ca^{2+} (Abe et al., 1992; Joly et al., 1995; Pin, Waeber, Prézeau, Bockaert, & Heinemann, 1992; Watabe, Carlisle, & O'Dell, 2002). (ii) The PI3K/Akt/mTOR cascade: phosphorylation of the phosphoinositide 3-kinase (PI3K) activates Akt (serine/threonine-specific protein kinase), which turns on the mammalian target of rapamycin (mTOR) (Hou & Klann, 2004; Ronesi & Huber, 2008). (iii) The extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) cascade: the tyrosine kinase Src phosphorylates and activates mitogen-activated protein kinase kinase (MEK), which in turn phosphorylates and activates ERK (also called microtubule associated protein kinase, MAPK) (Berkeley & Levey, 2003; Choe & Wang, 2001; Ferraguti, Baldani-Guerra, Corsi, Nakanishi, & Corti, 1999; Gallagher, Daly, Bear, & Huber, 2004; Grueter et al., 2006; Mao et al.,

2005). Interestingly, all three pathways have been directly or indirectly linked to the regulation of protein synthesis (Banko, Hou, Poulin, Sonenberg, & Klann, 2006; Davidkova & Carroll, 2007; Gallagher et al., 2004; Hou & Klann, 2004; Klann & Dever, 2004; S. Park et al., 2008; Waung, Pfeiffer, Nosyreva, Ronesi, & Huber, 2008) demonstrating that binding of glutamate to Group-I mGluR activates multiple intracellular second messenger cascades that regulate protein synthesis. This suggests that many of the long-term consequences of Group-I mGluR activation might be protein synthesis-dependent.

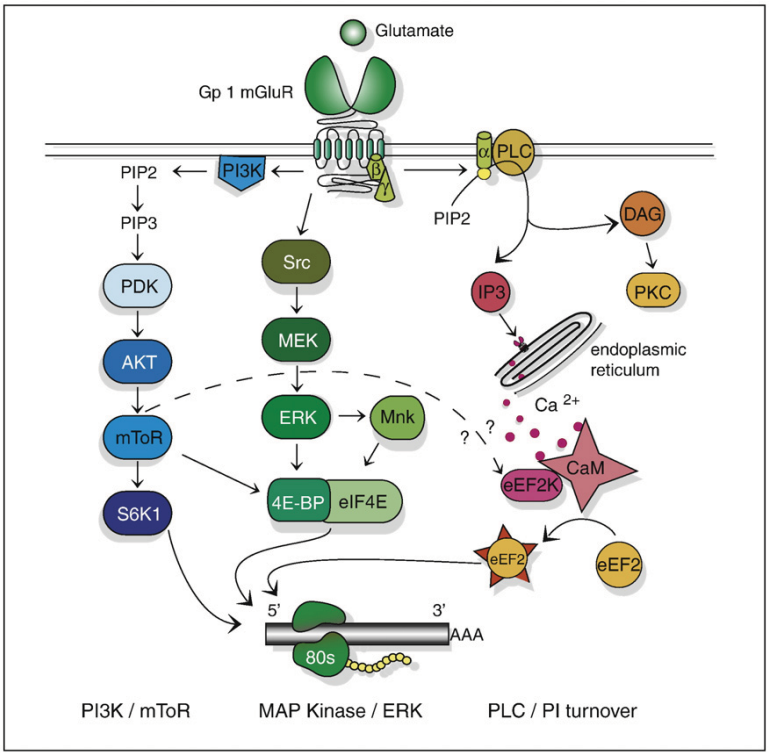

Figure 3. Signaling cascades coupling to mGluRs (PI3K, MAPK, PLC) converge to regulate protein synthesis. (Modified from (Dölen, Carpenter, Ocain, & Bear, 2010)).

3.1. Group-I mGluRs dependent synaptic plasticity

Activation of Group-I mGluRs implicates in many forms of brain plasticity, including learning and memory, drug addiction, and chronic pain (reviewed by (Dölen & Bear, 2008; Grueter, McElligott, & Winder, 2007)). As stated above, group-I mGluRs are localized postsynaptically within a perisynaptic zone surrounding the ionotropic

receptors (Lujan et al., 1996). Thus, they are well positioned for rapid and selective regulation of excitatory synaptic strength for example by redistribution of AMPAR and NMDAR (reviewed by (Lüscher & Malenka, 2012)). The best characterized form of synaptic plasticity induced by Group-I mGluRs is LTD of excitatory synaptic strength (mGluR-LTD). In hippocampal CA1 pyramidal neurons, mGluR-LTD is typically induced by either prolonged low frequency synaptic stimulation (1-3 Hz, 5-15 min) of the *Schaffer* collaterals or brief application of the Group-I mGluRs agonist, R,S-Dihydroxyphenylglycine (5-10 min; DHPG) both *in vitro* and *in vivo* (Bolshakov & Siegelbaum, 1994; Kemp & Bashir, 1999; Lüscher & Huber, 2010; Manahan-Vaughan, 1997; Naie & Manahan-Vaughan, 2005; Volk et al., 2007).

In addition to the hippocampal CA1 region, activation of group I mGluRs induces protein synthesis-dependent LTD in the DG of freely moving rats, and in the cerebellum at the mossy fiber-deep cerebellar nucleus synapses and granule cell-purkinje cell synapses, and in the ventral tegmental area (Karachot, Shirai, Vigot, Yamamori, & Ito, 2001; Mameli, Balland, Luján, & Lüscher, 2007; Naie & Manahan-Vaughan, 2005).

In mature CA1 neurons *in vitro*, mGluR-LTD requires rapid dendritic protein synthesis (within 15 min) from preexisting mRNA (Huber et al., 2000; Nosyreva & Huber, 2006). Indeed, induction of mGluR-LTD in hippocampal CA1 requires translation (protein synthesis inhibitors like cyclohexamide and anisomycin prevent the induction of paired-pulse-induced LTD) but not transcription (transcription inhibitor actinomycin prevents DHPG-induced LTD) (Hou & Klann, 2004; Huber et al., 2000; Huber, Roder, & Bear, 2001). This idea that local dendritic protein synthesis from pre-existing mRNAs is necessary and sufficient for mGluR-LTD is further supported by the occurrance of this plasticity form in dendrites that were mechanically isolated from their cell bodies (Aakalu, Smith, Nguyen, Jiang, & Schuman, 2001). It should be noted however, that in young rodents (<20 days postnatal) mGluR-LTD at hippocampal *Schaffer*-collateral synapses is not protein synthesis-dependent and is instead mediated by a change in presynaptic function (Bolshakov & Siegelbaum, 1994; Feinmark et al., 2003; Fitzjohn et al., 2001; Nosyreva & Huber, 2006; Zakharenko, Zablow, & Siegelbaum, 2002).

Determining the molecular mechanisms of mGluR-LTD is essential to understanding how newly synthesized proteins in dendrites mediate plasticity. Group-I mGluR activation triggers the rapid synthesis of new “LTD proteins”, meaning new proteins in dendrites that induce LTD at locally active synapses (Waung & Huber,

2009). mGluR-LTD in mature rodents is mediated by a persistent decrease in AMPAR surface expression. Activation of Group I-mGluRs causes a long-term decrease in surface AMPARs, both GluR1 and GluR2 subunits, lasting for at least one hour (Moult et al., 2006; Snyder et al., 2001). Endocytosis of AMPARs is triggered by *de novo* synthesis of "LTD proteins", such as the striatal enriched tyrosine phosphatase (STEP) that dephosphorylates the AMPAR subunit GluA2 (Moult et al., 2006). Other candidate proteins are Arc/Arg 3.1, which regulates AMPAR endocytosis by interacting with endophylin2/3 and dynamin (Chowdhury et al., 2006; S. Park et al., 2008), and MAP1B, which interacts with glutamate receptor interacting protein 1 (GRIP1) (Davidkova & Carroll, 2007). Proteins synthesized in response to Group-I mGluRs stimulation also include FMRP, EF1α, and αCaMKII. All of these proteins play an important role in the regulation of translation machinery (Huang et al., 2001; Weiler et al., 1997). Not surprisingly, the majority of proteins involved in the induction and expression of mGluR-LTD either regulate AMPA receptor trafficking/function or protein translation.

It is important to point out that there are distinct forms of LTD, independent of mGluRs that coexist at CA1 excitatory synapses (reviewed by (Lüscher & Huber, 2010)). These forms typically rely on activation of NMDARs. Interestingly, NMDAR-LTD is also expressed as a decrease in surface AMPARs, but unlike mGluR-LTD it is not associated with a persistent increase in AMPAR endocytosis rate (reviewed by (Waung & Huber, 2009)). These data suggest that mGluR-LTD, specifically, is induced by new proteins that increase AMPAR endocytosis rate. To maintain the steady state level of surface AMPARs observed during mGluR-LTD in the face of a persistently elevated endocytosis rate, the requisite exocytosis rate must also increase. This model also implies that the recycling of AMPARs is faster during mGluR-LTD.

The biochemical cascades linking Group-I mGluR activation to protein synthesis and mGluR-LTD have been extensively investigated (reviewed by (Bhakar, Dölen, & Bear, 2012; Waung & Huber, 2009)). Group-I mGluRs are Gq-coupled glutamate receptors, and as such, activate the canonical Gq-dependent PLC signaling cascade. PLC hydrolyzes PIP2 to form IP3 and DAG, which in turn release Ca^{2+} from internal stores and activate PKC, respectively. However, mGluR-LTD is unaffected by inhibition of PKC or chelation of intracellular Ca^{2+}, demonstrating that this classical Gq signaling pathway is not necessary for mGluR-dependent LTD (Fitzjohn et al., 2001). It has been demonstrated that activation of group-I mGluRs leads to protein synthesis through ERK and mTOR signalling pathways and that activation of both of these pathways are

required for mGluR-LTD (Gallagher et al., 2004; Hou & Klann, 2004). However, the mechanisms of specific mRNAs translation are not clear. A recently proposed model suggests that mTOR activation is required for the sustained increase in the overall rate of mRNA translation at the modified synapse, while ERK activation controls the translation of specific mRNAs, such as those encoding "LTD proteins" (Bhakar et al., 2012).

3.2. Group-I mGluRs modulation of NMDAR-dependent synaptic plasticity

Activation of Group-I mGluRs is involved in the induction of NMDAR-dependent synaptic plasticity. Activation of Group-I mGluRs can induce LTP or LTD depending on whether the currents are elicited by exogenous NMDAR agonist application or synaptic stimulation. Currents mediated by exogenous application of NMDAR agonist are transiently potentiated by Group-I mGluR activation (Benquet, Gee, & Gerber, 2002; Grishin, Gee, Gerber, & Benquet, 2004; Heidinger et al., 2002; Mannaioni, Marino, Valenti, Traynelis, & Conn, 2001; Skeberdis et al., 2001; Snyder et al., 2001). Mice lacking mGluR5 show a decrease in NMDAR-dependent LTP in the CA1 region and DG, and in hippocampal-dependent learning paradigms such as the water maze and contextual fear conditioning (Y. M. Lu et al., 1997). The importance of mGluR5 in potentiating NMDAR-evoked currents and its role in facilitating NMDAR-dependent LTP has been confirmed by several studies (Attucci, Carlà, Mannaioni, & Moroni, 2001; Awad, Hubert, Smith, Levey, & Conn, 2000; J.-H. Hu et al., 2010; Jia et al., 1998; Mannaioni et al., 2001; Pisani et al., 2001). This potentiation of NMDAR-evoked currents is mediated by activation of PLC (Skeberdis et al., 2001), release of Ca^{2+} from IP3-sensitive calcium stores, and the activity of PKC (Benquet et al., 2002; Skeberdis et al., 2001) and src family tyrosine kinases (Benquet et al., 2002; Heidinger et al., 2002). Activation of these kinases leads to tyrosine phosphorylation of NMDAR subunits (Huang et al., 2001; Kotecha & MacDonald, 2003; W. Y. Lu et al., 1999).It is not clear whether the potentiation of the NMDAR-evoked current results from an increase in the opening probability of the NMDAR channels (W. Y. Lu et al., 1999; Xiong et al., 1998) or an increase in the number of channels available in the membrane (Lan et al., 2001). Of note, mGluR5 activation is also required for *in vivo* LTP and the formation of working and reference memory in freely moving rats (Naie & Manahan-Vaughan, 2005).

Conversely, synaptically elicited NMDAR-mediated excitatory postsynaptic currents (NMDAR-EPSCs) undergo depression in response to Group-I mGluR activation (Baskys & Malenka, 1991; Snyder et al., 2001; Watabe et al., 2002). The reasons for this

disparity and the mechanisms underlying the mGluR-induced depression of the NMDAR-EPSCs are poorly understood. For example, one study showed that the mechanism for the transient acute depression is most likely pre-synaptic in origin (Watabe et al., 2002), but another study showed that Group-I mGluR-mediated rapid depression and LTD of the synaptically evoked NMDAR currents appears to be mediated post-synaptically by lateral movement of synaptic NMDARs via actin depolymerization (Ireland & Abraham, 2009). However, some evidences have shown that Group-I mGluR-mediated LTD of NMDAR-EPSCs is not dependent on protein synthesis, or tyrosine kinase or phosphatase activity, or an increase in intracellular calcium (Ireland & Abraham, 2009). Further studies are necessary in order to clarify the possible mechanisms responsible for the mGluR-induced depression of the NMDAR-EPSCs.

3.3. Group-I mGluRs in learning and memory

Group-I mGluRs have been identified as important interfaces in mechanisms of synaptic plasticity as well as in learning and memory paradigms. Mechanisms of LTP and LTD are believed to be the underlying cellular basis of learning and memory formations. Both *in vitro* and *in vivo* studies have demonstrated that the activation of Group-I mGluRs is an important interface in the cellular cascades leading to memory formation and learning.

Pharmacological blockade of either mGluR1 or mGluR5 have shown an impairment in aversive learning tasks and in hippocampal-dependent spatial learning (Balschun & Wetzel, 2002; Conquet et al., 1994; El-Kouhen et al., 2006). More specifically, the same treaments in the CA1 region have shown to impair inhibitory avoidance learning while blocking mGluR1 also impaired extinction learning of inhibitory avoidance memory (Simonyi et al., 2007). Further evidence comes from the mGluR5 KO mice. Indeed, it was shown a significant impairment in three different spatial learning tasks, which are known to depend on intact hippocampus (Phillips & LeDoux, 1992), namely the water maze, radial arm maze, and contextual fear conditioning, but not in the cue fear conditioning task (Phillips & LeDoux, 1992). These findings suggest that Group-I mGluRs signaling is required for different forms of adaptive and spatial learning.

Recently, it has been shown that selective positive allosteric modulators of mGluR5 can enhance adaptive learning in mice (J. Xu et al., 2013). These results suggest

that a positive modulation of mGluR5 may represent a viable strategy for treatment of maladaptive learning and for improving behavioral flexibility.

4. Homer proteins: focus on Homer 1

Group-I mGluRs mediated signalling is strongly modulated by the interaction with regulatory proteins at the intracellular C-terminal receptor domain. The distal proline-rich region of the C-terminus domain of mGluR1 and mGluR5 interacts with protein members of the Homer family, which function as scaffolds between the receptors and a number of post-synaptic adaptor and signalling proteins (reviewed by (Shiraishi-Yamaguchi & Furuichi, 2007)). The Homer protein family consists of three subtypes, Homer 1, Homer 2, and Homer 3, all of which have several isoforms as a result of alternative splicing (reviewed by (Shiraishi-Yamaguchi & Furuichi, 2007)). Homer 1 proteins exist in two different isoforms classified as long and short splicing variants. Homer 1a (186 amino acids, also called vesl-1s) is the short variant of Homer 1, and was the first Homer protein to be isolated (Brakeman et al., 1997). The expression of Homer 1a is very low under normal conditions and increases rapidly following neuronal activation via the activity of immediate-early genes (IEGs). The long splicing variant of Homer 1 is called Homer 1b/c (366 amino acids, also called vesl-1L). Unlike for Homer 1a, substantial levels of the mRNA and protein of Homer 1b/c can be detected *in vivo* and *in vitro* under basal (un-stimulated) conditions (reviewed by (Shiraishi-Yamaguchi & Furuichi, 2007)).

Both Homer 1 proteins are strongly expressed in the nervous system, and at lower levels in the heart, skeletal muscle, and other peripheral tissues. Homer 1b/c proteins have a brain region and cell-type dependent distribution in the mouse brain that changes during postnatal development (Shiraishi, Mizutani, Yuasa, Mikoshiba, & Furuichi, 2004). In the hippocampus, Homer 1b/c proteins are predominantly localized in the CA1 region (Shiraishi et al., 2004). In contrast, Homer 1a is found at very low levels in hippocampal cells (Kato, Fukazawa, Ozawa, Inokuchi, & Sugiyama, 2003; Sala et al., 2003). In fractionation studies on the rodent brain, Homer 1b/c proteins were mainly found in the subcellular fractions that are enriched with PSD proteins or postsynaptic membrane proteins (Shiraishi et al., 1999; B. Xiao et al., 1998). This suggests a crucial roles in synaptic plasticity and signal transduction (Petralia et al., 2001; Shiraishi et al., 2004; Shiraishi, Mizutani, Mikoshiba, & Furuichi, 2003; B. Xiao et al., 1998).

Homer 1 proteins share the highly conserved amino-terminal domain, which contains 175 amino acids and is very similar to the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) homology 1 (EVH1) domain. In addition, long Homer 1b/c

have a specific carboxy-terminal domain, which consists of a coiled-coil structure and two leucine zipper motifs (Kato et al., 1998; Sun et al., 1998; Tadokoro, Tachibana, Imanaka, Nishida, & Sobue, 1999; B. Xiao et al., 1998). This domain can mediate homomeric or heteromeric interactions between long Homer forms (Kato et al., 1998; B. Xiao et al., 1998). The short Homer 1a lacks the coiled-coil domain and therefore cannot form homodimers.

4.1. Dynamic distribution and synaptic function of Homer 1 proteins

The synaptic localization of long Homer forms is not static but dynamically regulated by synaptic activity. Two Homer 1b/c monomers form dimers via their carboxy-terminal domains. The exposed amino-terminal EVH1-like domains of the dimers selectively bind mGluR1 and mGluR5 linking also proteins that are functionally related to one another, including IP3 receptor (IP3R), NMDAR, Shank proteins, and receptor channels involved in the Ca^{2+} signaling pathways at the PSD (Brakeman et al., 1997; S. Lim et al., 1999; Okabe, Urushido, Konno, Okado, & Sobue, 2001; Yuan et al., 2003) (Figure 4). Clusters of Homer 1b/c and other proteins form different PSD signaling complexes in which Homer 1b/c facilitate the crosstalk among the target proteins. Homer 1b/c also affect the communication between these complexes and the intracellular signaling pathway through its dynamic distribution. Homer 1b/c redistribution is caused by their association with structural elements such as actin, tubulin and other cytoskeletal proteins (Okabe et al., 2001). On the other hand, lacking the carboxy-terminal multimerization domain, Homer 1a which is transcriptionally induced only upon neuronal stimulation, cannot form homodimers; instead, it shares with Homer 1b/c the amino-terminal EVH1-like domain and binds the target proteins of Homer 1b/c, resulting in the deformation of the complexes. Therefore, Homer 1a functions as a dominant-negative protein involved in synapse regulation modulating the scaffolding and signaling capabilities of the long forms. This property seems to be related to synapse and circuit regulation. Indeed, there are several reports demonstrating that Homer 1a is upregulated by various forms of activity and neuromodulation that induce synaptic activities. These include seizure and kindling (Bottai et al., 2002; Kato, Ozawa, Saitoh, Hirai, & Inokuchi, 1997), stimulation by light (Brakeman et al., 1997), dopaminergic stimulation (Bottai et al., 2002), exploration of a novel environment (Vazdarjanova, McNaughton, Barnes, Worley, & Guzowski, 2002), learning or long-term potentiation (Hennou et al., 2003; Matsuo, Murayama, Saitoh, Sakaki, & Inokuchi, 2000), and administration of psychoactive

stimulants or drugs (Ambesi-Impiombato et al., 2007; Tomasetti, Dell'Aversano, Iasevoli, & de Bartolomeis, 2007). The signaling cascades involved in the induction of Homer 1a expression include the MAPK cascade in cerebellar granule cells (Sato, Suzuki, & Nakanishi, 2001) and the ERK1/2 cascade in hippocampal DG cells (Rosenblum et al., 2002).

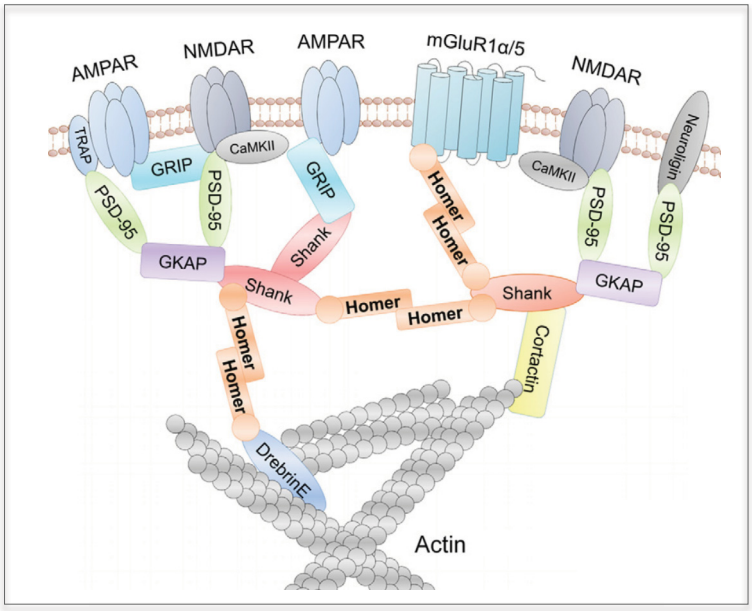

Figure 4. Schematic diagram of postsynaptic density structure. As a scaffold protein of the postsynaptic density (PSD), the Homer proteins interact with other core scaffold proteins of the PSD, such as PSD-95, GKAP, Shank, and GRIP. This forms a framework structure that serves as an assembly platform for postsynaptic membrane proteins (mGluRs, NMDARs, AMPARs, Neuroligin), signaling molecules (TRAP and CaMKII), and cytoskeleton proteins. (Modified from (Luo, Li, Fei, & Poon, 2012)).

4.2. Homer 1 proteins and Group-I mGluRs

The Homer 1 proteins selectively bind Group-I mGluRs regulating their postsynaptic localization and intracellular signaling in various ways. Homer 1 proteins modulate the trafficking and the surface dynamics of Group-I mGluRs and their targeting to the membrane (Ango et al., 2001; Coutinho, Kavanagh, Sugiyama, Tones, & Henley, 2001; Sergé, Fourgeaud, Hémar, & Choquet, 2002). It has been shown that Homer 1b/c inhibit cell-surface targeting of mGluR5 and induce its retention in the endoplasmic reticulum (Roche et al., 1999). Moreover, surface mGluR1a and mGluR5

are significantly increased in neurons from the Homer 1a KO mouse, whereas mGluR1 and mGluR5 are significantly reduced on the neuronal surfaces following the transfection of a virus expressing the Homer 1a gene (J.-H. Hu et al., 2010). In addition, exogenously expressed Homer 1b/c (but not Homer 1a) increase cell-surface clustering of mGluR5 and confines its movement within the membrane of cultured hippocampal neurons, thereby regulating the enrichment of receptors locally at the PSD (Sergé et al., 2002). These results indicate that the long and short Homer 1 proteins regulate neuronal surface targeting and dynamics of Group-I mGluRs in an opposite manner.

Homer 1 proteins also mediate Group-I mGluRs signaling and coordinate the regulation of protein translation initiated by the Group-I mGluRs. The activation of Group-I mGluRs signaling is tightly coupled to the release of intracellular Ca^{2+}, which, in turn, is regulated by the coupling of Group-I mGluRs and IP3R. Homer 1b/c have been shown to link Group-I mGluRs to IP3R and regulate intracellular Ca^{2+} release (Tu et al., 1998), and to mediate the association of mGluR1, PLCb4, and IP3R (Nakamura et al., 2004). In contrast to Homer 1b/c, Homer 1a has been implicated in reducing the intracellular Ca^{2+} responses mediated by Group-I mGluRs, without changing the subcellular distribution of the receptors (Kammermeier & Worley, 2007; Tu et al., 1998). This decrease in the activation of Group-I mGluRs can be explained by a dominant-negative effect of Homer 1a toward Homer 1b/c. Futhermore, Homer 1 proteins regulate Group-I mGluRs-stimulated translation by affecting the activation of ERK1/2 and PI3K pathway (Ronesi & Huber, 2008). Homer 1b/c form the central signaling pathway from mGluR5 to ERK1/2, whereas Homer 1a activates the mGluR-ERK pathway by disrupting the coordination between mGluR and Homer 1b/c (Mao et al., 2005; Ronesi et al., 2012; Tappe et al., 2006). Homer 1b/c facilitates the activation of the mGluR-PI3K pathway by linking with PI3K enhancer (PIKE), which activates PI3K in response to Group-I mGluRs activation (Rong et al., 2003). On the other hand, Homer 1a restricts the mGluR signaling to PI3K by reducing the mGluR-Homer 1b/c interaction (Ronesi et al., 2012). These signaling mechanisms are necessary for mGluR5-dependent LTD and LTP (Gerstein, O'Riordan, Osting, Schwarz, & Burger, 2012). The roles of mGluR/Homer complexes in plasticity underlie certain memory processes. Homer 1a specific KO mice have impaired fear memory formation (Inoue et al., 2009). In contrast, stress-induced interactions between Homer 1a and mGluR5 enhance context fear conditioning (Tronson et al., 2010).

Group-I mGluRs/Homer interaction is also able to modulate NMDAR function.

mGluR5 are physically linked to the NR2 subunit of the NMDAR through a chain of anchoring proteins including PSD-95, guanylate kinase-associated proteins (GKAPs), Shank, and Homer (Tu et al., 1998). This interaction can be disrupted by Homer 1a (Perroy et al., 2008). The functional interaction between the NMDAR and Group-I mGlu receptors has been extensively studied (Homayoun & Moghaddam, 2010), but whether the dynamic exchange of Homer 1 proteins controls functional crosstalk between these receptors has received little attention (Bertaso et al., 2010). The disassembly of the synaptic multimeric mGluR5/Homer complex by Homer 1a allows physical and functional interactions between NMDAR and mGluR5. Such a scaffold remodeling triggers a direct physical interaction between mGluR5 and NMDAR and inhibition of NMDA currents (Moutin et al., 2012). On the other hand, recent data suggest that a temporally coincident activation of Group-I mGlu and NMDAR result in synaptic potentiation and that this potentiation is critically dependent on the long Homer-mediated mGluR/NMDAR complex (Sylantyev, Savtchenko, Ermolyuk, Michaluk, & Rusakov, 2013). Therefore, the Group-I mGluRs-mediated facilitation of NMDAR activity may tightly depend on the stability of the mGluR/Homer complex.

5. mGluR5 dysfunction in Fragile X Syndrome: the "mGluR theory"

Human patients with FXS have significant cognitive impairments, with mental retardation in the moderate-to-severe range (R. J. Hagerman & Hagerman, 2001). A prevailing view in neuroscience is that the phenomenon of synaptic plasticity is a molecular mechanism underlying memory and cognition. Therefore, many studies have investigated whether the loss of FMRP results in impairments or alterations in synaptic plasticity. However, first investigations of NMDAR-dependent forms of plasticity failed to reveal a deficit in *Fmr1* KO mice (Godfraind et al., 1996; Huber et al., 2002; Paradee et al., 1999). The study of FMRP in Gropup-I mGluRs-synaptic plasticity was first initiated after the discovery that activation of mGluRs stimulates the rapid translation of pre-existing mRNAs and promotes synthesis of FMRP in synaptoneurosomes (Weiler et al., 1997). Since this finding, a growing number of studies have been carried out to support the role of Gropup-I mGluRs in the pathophysiology of FXS (Huber et al., 2002). Moreover, recently it has been reported that FMRP directly interact with the mRNA of mGluR5 (Darnell et al., 2011).

Huber et al. (2000) provided evidence for a link between Gropup-I mGluRs, protein synthesis and synaptic plasticity by showing that a Gropup-I mGluRs-specific agonist DHPG induces a form of hippocampal long term depression (mGluR-LTD) that requires rapid translation of pre-existing mRNA (Huber et al., 2000). This form of LTD was greater in *Fmr1* KO mice and caused by an increased internalisation of GluA1-containing AMPAR. Furthermore LTD in *Fmr1* KO was no longer dependent on protein synthesis, indicating that the proteins necessary for LTD induction and maintenance are already present in the *Fmr1* KO neurons as a result of increased basal protein synthesis (Huber et al., 2002). A direct prediction from this hypothesis is that in *Fmr1* KO mice mGluR-LTD, which is normally blocked by acute administration of protein synthesis inhibitors, should be insensitive to blockade of translation because the proteins necessary for mGluR-LTD expression are already present at the synapse. In support of this hypothesis, mGluR-induced LTD persists following acute application of protein synthesis inhibitors (Hou et al., 2006; Nosyreva & Huber, 2006).

These findings, combined with evidence for FMRP as a translational suppressor, suggested that FMRP acts to inhibit translation of proteins that are required for mGluR-LTD, termed "LTD proteins" (Hou et al., 2006; Liao, Park, Xu, Vanderklish, & Yates, 2008; Westmark & Malter, 2007; Zalfa et al., 2003). Because one of the proteins

synthesized in response to Gropup-I mGluRs activation is FMRP itself, it was suggested that FMRP might function as a negative feedback mechanism to limit Gropup-I mGluRs-stimulated translation. In support of this conclusion, regionally selective elevations in basal protein synthesis *in vivo* (Qin, Kang, Burlin, Jiang, & Smith, 2005) and increased basal rates of protein synthesis in hippocampal slices (Dölen et al., 2007; Osterweil, Krueger, Reinhold, & Bear, 2010) have been reported in *Fmr1* KO mice.

Taken together, these findings suggest that Gropup-I mGluRs and FMRP work in opposition to regulate mRNA translation at the synapse, and that in the absence of FMRP exaggerated mGluR-dependent protein synthesis occurs that leads to the pathogenesis of the disease (Bear et al., 2004). These ideas were outlined in what is now known as "the mGluR Theory of Fragile X Syndrome" (Bear et al., 2004) (Figure 5). The mGluR theory also predicts that a number of phenotypic features of FXS can be corrected by down-regulation of Group-I mGluRs.

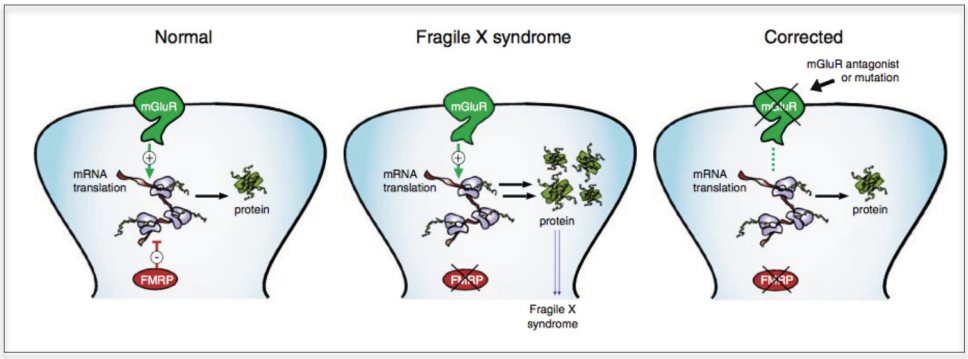

Figure 5. The "mGluR theory" of FXS. FMRP is a negative regulator of translation at the synapse. Stimulation of Group-I mGluRs with DHPG leads to the synthesis of proteins. Furthermore, many of the long-term consequences of Group-I mGluR activation are protein synthesis dependent. The mGluR theory posits that in the absence of FMRP the balance between FMRP and Group-I mGluRs is lost, and exaggerated protein synthesis at the synapse leads to the characteristic features of the disease. Furthermore, this balance could be restored by reducing Group-I mGluR activity at the synapse, by either knockdown or pharmacological blockade of the receptor. The therapeutic implication of the theory is that symptoms of FXS could be corrected by appropriate modulation of Group-I mGluR mGluR signaling. (Modified from (Dölen & Bear, 2009)).

5.1 Validation of the "mGluR theory" using pharmacological treatments

In the intervening decade, researchers have accumulated evidence in animal models of FXS that strongly supports the mGluR theory (Table 3). Dolen et al. (2007)

were able to prevent the appearance of multiple FXS phenotypes by genetically reducing levels of mGluR5 by 50% in *Fmr1* KO mice (Dölen et al., 2007). The corrected phenotypesincluded the increased spine density in L3 cells of binocular visual cortex, exaggerated hippocampal mGluR-LTD and enhanced ocular dominance plasticity. Importantly, the reduction in mGluR5 levels returned the increased basal protein synthesis to normal base-line levels (Dölen et al., 2007). Behavioural deficits, such as enhanced inhibitory avoidance extinction and increased susceptibility to audiogenic seizures were also corrected (Dölen et al., 2007). In addition, the prolonged persistent activity in the neocortex of *Fmr1* KO mice following thalamic stimulation was also restored to normal levels by reducing mGluR5 signalling (Hays, Huber, & Gibson, 2011). These studies provided proof-of-principle validation of the mGluR theory. However, for it to be applied as a clinical strategy, pharmacological studies aimed to specifically reduce the mGluR5 signalling were needed. Indeed, pharmacological inhibition of mGluR5 with negative allosteric modulators (NAMs) corrected many of the pathophysiological phenotypes of *Fmr1* KO mice. Acute application of 2-Methyl-6-(phenylethynyl)pyridine (MPEP), a mGluR5 NAM, was able to correct increased prepulse inhibition, audiogenic seizure incidence, locomotor activity in the open field, and learning and memory deficits in Fmr1 KO mice to WT levels (T. Chen et al., 2014; de Vrij et al., 2008; Gandhi, Kogan, & Messier, 2014; A. M. Thomas, Bui, Perkins, Yuva-Paylor, & Paylor, 2012; Yan et al., 2005). However, acute administration of MPEP did not correct the altered nociceptive phenotype in *Fmr1* KO mice (Price et al., 2007). The De Vrij et al. (2008) study also determined the effect of MPEP on dendritic spine morphology in *Fmr1* KO mice, showing a rescue of the ratio of normal spines to filopodia in *Fmr1* KO mice (de Vrij et al., 2008). At a cellular level, acute blockade with MPEP restored the low levels of mRNA granules in *Fmr1* KO mice to WT levels (Aschrafi, Cunningham, Edelman, & Vanderklish, 2005), and reduced the elevated protein synthesis in *Fmr1* KO mouse hippocampal slices (Osterweil et al., 2010) and in cortical synaptoneurosomes (Gross et al., 2010) (Table 3).

One of the consequences of increased protein synthesis in FXS is the alteration in synaptic plasticity (reviewed by (Sidorov, Auerbach, & Bear, 2013)). In *Fmr1* KO mice, the most characterized form of altered synaptic plasticity is enhanced mGluR-LTD in the *Schaffer* collaterals of the CA1 region of the hippocampus (Huber et al., 2001; 2002). To date, no studies have investigated the effects of MPEP on LTD anywhere in the brain. However, Nakamoto et al. (2007) observed that the increased internalization of AMPAR

in *Fmr1* KO dendrites was corrected by MPEP treatment (Nakamoto et al., 2007).

Despite these promising results, one should keep in mind that MPEP is not a specific inhibitor of mGluR5, and at high concentrations inhibits NMDA receptors (Lea, Movsesyan, & Faden, 2005). Most studies to date have used MPEP at concentrations low enough not to affect NMDA activity, but it is conceivable that some conclusions drawn from research involving MPEP could be due to its NMDA-inhibiting action rather than its ability to inhibit mGlu5. Moreover, MPEP cannot be used as a therapy in humans with FXS owing to its toxicity and very short half- life (~1 h in C57BL/6J mice (Anderson et al., 2003)). Thus, the development of novel selective mGluR5 NAMs was necessary.

AFQ056 (Mavoglurant) is a selective mGluR5 NAM developed by Novartis which could potentially be used to treat FXS in humans since it does not have the same issues as mentioned for MPEP. Levenga et al. (2011) showed that AFQ056 restored the PPI in *Fmr1* KO mice. AFQ056 was also effective in restoring the normal dendritic spine length when acutely administered to *Fmr1* KO mice (Levenga et al., 2011; Pop et al., 2014). Moreover, chronic administration of AFQ056 was able to restore sociability behaviour of *Fmr1* KO mice to base-line levels of WT littermates (Gantois et al., 2013) (Table 3).

Fenobam is another selective mGluR5 NAM developed by Neuropharm. *Fmr1* KO mice treated with Fenobam showed a rescue in associative motor learning and avoidance behavior deficits as well as spine morphology abnomatilies (de Vrij et al., 2008; Vinueza Veloz et al., 2012) (Table 3).

Yet, Michalon et al. (2012) provided compelling evidence that FXS may also be amenable to pharmacological intervention in adult mice (Michalon et al., 2012). Chronic oral administration of the selective long acting (half- life ~18 h) mGluR5 NAM 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine (CTEP), in young-adult *Fmr1* KO mice resulted in the correction of hippocampal protein synthesis, spine density and mGluR-LTD, and of behavioural phenotypes such as cognitive deficits, auditory hypersensitivity, inhibitory avoidance and extinction test (Michalon et al., 2012; Michalon et al., 2014). In addition, functional magnetic resonance imaging (fMRI) showed that chronic CTEP treatment normalized cerebral blood flow in the amygdala and the lateral hypothalamus in *Fmr1* KO mice. Furthermore CTEP decreased cerebral blood flow in the hippocampus and increased it in primary sensorimotor cortical areas (Michalon et al., 2014) (Table 3).

	mGluR5$^{+/-}$ mice	MPEP	AFQ056	Fenobam	CTEP	MTEP
AMPAR internaliz.	-	√	-	-	-	-
		Nakamoto et al., 2007				
Protein synthesis	√	√	-	-	√	-
	Dölen et al., 2007	Aschrafi et al., 2005; Osterweil et al., 2010; Gross et al., 2010			Michalon et al., 2012	
mGluR-LTD	√	-	-	-	√	-
	Dölen et al., 2007				Michalon et al., 2012	
Spines	√	√	√	√	√	-
	Dölen et al., 2007	De Vrij et al., 2008	Levenga et al., 2011; Pop et al., 2014	De Vrij et al., 2008	Michalon et al., 2012	
Audiogenic seizures / Epileptiform discharge	√	√	-	-	√	√
	Dölen et al., 2007;	Chuang et al., 2005; Yan et al., 2005; Min et al., 2009; Hays et al., 2011; Thomas et al., 2012; Pacey et al., 2011;			Michalon et al., 2012	Psychogenics.com
Hypersensitivity / startle response	-	√	√	-	√	-
		De Vrij et al., 2008;	Levenga et al., 2011		Michalon et al., 2012	
Nociception	-	**X**	-	-	-	-
		Price et al., 2007				
Repetition / perseveration	-	√	-	-	-	-
		Thomas et al., 2012; Gandhi et al., 2014				
Social behaviour	-	-	√	-	-	-
			Gantois et al., 2013			
Open field activity / locomotor behavior	-	√	-	-	√	-
		Yan et al., 2005; Min et al., 2009;			Michalon et al., 2012	
Anxiety	-	-	-	√	-	√
				Vinueza Veloz et al., 2012		Busquets-Garcia et al., 2013
Learning And memory	√	√ **X**	-	√	√	√
	Dölen et al., 2007	Gandhi et al., 2014; Chen et al., 2014; Franklin et al., 2014;		Vinueza Veloz et al., 2012	Michalon et al., 2012; Michalon et al., 2014	Busquets-Garcia et al., 2013

√ = corrected **X** = not corrected

Table 3. Pharmacological inhibition of mGluR5 with negative allosteric modulators (NAMs) in *Fmr1* KO mice.

A recent study with 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP), which is a last generation mGluR5 NAM, has shown that acute administration of the drug did not ameliorate the cognitive deficit of *Fmr1* KO mice. However, when chronically administered, MTEP was effective at preventing the cognitive deficit in *Fmr1* KO mice (Busquets-Garcia et al., 2013). Moreover, acute mGluR5 blockade with MTEP corrected the reduced-anxiety phenotype of *Fmr1* KO mice (Busquets-Garcia et al., 2013) (Table 3).

5.2 Clinical trials with mGluR5 negative allosteric modulators for FXS

Much of the evidence in preclinical animal models of FXS indicates that mGlu5 NAMs may eventually be used therapeutically. However, the major limitation within the preclinical studies is the fundamental problem of modelling human diseases in animals. The validity of the models depends on the extent to which the animal disease is analogous to the human disease. Overall, the prospect for the development of mGluR5 NAMs as a therapy appears good and the positive results of studies in preclinical animal models have led to the development of pharmaceuticals and clinical trials in humans with FXS that target mGluR5 (Table 4).

A pilot open label, single-dose trial of fenobam was conducted on 12 subjects (6 male, 6 female) with FXS (Berry-Kravis et al., 2009). The main aim of the experiment was to determine the safety of the drug and identify any significant adverse effects. Approximately 50% of the patients had correction of PPI after drug administration and none showed negative side-effects. It is known that the placebo effect is exaggerated in individuals with mental retardation (Sandler, 2005) and so the open label nature of this trial may have some influence on the results. The results also suffered from a low number of trial participants and the fact that they were only given a single dose. However, the lack of adverse effects was promising for future trials.

A double-blind of the selective mGlu5 inhibitor AFQ056 was conducted on 30 individuals with FXS (Jacquemont et al., 2011). Individuals were screened to determine the extent of the methylation of their *Fmr1* promoter. Interestingly, only individuals with a fully methylated promoter and no *Fmr1* mRNA detected in the blood showed significant improvement in various behavioural rating scales over the control group. This suggests that screening for full methylation could determine which patients would benefit from mGlu5 antagonists. The response to treatment in patients with partial methylation of the *Fmr1* promoter was varied. Jacquemont et al. (2011) propose that this

variation in response is due to different degrees of mGluR5 hyperactivity as a result of the methylation state (Jacquemont et al., 2011). The small number of trial patients (seven with fully methylated *Fmr1* promoters) makes it difficult to draw any solid conclusions from this study. Moreover, Jacquemont et al. (2011) chose to use behavioural response Aberrant Behavior Checklist (ABC) to measure the efficacy of AFQ056 (Jacquemont et al., 2011). Measurement of change in PPI or eye tracking would have been a more objective way to quantify the response. Also, to see a developmental improvement in the participants rather than just symptomatic improvements, future trials will have to be conducted over significantly longer time periods, particularly in older patients.

Despite all hopes, the drug tests of mGluR5 NAMs in humans showed much less effect than expected, suggesting that FXS might be more complex than initially thought. Recently Novartis has announced that the company will be discontinuing its development program in FXS for AFQ056 following negative results in a large international clinical trial in adults (reported in 2014) and in a trial in adolescents. In both placebo-controlled trials, patients taking AFQ056 did not show improvement over placebo in any outcome measure. The reasons why these clinical trials failed may be an inadequate dose range for Fragile X patients and an unexpected development of tolerance (www.fraxa.org). Dosage problems are relatively easy to correct, but tolerance may be something intrinsic to mGluR5 physiology. Indeed, in a preclinical study using high doses of mGluR5 antagonists, Yan et al., (2005) observed the development of tolerance and then the loss of drug activity in *Fmr1* KO mice.

Very recently (September 2014), Hoffmann La Roche also discontinued the FXS development programme for another mGluR5 NAM, RG7090 (RO4917523), based on the negative phase II clinical study results in adults and adolescents, and on a clinical study involving children.

Despite the repeated clinical failures, the mGluR5 pathway remains one of the most promising approaches for the treatment of FXS. Short term trials might be not sufficient to prove or disprove the efficiency of mGluR5 NAMs for the treatment of FXS. Future trials need to be carried out over a much longer time period and with many more participants to find out to what extent these drugs can be beneficial. Moreover, clinicians need to look at much younger participants in the study to really have a chance of impact, when there is the ability to change a growing brain, to alter pathways that are being laid down much earlier in life. In addition, the quality of the outcome measures available for Fragile X clinical trials seems to be inadequate. These outcome measures,

Table 4. Clinical trials with mGlu5-negative allosteric modulators (NAMs) for FXS.

Drug	Study Design	Sample size	Identification of the study	Reversed phenotypes	Issue	References
AFQ056 (Mavoglurant) Novartis pharmaceutical	Not randomized, open label	18 to 46 (males and females), **estimated 200 patients**	NCT01348087 First received: 2011/05 Last verified : 2014/05	Not released	Ongoing: Phase II	www.clinicaltrials.gov
AFQ056 (Mavoglurant) Novartis pharmaceutical	Not randomized, open label	12 to 18 (males and females), **Estimated 170 patients**	NCT01433354 First received : 2011/08 Last verified : 2014/05	Not released	Ongoing: Phase II and phase III	www.clinicaltrials.gov
AFQ056 (Mavoglurant) Novartis pharmaceutical	Randomized, double blind, placebo controlled	18 to 45 years (males and females), **175 patients**	NCT01253629 First received: 2010/12 Last verified: 2014/07	Not released	Completed: phase IIb	www.clinicaltrials.gov
AFQ056 (Mavoglurant) Novartis pharmaceutical	Randomized, double blind, placebo controlled	12 to 17 years (males and females), **139 patients**	NCT01357239 First received: 2011/05 Last verified: 2014/06	Not released	Completed: phase IIb	www.clinicaltrials.gov
AFQ056 (Mavoglurant) Novartis pharmaceutical	Randomized, parallel assessment, open label	3 to 11 years (males and females), **21 patients**	NCT01482143 First received 2011/11 Last verified: 2014/09	Not released	Completed: phase I	www.clinicaltrials.gov
AFQ056 (Mavoglurant) Novartis pharmaceutical	Open label pilot study, single dose	30 males (18-35 years), **30 patients** (7 patients with full Fmr1 promoter methylation)	NCT00718341 First received: 2008/07 Last verified: 2010/05	No significant effects of treatment on the primary outcome measure	Suspended for negative results (April 2014) Inadequate dose range; unexpected development of tolerance	www.clinicaltrials.gov www.fraxa.org Jacquemont et al., 2011
NPL-2009 (fenobam) Neuropharm	Randomized, open label, single dose	18 to 45 years (males and females), **12 patients**	NCT00637221 First received: 2008/03 Last verified: 2012/04	Improvement in communication and eye contact; Improvement in the PPI	Completed: Phase I and Phase II	www.clinicaltrials.gov Hessl et al. 2008; Berry Kravis et al. 2009
STX107 Seaside Therapeutics	Randomized, double blind, placebo controlled	18 to 50 years (males), **estimated 16 patients**	NCT01325740 First received: 2011/03 Last verified: 2012/12	Not released	Suspended: Phase II	www.clinicaltrials.gov www.fraxa.org
STX107 Seaside Therapeutics	Randomized, double blind, placebo controlled, single dose	18 to 50 years (males), **estimated 40 patients**	NCT00965432 First received: 2009/08 Last verified: 2010/02	Not released	Completed: Phase I	www.clinicaltrials.gov
RG7090 (RO4917523) Hoffmann-La Roche	Randomized, double blind, placebo controlled	14 to 50 years (males and females), **185 patients**	NCT01517698 First received: 2012/01 Last verified: 2014/10	Not released	Suspended: Phase II Closed for negative results	www.clinicaltrials.gov www.fraxa.org
RG7090 (RO4917523) Hoffmann-La Roche	Randomized, double blind, placebo controlled	5 to 13 years (males and females), **47 patients**	NCT01750597 First received: 2012/12 Last verified: 2014/10	Not released	Completed: Phase II Closed for negative results	www.clinicaltrials.gov www.fraxa.org
RG7090 (RO4917523) Hoffmann-La Roche	Randomized, double blind, placebo controlled	18 to 50 (males and females), **40 patients**	NCT01015430 First received: 2009/11 Last verified: 2014/10	Not released	Completed: phase II	www.clinicaltrials.gov

like the ABC and the Clinical Global Impression (CGI) scales, are insufficient instruments. There is little doubt that the "resolution" of these measurements is poor, and there is quite a bit of room for improvement in outcome measures for Fragile X clinical trials.

5.3. Other potential treatments

Increased knowledge about the role of FMRP has led to different therapeutic strategies for FXS. γ-aminobutyric acid (GABA) agonists are emerging as contenders for treating FXS. GABA is the main inhibitory neurotransmitter in the adult central nervous system, and binds to two types of receptors, i.e. the ionotropic $GABA_A$ receptor ($GABA_AR$) and the metabotropic $GABA_B$ receptor ($GABA_BR$). In FXS, inhibitory neurotransmission involving GABA is insufficient, and treatments can target either type of receptor. Deficits in GABA inhibition may underlie symptoms such as seizures, anxiety, and autistic-like behaviors in FXS (R. Hagerman, Hoem, & Hagerman, 2010). Recent findings indicate that *Fmr1* KO mice express lower mRNA and protein levels of $GABA_AR$ subunits, which is not surprising given that mRNAs encoding the $GABA_AR$ subunits are targets of FMRP (Curia, Papouin, Séguéla, & Avoli, 2009; D'Hulst et al., 2006; Gantois et al., 2013). Moreover, $GABA_AR$ agonists compensate for deficiencies in $GABA_AR$ subunits (R. Hagerman, Lauterborn, Au, & Berry-Kravis, 2012).

Drugs that bind to $GABA_AR$ include diazepam (benzodiazepine), ganaxolone (neuroactive steroid), and acamprosate (alchool deterrent). Administration of diazepam or ganaxolone, as been found to rescue audiogenic seizures in *Fmr1* KO mice (Heulens, D'Hulst, Van Dam, De Deyn, & Kooy, 2012). Open-label treatment with acamprosate, which inhibit NMDA receptors while activating $GABA_AR$, remarkably improved communication in adult patients with FXS and comorbid autism, and social behavior, attention, and hyperactivity in young-adult patients without causing significant adverse effects or changes in vital signs (C. A. Erickson et al., 2013; M. A. Erickson, Maramara, & Lisman, 2010).

$GABA_BR$ regulates cell excitability directly by enhancing K^+ channels (Lüscher, Jan, Stoffel, Malenka, & Nicoll, 1997; Newberry & Nicoll, 1984), and blocking Ca^{2+} channels (Lambert & Wilson, 1996; Mintz & Bean, 1993; Pfrieger, Gottmann, & Lux, 1994), and indirectly by affecting the release of glutamate (Sohn, Lim, Lee, & Ho, 2007). For instance, $GABA_BR$ on presynaptic glutamatergic neurons inhibits the release of glutamate, and consequently, the signaling downstream of mGluR5 (Isaacson & Hille,

1997). Therefore, the use of $GABA_BR$ agonists can indirectly restore the aberrant basal protein levels in FXS. Arbaclofen (STX209) is a $GABA_BR$ selective agonist. Its racemic mix, baclofen, has been used safely in the clinic for over thirty years, and the R-enantiomer shows promising results in treating FXS. Studies have shown that treatment with arbaclofen reduces mRNA translation and corrects the elevated basal protein synthesis in *Fmr1* KO mice, and corrects increased spine density in juvenile *Fmr1* KO mice (Berry-Kravis et al., 2012). It also reduces AMPAR internalization in cultured *Fmr1* KO neurons (Berry-Kravis et al., 2012). A randomized, double-blind, placebo-controlled cross-over trial showed that arbaclofen had a positive effect on social function as tested by the ABC social avoidance scale in the whole study population (Berry-Kravis et al., 2012). Together, the mGluR theory and the GABA hypothesis suggest that the imbalance between excitation and inhibition favors overall excitation in the FXS brain and that targeting either mGluR5 or GABARs can treat FXS symptoms.

In addition to the two major theories, others propose that disruptions at several other levels, among which the matrix metalloproteinase-9 (MMP-9), could also account for the abnormal phenotypes observed in FXS (Bilousova et al., 2009; Goebel-Goody et al., 2012; Rotschafer, Trujillo, Dansie, Ethell, & Razak, 2012). Minocycline treatment (which is thought to mediate its actions via matrix metalloproteinases (MMPs)), either *in vitro* or *in vivo*, corrects the hippocampal dendritic spine deficits in *Fmr1* KO mice. There are also behavioral improvements with minocycline in *Fmr1* KO mice in paradigms such as elevated plus maze indicating decreased anxiety as well as in hippocampal dependent Y-maze task showing improved spatial memory (Bilousova et al., 2009). Furthermore, the ultrasonic vocalizations produced by adult male mice during mating, which are reduced in *Fmr1* KO mice, can be rescued by minocycline treatment (Rotschafer et al., 2012). In a preliminary open-label study 50 children and adults with FXS receiving minocycline treatment for at least 2 weeks showed improvements in cognition, language, and behavior (Utari et al., 2010). In an open-label trial with minocycline in 20 patients with FXS aged 13-32 years, improvements were observed in the ABC-irritability subscale, global clinical improvements, and visual analog scale for behavior (Paribello et al., 2010). A double-blinded, placebo controlled, crossover trial with minocycline in children aged 3.5-16 years with FXS is now underway at the MIND institute, UC Davis.

Yet, another approach for treatment was tried by the group of Bear who show the 3-idrossi-3-metilglutaril-coenzima A (HMG-CoA) reductase inhibitor lovastatin can

inhibit Ras-ERK1/2 signaling in hippocampal neurons (W. Li et al., 2005) and the ERK1/2 signaling pathway lies upstream of the excessive hippocampal protein synthesis in the *Fmr1* KO mice (Osterweil et al., 2010; 2013). Moreover, lovastatin administered either orally or by injection was able to inhibit the expression of audiogenic seizures in *Fmr1* KO mice (Osterweil et al., 2013). It will be of interest to assess the effect of lovastatin treatment on the full spectrum of FXS phenotypes. Lovastatin is particularly exciting as a potential therapy in FXS because the drug has already been widely used for years and is approved for use in children with hypercholesterolemia, thus expediting the tests of its effectiveness in FXS patients.

Very recent studies proposed the rescue of several core hyperexcitability phenotypes of FXS by targeting BKCa channels. Acute pharmacological treatment with a BKCa channel opener, BMS-204352, corrected dendritic and cellular hyperexcitability defects, as well as sensory hypersensitivity in the somatosensory neocortex of *Fmr1* KO mice (Zhang et al., 2014). Moreover, acute treatment with BMS-204352 restored a normal phenotype in social, cognitive and emotional components by improving sociability, social and spatial recognition, and social/non-social anxiety in *Fmr1* KO mice (Hébert et al., 2014).

Although there is presently no cure for FXS, there are a variety of medications that can improve the behavior problems and neurological deficits seen in FXS. Not every Fragile X patients show the same phenotype, and not every patients will respond to each medication in the same way. Sometimes more than one medication is used to treat a combination of symptoms and sometimes medications are used together because of a synergistic effect. It seems that monotherapy could have relatively little effect, while a rational drug combination could be quite effective in a complex desease like FXS. However, it is important to remember that medication is not the only treatment for FXS. The use of therapy in the motor and language areas and special education intervention are also essential in the treatment program for Fragile X patients, especially for the young ones. Most children with FXS qualify for special education services. Education can be complemented by a variety of therapies that will help your child become more independent in the transitions from childhood through adolescence and into adulthood. Early intervention is important. Because a young child's brain is still forming, early intervention gives children the best start possible and the greatest chance of developing a full range of skills. The sooner a child with FXS gets treatment, the more opportunity there is for learning.

6. mGluR5/Homer crosstalk dysfunction in Fragile X Syndrome

Understanding the molecular mechanisms of the altered Group-I mGluR activation/signalling is important because mGluR5 antagonists are promising therapeutical agents in FXS (reviewed by (D'Antoni et al., 2014)). The core of pathophysiological mechanisms underlying FXS has been related to dysfunctional protein synthesis at synapses, which interferes with neuronal plasticity and cognitive functions. However, recent evidence indicates that some relevant phenotypes might be dependent also on other mechanisms suggesting that altered mGluR5 function is upstream of protein translation in FXS. The first studies aimed at investigating the expression of mGluR5 in the brain of FXS mouse models revealed no changes in protein expression and mRNA levels in hippocampal homogenates (Huber et al., 2002) and forebrain synaptosomes (Giuffrida et al., 2005). However, this data not esclude that an altered organization of the mGluR5 at the synapses may underlie the functional abnormalities in *Fmr1* KO mouse. Indeed, it has been found that although total mGluR5 levels are normal in *Fmr1* KO mouse, there is less mGluR5 in the PSD fraction due to the altered balance of mGluR5 association with short and long isoforms of the postsynaptic scaffolding protein Homer 1. mGluR5 is less associated with the long Homer isoforms although the basal levels of Homer 1 are not changed (Giuffrida et al., 2005).

An extensive litterature describes the association of mGluR5/Homer and the importance of this interaction for the regulation of several function of mGluR5, such as the constitutive activity (Ango et al., 2001), surface expression and trafficking (Ango et al., 2001; Coutinho et al., 2001), lateral diffusion (Sergé et al., 2002), and physical and functional crosstalk with NMDAR (Bertaso et al., 2010; Moutin et al., 2012). However, very little is known about the consequences of mGluR5/Homer crosstalk disruption in the context of FXS.

The altered balance in mGluR5 interactions with Homer isoforms might contribute to the mGluR5 dysfunction and pathophysiology of FXS. Disruption of mGlu5/Homer interaction by a cell-permeable TAT fused-peptide containing the proline-rich motif of the mGluR5 C-terminal inhibits Group-I mGluR activation of the PI3K-mTOR pathway, but does not affect ERK pathway, and inhibits mGlu-LTD (Ronesi & Huber, 2008). In *Fmr1* KO mice, activation of Group-I mGluR fails to activate mTOR pathway and induces LTD independently of Homer interaction (Ronesi

& Huber, 2008). Interestingly, mGlu5/Homer interaction exerts an inhibitory control on eEF2K, which in turn phosphorylates EF2, thus slowing the elongation step of translation and inhibiting general protein synthesis. This step is believed to favour the rapid synthesis of specific proteins (Parket al., 2008).

Moreover, Ronesi et al., (2012) crossed *Fmr1* KO mice with mice selectively lacking the Homer 1a isoform of Homer 1 and determined whether Homer 1a deletion restored mGluR5 function and Homer interactions, as well as neurophysiological and behavioral phenotypes of *Fmr1* KO mice (Ronesi et al., 2012). Deletion of Homer 1a, which shifts the equilibrium towards mGlu5-Homer association, restored increased rate of total protein synthesis in *Fmr1* KO mice to WT levels, but did not correct increased mGluR-LTD nor increased levels of "LTD proteins". Thus, disruption of mGlu5/Homer crosstalk is not involved in the abnormal translationalcontrol of FMRP target mRNAs. In contrast, Homer 1a deletion corrected prolonged UP states and open field activity phenotypes and reduced susceptibility to audiogenic seizures in *Fmr1* KO mice (Ronesi et al., 2012).

However, how the loss of FMRP leads to altered mGluR5/Homer crosstalk is still not clear. Protein levels of long Homers and Homer 1a are unchanged in total homogenates of *Fmr1* KO hippocampi (Giuffrida et al., 2005), and FMRP is not reported to interact with mRNA for any Homer isoforms (Darnell et al., 2011). Previous work reported a decrease in tyrosine phosphorylation of long Homer in *Fmr1* KO forebrain (Giuffrida et al., 2005), but it is unknown whether or how this affects interactions with mGluR5. Phosphorylation of Homer 3 regulates interactions with other Homer effectors (Mizutani, Kuroda, Futatsugi, Furuichi, & Mikoshiba, 2008). Similarly, phosphorylation of mGluR5 at the C-terminal Homer interaction domain reduces the affinity of mGluR5 for Homer (Orlando et al., 2009). Therefore, post-translational modification of mGluR5 and/or Homer in *Fmr1* KO mice may underlie the decreased interactions.

Recent data suggest an involvement of Homer 1 in autism. Kelleher et al., (2012) identified rare and potentially deleterious Homer 1 single-nucleotide variants (SNV) exclusively in a population of non syndromic autism cases compared to ethnically-matched controls, by high-throughput multiplex sequencing (Kelleher et al., 2012). Interestingly, all of the identified missense mutations alter residues, which are conserved among mammalian species. Two of these SNV localize to the EVH domain of Homer 1, one is located in a proline rich domain which is also important for interaction with mGluR or Homer 1 homo-multimerization, and a fourth one is located in the 3'

untranslated region within a cluster of predicted microRNA binding sites, with possible consequences in Homer 1 mRNA translation and protein expression (Kelleher et al., 2012).

All together these findings suggest that Homer 1 dysfunction may play a key role in the pathophysiology of FXS and related autistic disorders. Works in FXS show that while some phenotypes are critically dependent on mGluR-activated protein synthesis, others implicate additional mechanisms such as mGluR5/Homer dysfunction. Thus, the modulation and restoration of mGluR5/Homer interactions may represent a new therapeutic strategy for FXS and related cognitive and autistic disorders. However, future experimets are required for a deep undertanding of the role of mGluR5/Homer crosstalk disruption in the pahtophysiology of FXS.

7. Aim of the study

Aletred mGluR5 function is causally associated with the pathophysiology of FXS (reviewed by (D'Antoni et al., 2014)). Accumulating evidence indicates that many of the protein-synthesis-dependent functions of mGluR5 are exaggerated in *Fmr1* KO mice. These findings motivated the "mGluR theory of FXS" which posits that multiple FXS phenotypes are rescued in the *Fmr1* KO mouse by pharmacological inhibition or genetic reduction of mGluR5. Based on these promising results, mGluR5 antagonists were introduced in clinical trials in FXS patients. However several clinical trials employing novel mGluR5 antagonists have recently been cancelled due because of lack of efficacy of the treatment.

An improved understanding is thus needed to determine the core molecular deficits of FXS and provide novel targets for pharmacological intervention. Indeed, the cellular mechanisms of mGluR5 dysfunction in FXS have been elusive and most of the studies have been focused just on the altered translational processes at the synapse initiated by mGluR5 activation. A clue comes from the findings that in *Fmr1* KO mice mGluR5 is less associated with the synaptic scaffolding molecule Homer suggesting alteration in mGluR5 targeting and signalling (Giuffrida et al., 2005; Ronesi et al., 2012). Therefore, the goal of my thesis is to study the consequences of mGluR5/Homer crosstalk disruption in *Fmr1* KO mice, in term of properties and functions of mGluR5, such as distribution during development, surface expression and axonal/dendritic targeting, agonist-induced internalization, surface dynamics and mGluR5-mediated modulation of NMDAR function. To achieve this purpose we used a powerful combination of techniques such as immunofluorescence and biochemical approaches together with live-cell imaging and single-molecule tracking and, electrophysiological methods in hippocampal neurons from *Fmr1* KO and WT mice.

This study will lead to the identification of new mechanisms that are altered in the brain of *Fmr1* KO mice and will therefore be helpful for a better understanding of the pathophysiology of FXS. Results of this work might help to develop new selective pharmacological strategies for the treatment of FXS.

CHAPTER II

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Metabotropic Glutamate subtype 5 receptors are increased at synapses and do not undergo agonist-induced internalization in the Fmr1 KO mouse model of Fragile X Syndrome

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ABSTRACT

Fragile X syndrome (FXS) is a common form of inherited intellectual disability and autism caused by the lack of Fragile X Mental Retardation Protein (FMRP), an RNA binding protein involved in RNA metabolism and protein synthesis. A substantial number of studies supports a key role of group I metabotropic glutamate receptor subtype 5 (mGlu5) in the pathophysiology of FXS. In particular, an exaggerated response to activation of mGlu5 receptors may underlie synaptic dysfunctions in this disorder. However, the expression of mGlu5 receptors in the brain of *Fmr1* knockout (KO) mice, the animal model of FXS, has not been systematically investigated. We have previously reported that in the brain of *Fmr1* KO mice, mGlu5 receptors are less associated with the constitutive forms of Homer proteins, suggesting alterations in mGlu5 receptor targeting and signalling. Here we report that a) mGlu5 receptors are more expressed in hippocampal synaptosomes of juvenile, but not adult *Fmr1* KO mice, whereas only a trend towards an increased expression was found in the cortex at young

age; b) mGlu5 receptors are more expressed on the cell surface and differently targeted to dendrites and axons in *Fmr1* KO hippocampal neurons in culture; c) mGlu5 receptors do not undergo internalization after a sustained exposure to the group I mGlu receptor agonist, (S)-3,5-Dihydroxyphenylglycine (DHPG), in *Fmr1* KO neurons; d) mGlu5 receptor-stimulated polyphosphoinositide hydrolysis is enhanced in young *Fmr1* KO mice with respect to wild type mice. These results suggest that mGlu5 receptors are dysregulated in the absence of FMRP, with a major effect during development.

INTRODUCTION

Fragile X syndrome (FXS) is an X-linked developmental disorder, which represents the most common form of inherited cognitive disability and a leading genetic cause of autism (Abrahams and Geschwind, 2008; Kelleher and Bear, 2008). In most cases FXS is caused by transcriptional silencing of *FMR1* gene which leads to the lack of the Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein involved in the regulation of transport and translation of target mRNAs (Bassel and Warren, 2008; Pfeiffer and Huber, 2009; Bagni and Oostra, 2013).

Over the last fifteen years, a remarkable number of studies have established a crucial role for metabotropic glutamate receptor subtype 5 (mGlu5) in the pathophysiology of FXS (reviewed in Dölen et al., 2010; Krueger and Bear 2011; Bhakar et al., 2012; D'Antoni et al., 2014). Based on the pivotal observation that mGlu5 receptor-mediated, protein synthesis-dependent, long-term depression (LTD), is enhanced in the *Fmr1* knockout (KO) mouse model of FXS, it was proposed that interventions aimed at reducing mGlu5 receptor signalling might be useful in the treatment of FXS (Bear et al., 2004). Accordingly, negative allosteric modulators or genetic deletion of mGlu5 receptors have been found to correct most of the pathological hallmarks of *Fmr1* KO mice (Dölen et al., 2007; Chang et al., 2008; Hays et al., 2011; Levenga et al., 2010; Pop et al., 2014).

Despite the number of studies looking at the possible therapeutic effect of mGlu5 receptor blockade in FXS, the expression of mGlu5 receptors has not been systematically investigated. We decided to re-examine this question because, of the 842 FMRP mRNA targets recently identified using a stringent high-throughput sequencing–cross-linking immunoprecipitation (HITS-CLIP) method, a significant fraction is represented by mGlu5 mRNAs (Darnell et al., 2011). Thus, mGlu5 receptor protein

expression might be abnormal in FXS as a consequence of the lack of FMRP control on mGlu5 mRNA translation.

mGlu5 receptor properties might also be affected in FXS as a consequence of a disrupted interaction with Homer proteins, a class of scaffolding proteins which anchor mGlu5 receptors to post-synaptic density (Giuffrida et al., 2005). Recent data have shown that disrupted Homer scaffolds may cause several phenotypes of *Fmr1* KO mice, including changes in mGlu5 receptor signaling, neocortical circuit dysfunction, and behavior abnormalities, supporting the view that an abnormal interaction between mGlu5 receptors and Homer proteins contributes substantially to the pathophysiology of FXS (Ronesi et al., 2012). Here, we examined whether different properties of mGlu5 receptors that at least partially depend on receptor interaction with Homer proteins, such as surface expression, axonal distribution, and agonist-induced internalization (Coutinho et al., 2001; Ango et al., 2002) are altered in *Fmr1* KO mice.

We found that (i) both synaptic expression of mGlu5 receptors and mGlu5 receptor-mediated polyphosphoinositide (PI) hydrolysis were enhanced in young *Fmr1* KO mice as compared to age-matched wt mice; (ii) surface distribution and axonal targeting of mGlu5 receptors were increased in *Fmr1* KO hippocampal neurons, as a consequence of mGlu5/Homer disruption; and, (iii) agonist-induced internalization of mGlu5 receptors was absent in *Fmr1* KO neurons, but independently of mGlu5/Homer interaction.

MATERIALS AND METHODS

Animals

We used brains of *Fmr1* KO mice (FVB strain) and their WT littermates, which derive from a colony of *Fmr1* KO mice originally provided by Prof. Ben Oostra. Wild-type and *Fmr1* KO mice were kept under environmentally controlled conditions with food and water ad libitum. Genotypes were determined by PCR analysis of DNA extracted from tails. The primers used were the same as those indicated in the original paper describing these animals (Dutch-Belgian Fragile X Consortium, 1994). All experiments were conducted in compliance with the European Council Directive (86/609/EEC) and the Italian Animal Welfare Act for the use and care of laboratory animals.

UV-crosslinking and immunoprecipitation mRNA interaction confirmation assay

To isolate mRNAs associated with FMRP *in vivo*, UV-crosslinking and immunoprecipitations were performed on total brain extracts obtained from WT and *Fmr1* KO mice at post natal day 12 (P12) using the protocol described by Ule et al. 2005. For each assay, 10µg of affinity-purified anti-FMRP antibody (R60 polyclonal antibody directed against the C terminus of FMRP) was used to immunoprecipitate 1mg of brain lysate. Approximately $1/100^{th}$ of the homogenate and $1/20^{th}$ of the immunoprecipitate were loaded on an 11% SDS-PAGE gel. Proteins transferred onto a 0.45µm nitrocellulose membrane were revealed using the mAb1C3 recognizing FMRP and the 3Fx antibody recognizing both FXR1P and FXR2P. mRNA was extracted from brain homogenate and immunoprecipitates using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and reverse-transcribed (RT) using the SuperscriptScriptII RT-PCR system (Invitrogen). RT products were subjected to polymerase chain reaction (PCR), using a PCR Master Kit (Promega) and primers, specific for Mtap1b (microtubule-associated protein 1B) (mRNA target of FMRP), Tubb3 (Tubulin beta-3) (negative control, Davidovic et al., 2011) and mGlu5 receptors cDNAs. The PCR program consisted in 10 min of initial denaturation at 95°C, followed by n cycles of: 30 sec at 95°C, 30 sec at 58°C, 30 sec at 72°C and a final elongation step of 10 min at 72°C. PCR products were visualized on a 1.5% TAE agarose gel and amplicon size was verified using the 1 Kb + DNA ladder (Invitrogen).

Crude synaptosomes

Crude synaptosomes were prepared according to Grilli et al. (2012), with minor modifications. Briefly frozen hippocampi and cortex from WT and *Fmr1* KO mice at different ages (P21, P45 and 3 or 8-15 months) were allowed to thaw on ice, weighed and homogenized in 10% (w/v) 0.32 M sucrose buffer containing Ethylenediaminetetraacetic acid (EDTA 1 mM, Sigma), Tris-HCl (10 mM, pH 7.4, Sigma), phenylmethanesulfonyl fluoride (PMSF 0.5 mM, Sigma) and Protease Inhibitor Cocktail (Roche). The homogenate was centrifuged for 10 min at 1000 × g (4°C) to separate the nuclear pellet. The supernatant was then centrifuged for 40 min at 20,000 × g (4°C), and the resulting pellet was resuspended in Tris-HCl (40 mM), pH 6.8, containing PMSF (0.5 mM). Protein content was determined by using the bicinchoninic acid method (BCA kit, Pierce Chemical Company).

Western Blot analysis

Proteins were denatured in denaturating sample buffer (4X) at 37°C for 5 min and then loaded onto 8% SDS-polyacrylamide gels. Gels were electroblotted to nitrocellulose membranes (Hybond C-extra, 0.45 mM, Amersham Biosciences). Filters were blocked in 5% non-fat dried milk in TBST [Tris (100 mM, Sigma), NaCl (0.9%, Sigma) and Tween 20 (1%, Sigma)], and then incubated overnight with the following primary antibodies: anti-mGlu5 (rb, 1:2000, Millipore), anti-actin (rb, 1:1000, Cell Signaling), anti-GAPDH (rb, 1:1000, Cell Signaling) and anti-tubulin (rb, 1:1000, Cell Signaling). After washing with TBST, filters were incubated with the anti-rb secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences). The signal was revealed by a chemiluminescent detection method (ECL plus, Amersham Biosciences) and quantified by computer-assisted densitometry, using the VersaDoc 4000 Imaging System (Biorad). The percentage of mGlu5 receptor surface expression was determined by normalising the bound optical density value to the unspecific band density values. Data were statistically analysed using t-test.

Hippocampal cultures

For immunocytochemistry hippocampal cultures were prepared from P0-P1 newborn pups of litters obtained by mating a heterozygous female with a *Fmr1* KO male. All experiments were performed without previous knowledge of the genotype of the culture being tested. The genotype of each pup was defined by PCR (Musumeci et al., 2007). Hippocampi from individual newborn pups were dissected, trypsinized with Trypsin 0,25% (Sigma) and plated (≈60.000 cells/cm^2) onto 35 mm cell culture dishes (Nunc) coated with poly-L-ornithine (10 μg/ml, Sigma). Hippocampal neurons were plated in Neurobasal medium (GIBCO) supplemented with B27 (2%, GIBCO). AraC (5 μM, Sigma) was added on DIV 4. Culture medium was changed every 7 days. Only cultures from WT and Fmr1 homo- and hemyzygous pups only were used.

For biotynilation assay, cultures were prepared separately from litters of WT and Fmr1 KO mice as described above and plated at high density (see below).

Immunocytochemistry

The expression of mGlu5 receptors was studied by using antibodies which recognize the -NH_2 terminal of the mGlu5 subunit in non-permeabilized cells (surface) or antibodies which recognized its -COOH terminal in permeabilized cells

(intracellular). After removing the medium, cell cultures were incubated, without permeabilization, with a primary monoclonal antibody anti-mGlu5-NH_2 (ms, 1:5, gift of Prof. Shigemoto) for 45 min at 37°C. Subsequently, cultures were fixed with 4% paraformaldeyde (PFA, Merck) and 4% sucrose (Sigma) for 15 min at room temperature (RT) and then incubated for 45 min at RT with the secondary Cy3 fluorescent antibody (anti-ms, 1:500, Jackson Immunoresearch). After incubation with secondary antibodies, cultures were fixed again for 5 min, permeabilized in PBS containing Triton (2%, Sigma) for 10 min and then incubated with blocking solution containing NGS (4%, Vector) for 20 min at RT. Afterwards, cultures were incubated for 1 hour and 30 min at RT with the following primary antibodies: anti-MAP-2 (rb, 1:1000, Millipore) and anti-Tau-1 (ms, 1:150, Millipore). After washing, cultures were incubated for 45 min at RT with the appropriate secondary fluorescent antibodies (FITC DyLight488 anti-rb, 1:250, Jackson Immunoresearch; Cy5 anti-ms, 1:500, Jackson Immunoresearch). For double-labelling experiments using the anti-mGlu5-COOH antibody, cells were fixed and permeabilized as above indicated and then incubated with anti-mGlu5-COOH (rb, 1:4000, Millipore; secondary antibody Cy3 anti-rb, 1:500, Jackson Immunoresearch) and anti-Tau-1 (ms, 1:150, Millipore; secondary antibody FITC anti-ms, 1:500, Jackson Immunoresearch).

Surface Biotinylation assay

Experiments were carried out on high density (≈150.000 cells/cm^2) hippocampal cultured neurons at 13 DIV from WT and *Fmr1* KO mice. After removing the medium, cells were washed in PBS (Gibco) containing Ca^{2+} (0.1 mM) and Mg^{2+} (1 mM) and treated with PBS containing 1 mg/ml sulfo-NHS-LC-biotin (Pierce Chemical Company) on ice for 30 min. Then, cells were quenched with glycine (100 mM, Sigma), washed in PBS and then lysed in lysis buffer containing Triton (1%, Sigma), sodium dodecyl sulphate (1%, Invitrogen), EDTA (1 mM, Sigma), sodium chloride (NaCl 50 mM/L, Sigma), Tris (50 mM/L, Sigma), PMSF (1 mM/L, Sigma), sodium fluoride (50 mM/L, Sigma), sodium orthovanadate (1 mM/L, Sigma), sodium deoxicolate (5%, Sigma) and Protease Inhibitor Cocktail (Roche). Cell lysates were clarified by centrifugation (14000 × g for 15 min) and protein content was determined using the BCA method. 10 μg were used as control (input). 80 μg of biotinylated surface proteins were incubated with agarose-conjugated NeutrAvidin (Thermo Scientific) on a head-over-head shaker for 5

hours at 4°C, washed with lysis buffer, eluted with sample buffer (4X) and processed for Western blotting analysis.

Agonist-induced internalization of mGlu5 receptors

After two weeks of culturing, cultures were incubated with primary antibody mGlu5-NH_2, without permeabilization, for 45 min at 37°C and, after two quick washes, were incubated with the agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG, 100 μM, Tocris) for 10 and 30 min. Afterwards, the cultures were washed three times in artificial cerebrospinal fluid (ACSF) [in mmol/L: sodium chloride 124 (MP Biomedicals); potassium chloride 3.0 (Carlo Erba); sodium phosphate 1.2 (Merck); magnesium sulfate 1.2 (Merck); calcium chloride 2.0 (Merck); sodium bicarbonate 26 (Sigma); D-glucose 10 (Merck), pH 7.3] and then cells were fixed using 4% PFA and 4% sucrose for 15 min at RT and incubated with the secondary fluorescent Cy3 anti-mouse antibody (1:500; Jackson Immunoresearch).

Experiments of internalization in the presence of Tat-fused peptides

We used the following Tat-fusion peptides, which were kindly provided by Dr. K.M.Huber, University of Texas-Southwestern Medical Center: the membrane permeable mGlu5 C-terminal Homer-binding (decoy) peptide, mGluR5CT (YGRKKRRQRRRALTPPSPFR), containing a proline-rich binding motif (PPxxF) that can disrupt mGlu5/Homer interaction, and its control peptide, the mGluR5MU (YGRKKRRQRRRALTPLSPRR) with a mutated Homer binding motif that is unable of binding Homer (Tu et al., 1998; Mao et al., 2005; Ronesi et al., 2012). After two weeks of culturing, 5 μM of both the Tat-fused mGluR5CT and mGluR5MU peptides were applied for 1 hour, after which coltures were incubated, always in presence of mGluR5CT and mGluR5MU peptides, with the primary antibody mGlu5-NH_2 without permeabilization for 45 min at 37°C. Cultures were then quickly washed with ACSF and exposed to DHPG (100 μM) for 30 min in incubation with mGluR5CT and mGluR5MU peptides. Afterwards, cultures were washed three times in ACSF and cells were fixed using 4% PFA and 4% sucrose for 15 min at RT and incubated with the secondary fluorescent Cy3 anti-mouse antibody (1:500; Jackson Immunoresearch). After incubation with secondary antibody, cultures were fixed again for 5 min, permeabilized in PBS containing 0.2% Triton for 10 min and then incubated with blocking solution containing NGS 4% for 20 min at RT. Afterwards, cultures were incubated for 1 hour

and 30 min at RT with the primary antibody anti-Tau-1 (ms, 1:150, Millipore) and then for 45 min with the secondary fluorescent FITC anti-ms antibody (1:500, Jackson Immunoresearch).

Microscope analysis

Images were obtained with the LSM-510 Meta confocal microscope (Zeiss) using a 63X lens and the quantitative analysis was performed blind using the free online software NIH ImageJ (http://rsb.info.nih.gov/ij/index.html). The threshold for mGlu5-positive fluorescent clusters (or puncta) was fixed choosing the values so that the objects to be counted are distinct. Then, with the function Analyze Particles we choose the limits for the dimension of particles to be counted and this dimension is calculated in pixel^2. Our clusters were ≈ 1–2.5 μm in size and only puncta lying along soma and proximal processes interpreted as dendrites or axons were counted. The results are presented as mean + SEM and were evaluated using a One- or Two-Way ANOVA test, as appropriate, followed by post hoc Holm-Sidak method for multiple comparison. For quantitative comparisons, the number of puncta per 50 μm length of dendrite or axon was expressed for the mGlu5 antibody within a given field.

Measurement of PI hydrolysis in cortical slices

Receptor agonist-stimulated PI hydrolysis was measured in cortical slices, as described by Nicoletti et al. (1986). Male WT (FVB strain) at PND8, 1 month, and 4-5 months of age (Charles River, Calco, Italy) and age-matched *Fmr1* KO mice, were killed by decapitation and fresh cortices were sliced (350×350 μm) using a Mc Ilwain tissue chopper. Slices were incubated at 37°C under constant oxygenation for 30-45 min in Krebs-Hensleit buffer equilibrated with 95% O_2, 5% CO_2 to pH 7.4. 40 ml of gravity packed slices were then incubated for 60 min in 250 μl buffer containing 1 μCi of myo-[3H]inositol. [3H]-Myo-inositol (18 Ci/mmol) was purchased from GE Healthcare (Milano, Italy); DHPG was purchased from Tocris Coockson (Bristol, UK).

Slices were incubated with LiCl (10 mM, for 10 min) followed by the mGlu1/5 receptor agonist, DHPG (3, 10, 50 and 100 mM). After 1 h,, the incubation was stopped by addition of 900 μl of methanol:chloroform (2:1), after washing the slices with ice-cold buffer. After further addition of 300 μl chloroform and 600 μl water, samples were centrifuged at 10,000 × g for 2 min to facilitate phase separation. The [^{3}H]InsP present in the supernatant was separated by anion exchange chromatography in 10 ml columns

containing 1,5 ml of Dowex 1-X-8 resin (formate form, 100-200 mesh, BioRad) using increasing concentrations of formate salts as mobile phase (Nicoletti et al., 1986). Data were statistically analysed using by Two-way ANOVA followed by post-hoc Holm Sidak method.

RESULTS

Expression of mGlu5 receptors was higher in hippocampal synaptosomes of juvenile *Fmr1* KO mice

Using the CLIP assay (Ule et al. 2005), we confirmed that the mRNA encoding for the mGlu5 receptor is bound to FMRP (see Darnell et al., 2011) (data not shown). We therefore wondered whether mGlu5 receptor protein expression was altered *Fmr1* KO mice. Because FMRP expression is developmentally regulated, we measured the expression of mGlu5 receptors in crude synaptosomes prepared from the hippocampus and cerebral cortex of wt and *Fmr1* KO mice at different ages (PND21, PND45, 3 months, and 8-15 months) by Western blotting. mGlu5 receptor expression was significantly higher in hippocampal synaptosomes of juvenile *Fmr1* KO (PND21), as compared to age-matched wt mice (+ 47%). A trend to an increase was found in hippocampal synaptosomes of *Fmr1* KO mice at PND45 and cortical synaptosomes at PND21 and PND45 of *Fmr1* KO mice as compared to age-matched wt mice. No changes were found in older *Fmr1* KO mice (Figure 1A-D). These results suggested that the lack of FMPR caused changes in synaptic expression/targeting of mGlu5 receptors that vanished with age.

Expression of mGlu5 receptors was higher in cultured *Fmr1* KO mouse hippocampal neurons at early developmental stages

The expression of mGlu5 receptors was also examined by ICC with an C-ter antibody in hippocampal cultured neurons at different DIV (3, 7, 13 and 17) (Figure 2). mGlu5-C-ter staining was mostly present in cell bodies and dendrites in the vast majority of neurons of both wt and *Fmr1* KO neurons. Staining was clearly detectable in the proximal portion of axons in wt neurons, whereas it was present along the total length of the axon in *Fmr1* KO neurons (Figure 2A,C). Semiquantitative analysis of the signal in the cell body and proximal dendrites showed that mGlu5 receptor expression was significantly increased in *Fmr1* KO cultures at 3 and 7 DIV (+19% and +23%,

respectively) but not at 13 and 17 DIV (Figure 2B). This developmental profile of expression was reminiscent of that seen in hippocampal synaptosomes (see above). Interestingly, FMRP also showed a decremental pattern of expression across neuronal maturation in culture. At 7 DIV, when the total length of axons could be analysed, expression of mGlu5 receptors declined from the proximal to the distal portion of the axons in wt neurons, whereas it was homogeneously high across the whole axon lenght in *Fmr1* KO neurons (Figure 2C).

Surface expression of mGlu5 receptors was higher in hippocampal neurons of *Fmr1* KO mice

We found previously that, although total receptor levels were similar in synaptic membranes prepared from wt and *Fmr1* KO mice, mGlu5 receptors were more easily extractable with mild detergents in *Fmr1* KO mice because of a reduced association with the long isoforms of the postsynaptic scaffolding protein Homer (Giuffrida et al., 2005). Knowing that mGlu/Homer interaction facilitates intracellular retention of mGlu5 receptors (Coutinho et al., 2001; Ango et al., 2002), we tested the hypothesis that surface expression of mGlu5 receptors could be increased in *Fmr1* KO mice. We studied the surface expression of mGlu5 receptors in hippocampal cell cultures prepared from wt and *Fmr1* KO mice by ICC with an antibody recognizing the N-terminal region of mGlu5 receptors, and with a biotinylation assay followed by Western blotting analysis.
ICC experiments performed in non-permeabilized cultures at 3, 7, 13 and 17 DIV (Figure 3A) showed a clear surface expression of mGlu5 receptors, which appeared clustered along the total surface of neurons, and were more concentrated in the cell body and dendrites than in axons. Staining analysis in the cell body and proximal dendrites showed a significant increase in the cell surface expression of mGlu5 receptors in *Fmr1* KO mouse cultures at all maturational stages, including 13 and 17 DIV (Figure 3C) These data were confirmed with the biotinylation assay carried out at 13-16 DIV, showing an increased surface expression of mGlu5 receptors in *Fmr1* KO neurons with no changes in total expression (input) (Figure 3B, D).

mGlu5 receptors are refractory to agonist-induced internalization in *Fmr1* KO neurons

We examined whether alterations in the process of agonist-induced internalization of mGlu5 receptors could contribute to the observed increase in surface

expression of mGlu5 receptors in *Fmr1* KO neurons. Exposure of cultured hippocampal neurons to the mGlu1/5 receptor agonist, DHPG (100 μM), for 10 or 30 min caused the internalization of surface mGlu5 receptors in wt neurons, but not in *Fmr1* KO neurons, as assessed by the analysis of puncta in the cell body and proximal dendrites (Figure 4A-C).

Disruption of mGlu5/Homer binding is responsible for increased mGlu5 surface expression but does not affect agonist-induced internalization

To investigate whether the high surface expression of mGlu5 receptors in *Fmr1* KO neurons was caused by an altered mGlu5 receptor-Homer interaction, we incubated both wt and *Fmr1* KO hippocampal cultures with the cell-permeable (Tat-fused) peptide mGlu5CT, which contains the prolin-rich motif of the mGlu5 receptor C-terminal tail that binds the EVH1 domain of Homer and is is able to disrupt the mGlu5/Homer interaction (Tu et al., 1998, Mao et al. 2005, Ronesi et al., 2008, 2012). As control, we used a peptide with a mutated Homer binding motif, mGlu5MU, which has no effect on mGlu5/Homer interaction (Mao et al., 2005; Ronesi et al., 2012). As expected, an increased surface expression of mGlu5 receptors was observed in wt cultures incubated with the mGlu5CT peptide, but not with the mGlu5MU control peptide (Figure 5A). In contrast, no changes in the surface expression of mGlu5 receptors were caused by the mGlu5CT peptide, in *Fmr1* KO neurons (Figure 5B).

Interestingly, incubation with the mGlu5CT peptide did not affect agonist-induced internalization of mGlu5 receptors in wt neurons and had no influence on the refractoriness of *Fmr1* KO neurons to agonist-induced internalization (Figure 5A, B).
The effect of incubation with the mGlu5CT peptide was also examined in the axons of wt and *Fmr1* KO neurons using the N-ter mGlu5 antibody (see above). Incubation with the mGlu5CT peptide enhanced surface expression of mGlu5 receptors along the total axonal length in wt neurons (Figure 5C), but not in *Fmr1* KO neurons (Figure 5D).
These data suggest that the increased surface expression and the increased axonal targeting of mGlu5 receptors in *Fmr1* KO neurons are secondary to a disrupted mGlu5/Homer interaction, whereas other mechanisms may account for the lack of mGlu5 receptor internalization.

Enhancement of mGlu5 receptor-mediated PI hydrolysis in the cerebral cortex of juvenile *Fmr1* KO mice

DHPG-stimulated PI hydrolysis was examined in the cortical slices of wt and *Fmr1* KO mice at different ages (PND8, 1 month, and 4 months). We could not perform the analysis in hippocampal slices because of the limited amount of tissue for slice preparation. As expected, DHPG stimulated PI hydrolysis to a much greater extent in neonate mice (Nicoletti et al., 1986). At PND8, maximally effective concentrations of DHPG (100 μM) increased [^{3}H]InsP formation by 5 and 7 fold in wt and *Fmr1* KO mice, respectively. The action of DHPG was entirely antagonized by the mGlu5 receptor NAM, MPEP (10 mM). The concentration-response curve at PND8 showed that DHPG had an increased efficacy in stimulating PI hydrolysis in *Fmr1* KO mice (Figure 6A). At 1 month of age, maximally effective concentrations of DHPG stimulated [3H] InsP formation by about 60% and 80% in wt and *Fmr1* KO mice, respectively. At this age, DHPG displayed both an increased potency and efficacy I stimulating PI hydrolysis in cortical slices from *Fmr1* KO mice. At 4 months of age, DHPG lost its ability to stimulate PI hydrolysis in cortical slices, and no difference was found between wt and *Fmr1* KO mice (Figure 6C).

DISCUSSION

In the present paper we provide evidence that mGlu5 receptors are dysregulated in the absence of FMRP in a complex manner. Overall our data suggests that mGlu5 receptor expression is up-regulated in FXS, an effect which may results from a combination of convergent and different mechanisms: 1) a FMRP-mediated inhibition of protein synthesis, whose consequence is more pronounced early during development; 2) a mGlu5/Homer disruption which cause an increased surface and axonal targeting of receptors; 3) lack of agonist induced desensitization, which is independent from mGlu5/Homer interaction.

We observed that the expression of mGlu5 receptors is higher in synaptic preparation from *Fmr1* KO than WT mice, with a major effect in the juvenile hippocampus. We have confirmed that mGlu5 mRNA is a FMRP target in the hippocampus at P13 and thus we believe that this early effect is caused by the lack of FMRP-mediated inhibitory control on mGlu5 receptor synthesis, which is more evident in developmental windows when both FMRP and mRNA encoding mGlu5 receptor are highly expressed (Catania et al., 1994; Lu et al., 2004; Davidovic et al., 2011; Bonaccorso et al., submitted). Accordingly, in hippocampal cell cultures the increased

expression of mGlu5 receptor protein in *Fmr1* KO neurons was observed using the anti-mGlu5-COOH antibody at early developmental stages (P3-P7), when also FMRP is highly expressed (Bonaccorso et al., submitted). In line with a major effect during development, we also found that DHPG-induced PI hydrolysis, the canonical signal transduction mechanism coupled to the activation of mGlu5 receptors, was higher at P8 and 1 month in the cortex from *Fmr1* KO mice, but was not affected in the adult, when mGlu5-mediated PI hydrolysis is negligible (Nicoletti et al., 1986). Although mGlu5 receptor-mediated signalling has been found to be dysregulated in *Fmr1* KO mice (Hou et al., 2006; Ronesi et al., 2008, 2012), no change in the expression of mGlu5 receptors has been detected before (Huber et al., 2002; Giuffrida et. 2005). It is possible that small differences were missed in previous studies because this issue was addressed in homogenates (Huber et al., 2002) or in synaptic preparation from total forebrain where a specific regional effect might be masked (Giuffrida et al., 2005). Intriguingly, in Drosophila, which contains a single *Fmr1* ortholog (Zarnescu et al., 2005) and a single functional *mGluR* (DmGluRA) ortholog of vertebrate group II mGluRs (Parmentier et al. 1996), an increase of DmGluRA has been detected, suggesting a FMRP-mediated inhibitory control on DmGluRA synthesis (Kanellopoulos et al., 2012). More importantly, an increased mGlu5 receptor binding density and protein expression have been detected in pre-frontal cortex of FXS patients (Lohith et al., 2013). In addition, an inverse correlation between levels of FMRP and mGlu5 receptors have been found in the cortex and cerebellum of children with autism corroborating the idea that FMRP might be a key regulator of mGlu5 expression and that mGlu5 over-expression might be related to autism (Fatemi et al., 2011a,b).

In addition to an increased expression at juvenile age in the hippocampus and in hippocampal neurons at 3-7 DIV, we detected an increased surface expression of mGlu5 receptors, which was detected at any developmental stages in cultures and was critically dependent on disruption of mGlu5-Homer binding. mGlu5/Homer interaction is responsible for mGlu5 receptor cytoplasmic retention in HEK and neurons (Ango et al., 2002), although underlying mechanisms have remained elusive. The lack of effect of mGluR5CT peptide in *Fmr1* KO neurons, where mGlu5 are more expressed on the surface confirm that a disrupted mGlu5/Homer interaction is responsible for the increased surface expression of mGlu5 receptors in *Fmr1* KO neurons. Interestingly, we observed that mGlu5 staining is present not only in the soma and dendrites, but also in the axons with both anti-mGlu5-COOH and anti-mGlu5-NH_2 antibodies. Axonal puncta

were detected in distal parts of axons in *Fmr1* KO neurons, whereas only the more proximal segments were labelled in WT neurons. The detection of mGlu5 staining in the distal axonal segments was mimicked by disruption of mGlu5/Homer interaction in WT, whereas it was not affected in *Fmr1* KO mice, as expected. This result was predicted since mGlu5/Homer binding has been shown to preclude axonal targeting in neurons (Ango et al., 2000). It is difficult to speculate on the functional significance of the increased axonal mGlu5 expression in FXS. Functional pre-synaptic mGlu5 have been detected in nerve terminals where they function by facilitating glutamate release upon depolarization (Thomas et al., 2000; Musante et al, 2008). It is possible that an increased mGlu5 presynaptic expression in FXS, particularly early in development may influence neuronal excitability, synaptic plasticity and activity-dependent synaptic stabilization.

Another finding that suggests a dysregulation of mGlu5 receptors in FXS that goes in the direction of an increased mGlu5 activity is the lack of agonist-induced internalization in *Fmr1* KO neurons. Differently than increased surface expression, lack of induced internalization was not mimicked by mGlu5/Homer disruption, suggesting additional mechanisms. A possible mechanism is an altered regulation of the protein kinase C mediated phosphorylation of mGlu5 receptors which inhibits binding to calmodulin, a critical step for mGlu5 receptor internalization (Lee et al., 2008). Another possibility is a dysregulation of the GRK/b arrestin mediated pathway involved in receptor desensitization (Ferguson et al., 2002). Several proteins involved in these pathways are encoded by mRNAs which are bound to FMRP (Darnell et al., 2011), and indeed 62% of proteins identified as an mGlu5 complex by a synaptic mouse proteome data base (Croning et al., 2009) are FMRP targets (Darnell et al., 2011).

Increasing evidence suggests that an increased expression/activity of group I mGlu receptors might be implicated in the pathophysiology of different autism spectrum disorders (see D'Antoni et al., 2014). In particular, an increased expression of mGlu1 and mGlu5 receptors has been found in different brain regions in several models of syndromic and non syndromic autism spectrum disorders (Chen et al., 2011, Wan et al., 2011, Baudouin et al., 2012). In line with this view, a recent paper reports a striking up-regulation of mGlu5 receptors in the hippocampal synaptosome fraction from mice carrying a homozygous Shank3 mutation that deletes exon 21, including the Homer binding domain, which is a particular mutation found in autistic patients (Kouser et al., 2013). In addition to an increased expression of mGlu5 receptors, it is possible that lack of agonist-induced mGlu5 receptor internalization can affect the fine tuning of synaptic

receptor signalling which is critical for normal brain function, and can therefore participate to synaptic dysfunctions responsible for cognitive impairment in FXS. In this respect, specific changes in mGlu5 receptors surface expression can be associated with alteration in mGlu-dependent form of synaptic plasticity, as found in mice carrying a deletion of norbin, a modulatory protein which positively regulates mGlu5 surface expression (Wang et al., 2009). Even more relevant in the context of our study, surface expression of mGlu5 receptors have been found to be altered in *Homer1a* KO mice, although in a different direction than in our study, and parallel those of AMPA receptors after induction of synaptic scaling (Hu et al., 2010). However, differently than AMPA receptors mGlu5 receptors changes during synaptic scaling do not depend on mGlu5/Homer interaction (Hu et al., 2010), similarly to what we observed for agonist-induced internalization. Our data support previous data indicating that mGlu5/Homer interaction is disrupted in FXS and that this mechanism may be responsible for an altered surface expression of mGlu5 receptors. Other studies are necessary to establish whether functional consequences of mGlu5/Homer disruption, including synaptic scaling or mGlu5 modulation of NMDA receptor functions, which can affect activity-dependent plasticity.

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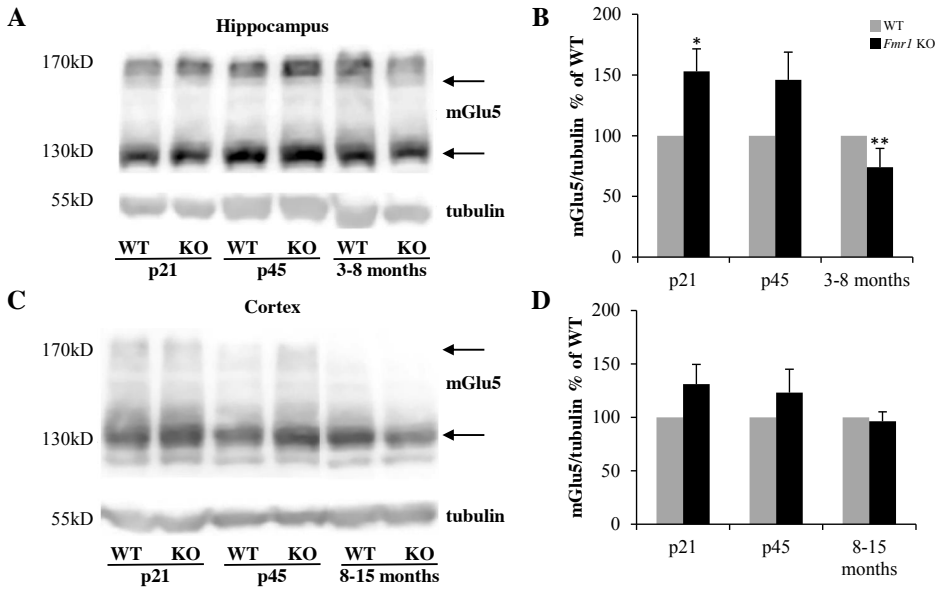

Figure 1. mGlu5 receptors are differently expressed at synapses in Fmr1 KO mice at different developmental stages. Western blots of synaptosomes obtained from hippocampi (**A**) and cortices (**C**) of WT and *Fmr1* KO mice at different ages (P21, P45 and 3 or 8-15 months). 60 mg of proteins were loaded. Semiquantitative analysis of mGlu5 receptors in hippocampal (**B**) and cortical (**D**) synaptosomes. *p = 0.008 versus respective WT; **p=0.029 versus respective WT by t-test. Data represent mean ± SEM of 4-5 experiments each performed in duplicate.

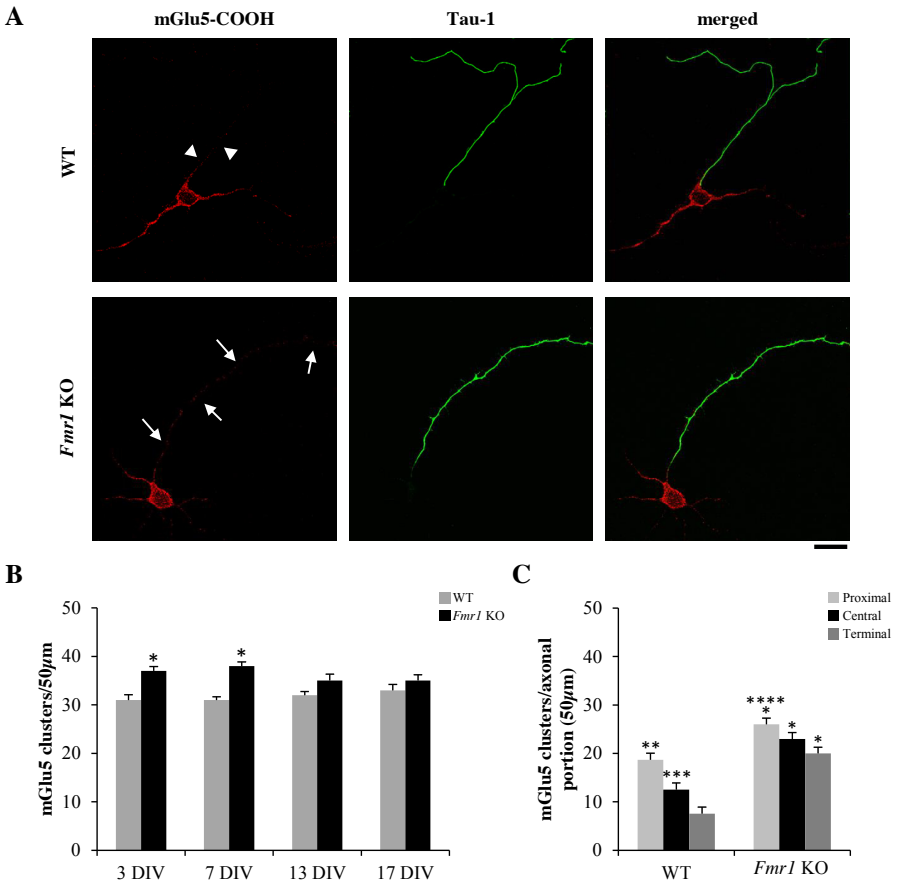

Figure 2. Expression and distribution of mGlu5 receptors in WT and Fmr1 KO neurons at different developmental stages (A) The panel shows representative confocal images of cultured hippocampal neurons from WT and *Fmr1* KO mice at 7 DIV stained with the primary antibodies anti-mGlu5-COOH and anti-Tau-1. Scale bar 20 μm. Arrowheads and arrows indicate the staining along the axon in WT and in *Fmr1* KO neurons respectively. (**B**) Graph shows semiquantitative analysis of anti-mGlu5-COOH staining in WT and *Fmr1* KO hippocampal neurons at different DIV (3, 7, 13, 17). *p = 0.05 versus WT by One Way ANOVA followed by post hoc Holm-Sidak method. Data represent mean ± SEM, n = 30-40 cells/condition in 3-4 experiments. (**C**) Graph shows semiquantitative analysis of mGlu5-COOH staining in the proximal, central and terminal part (50 μm of length for each part) of axons in WT and *Fmr1* KO hippocampal neurons at 7 DIV. *p = 0.05 versus respective portion of WT, **p = 0.05 versus central and terminal portion of axon of WT, ***p = 0.05 versus terminal portion of axon of WT and ****p = 0.05 versus terminal portion of axon of *Fmr1* KO with Two Way Anova test followed by post hoc Holm-Sidak method. Data represent mean ± SEM, n = 30 cells in 3 experiments.

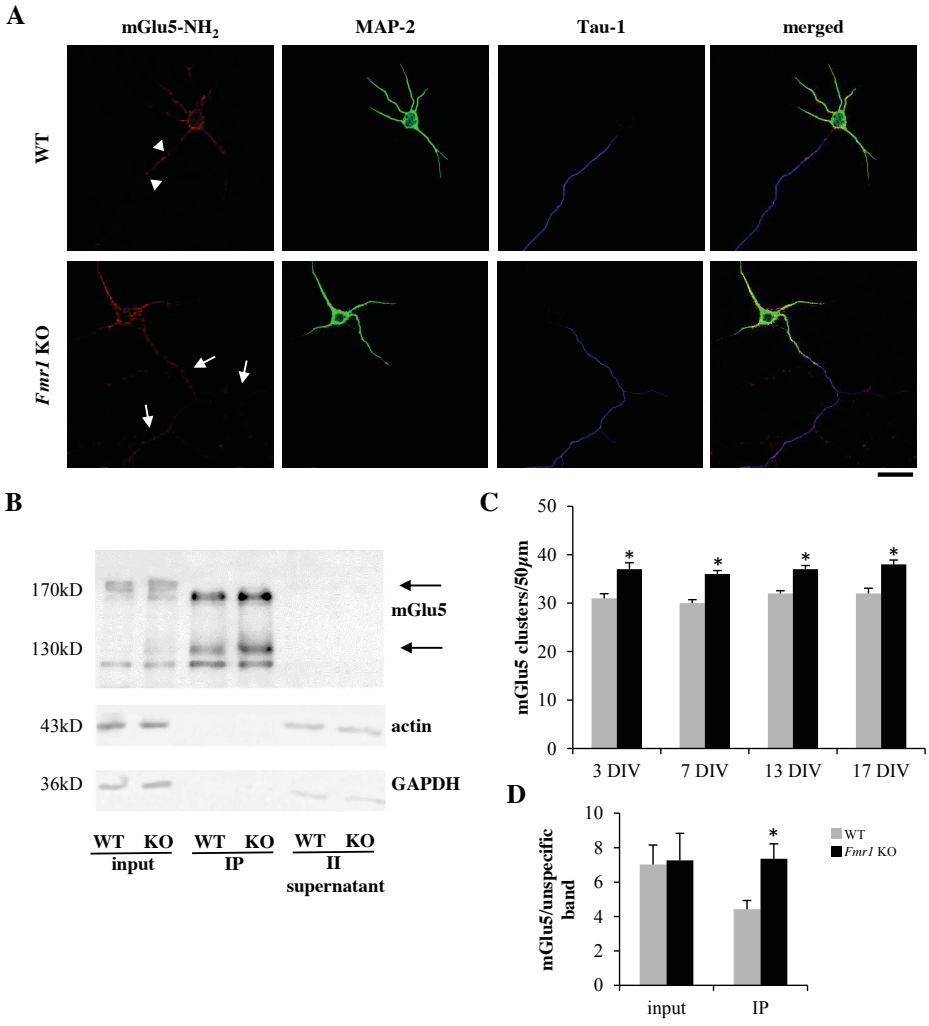

Figure 3. Surface expression of mGlu5 receptors in WT and *Fmr1* KO neurons at different developmental stages. (A) The panel shows representative confocal images of cultured hippocampal neurons from WT and *Fmr1* KO mice at 7 DIV triple-stained with the primary antibodies anti-mGlu5-NH_2, anti-MAP-2 and anti-Tau-1. Scale bar 20 µm. Arrowheads and arrows indicate the staining along the axon in WT and in *Fmr1* KO neurons respectively. (**B**) Western blots of total proteins (input) and surface proteins (biotinylated) obtained from cultured hippocampal neurons of WT and *Fmr1* KO mice at 13 DIV. 10 mg of input and 80 mg of biotinylated precipitated with agarose-conjugated NeutrAvidin were loaded. (**C**) Graph shows semiquantitative analysis of surface mGlu5 receptors in WT and *Fmr1* KO hippocampal neurons at different DIV (3, 7, 13, 17). *p = 0.05 versus respective WT by One Way Anova test followed by post hoc Holm-Sidak method. Data represent mean ± SEM, n = 30-40 cells/condition in 3-5 experiments. (**D**) Semiquantitative analysis of mGlu5 receptors in hippocampal neurons. *p = 0,005 by t-test. Data represent mean ± Standard Deviation (SD) of 3-4 experiments each performed in duplicate.

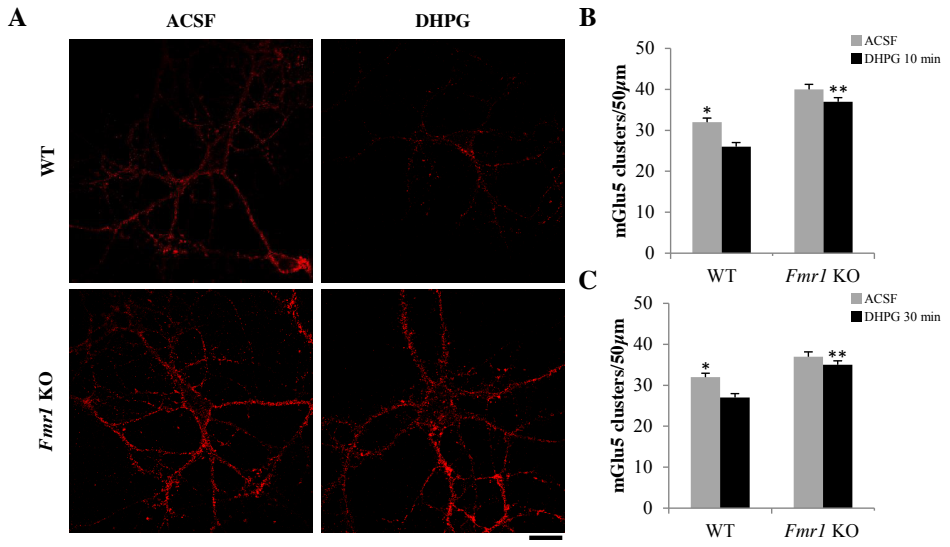

Figure 4. Lack of agonist-induced internalization in *Fmr1* KO neurons. (**A**) The panel shows representative confocal images of cultured hippocampal neurons from WT and *Fmr1* KO mice (15 DIV) stained with the primary antibody anti-mGlu5-NH_2 after exposure to ACSF or DHPG (100 μM) for 30 min. Scale bar 20 µm. (**B**) Graph shows semiquantitative analysis of surface mGlu5 receptors in WT and *Fmr1* KO neurons after exposure to ACSF or DHPG (100 μM) for 10 min. *p = 0.05 versus WT DHPH and *Fmr1* KO ACSF, **p = 0.05 versus WT DHPG (**C**) Graph shows semiquantitative analysis of surface mGlu5 receptors in WT and *Fmr1* KO neurons after exposure to ACSF or DHPG (100 μM) for 30 min. *p = 0.05 versus WT DHPG and *Fmr1* KO ACSF, **p = 0.05 versus WT DHPG with Two Way Anova test followed by post hoc Holm-Sidak method. Data represent mean $\pm$ SEM, n = 30-40 cells/condition in 3 experiments.

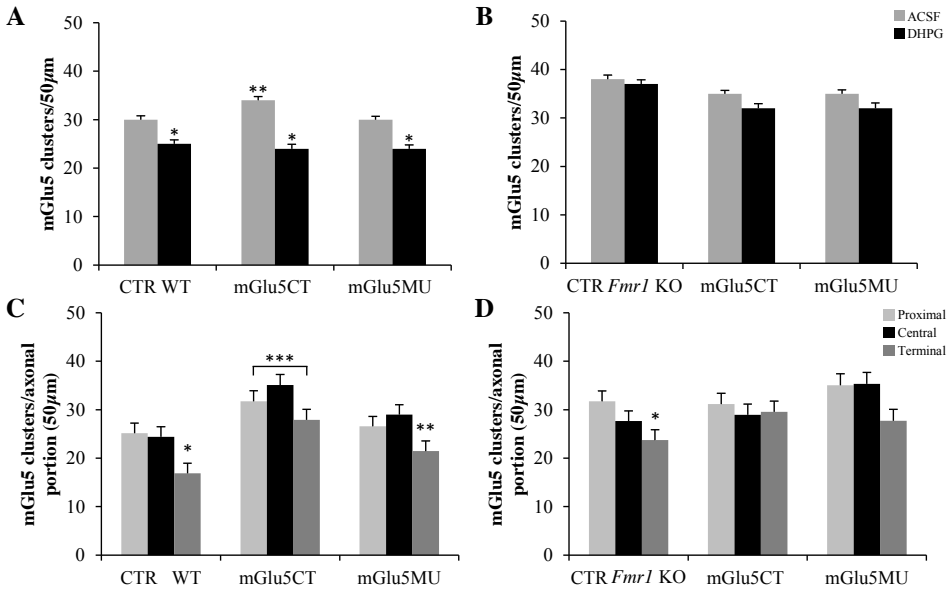

Figure 5. mGlu5/Homer distruption increases surface expression and axonal targeting of mGlu5 receptor in WT mice but has no effect in *Fmr1* KO neurons. Upper graphs show semiquantitative analysis of surface mGlu5 receptors in WT (A) and *Fmr1* KO (B) hippocampal neurons (15 DIV) incubated with a cell-permeable Tat fusion peptide which disrupts mGlu5/Homer interaction (mGlu5CT, 5 μM) or a control peptide (mGlu5MU, 5 μM), which has no effect, after exposure to ACSF or DHPG (100 μM) for 30 min. Lower graphs show semiquantitative analysis of surface mGlu5 receptors in the proximal, central and terminal part (50 μm of length for each part) of axons in WT (C) and *Fmr1* KO (D) hippocampal neurons (7 DIV) incubated with the cell-permeable Tat fusion peptides mGlu5CT (5 μM) and mGlu5MU (5 μM). Data represent mean $\pm$ SEM, n= 40-50 cells/condition in 3 experiments. (**A**) *p = 0.05 versus respective ACSF and **p = 0.05 versus CTR ACSF and mGlu5MU ACSF with One Way Anova test followed by post hoc Holm-Sidak method. (**B**) There is not a statistically significant difference whitin *Fmr1* KO data with One Way Anova test followed by post hoc Holm-Sidak method. (**C**) *p = 0.05 versus Proximal and Central portion of axon of CTR WT, **p = 0.05 versus Central portion of axon of mGlu5MU WT, ***p = 0.05 versus all respective portions of axon of CTR WT with Two Way Anova test followed by post hoc Holm-Sidak method. (**D**) *p = 0.05 versus Proximal portion of axon of CTR *Fmr1* KO with Two Way Anova test followed by post hoc Holm-Sidak method.

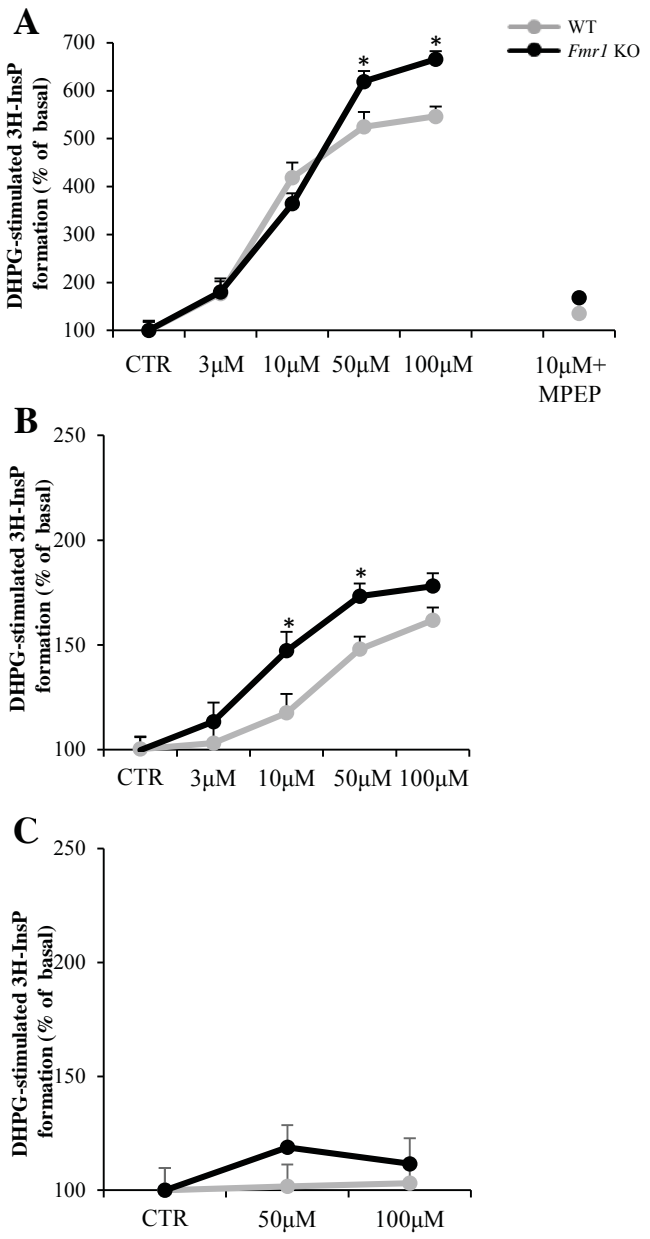

Figure 6. mGlu5-activated PI hydrolysis is increased in Fmr1 KO mice during development. Concentration-dependent stimulation of [3H]Ins-1-P formation elicited by DHPG in cortical slices from 8 day (A), 1 month (B) and 4 month (c) old WT and Fmr1 KO mice. Each point is the mean of at least 4 determinations * < 0.05 by Two-way ANOVA followed by post-hoc Holm Sidak test.

CHAPTER III

In preparation.

mGlu5 receptor surface dynamics are linked to abnormal NMDA receptor function and plasticity in Fragile X Syndrome

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ABSTRACT

Altered function of metabotropic glutamate receptor subunit 5 (mGluR5) is strongly implicated in the pathophysiology of Fragile X Syndrome (FXS), a leading inherited cause of intellectual disability and autism. Previously, we demonstrated that in *Fmr1* knockout (KO) mice (a model of FXS) mGluR5 is less associated with long Homer proteins, which are structural molecules of the postsynaptic density (PSD). Here we investigated the consequences of the disrupted mGluR5/Homer crosstalk for the surface dynamics of mGluR5 and consequently for the function and plasticity of NMDA receptors (NMDARs). To achieve this we used a powerful combination of innovative techniques such as live-cell imaging and single-molecule tracking together with electrophysiological and immunocytochemical approaches in hippocampal neurons from wild-type (WT) and *Fmr1* KO mice. We found that the disruption of the mGluR5/Homer crosstalk specifically increased the lateral diffusion of mGluR5 within the synapse of *Fmr1* KO mice. The altered mGluR5 mobility resulted in an increased probability of transient physical interactions between mGluR5 and NMDAR in the PSD of *Fmr1* KO neurons. As a consequence, the physical interaction between mGluR5 and NMDAR

altered the mGluR5-mediated modulation of NMDAR currents. Our findings demonstrate for the first time an alteration of the mGluR5 dynamics at synapses of *Fmr1* KO neurons and provide a new cellular mechanism by which mGluR5 dysfunction disrupts normal NMDAR function and synaptic plasticity.

INTRODUCTION

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and best-known cause of autism (reviewed by (Abrahams & Geschwind, 2008; Bassel & Warren, 2008). FXS is caused by transcriptional silencing of the *Fmr1* gene, which encodes fragile X mental retardation protein (FMRP), an RNA-binding protein that regulates translation, stability and trafficking of its interacting mRNAs in dendrites and dendritic spines (Antar et al., 2004; Bassell & Warren, 2008; de Diego Otero et al., 2002; Ferrari et al., 2007; Zalfa et al. 2007). FXS patients exhibit a number of neurological and behavioral deficits, including intellectual impairment, seizures, sensory hypersensitivity, social anxiety, hyperactivity and autism-like symptoms (Berry-Kravis, 2002; Hagerman, 2002).

In the mouse model of FXS, the *Fmr1* knockout (KO) mouse, alterations in synaptic plasticity have been suggested to underlie the cognitive aspects of this disorder (reviewed by (Pfeiffer and Huber, 2009)). Indeed, several studies have shown alterations in Group-I metabotropic glutamate receptor subtype 5 (mGluR5) signalling and mGluR-dependent synaptic long-term depression (LTD) in the hippocampus (Bear et al., 2004; Huber et al., 2002; Nosyreva & Huber, 2006). These findings motivated the "mGluR theory of FXS" which posits that altered mGluR5-dependent signalling is a core pathophysiological mechanism of the disease (Bear et al., 2004; Dolen et al., 2007, 2010). In agreement with this notion, pharmacological treatment or genetic reduction of mGluR5 reversed many phenotypes in the animal models of FXS, such as altered protein synthesis, mGluR-LTD, audiogenic seizures and cognitive functions (Bear et al., 2004; Dolen et al., 2007, 2010; Michalon et al., 2012). However, the precise mechanisms underlying the defective mGluR5 signalling are not clear. Moreover, despite the prominent role of mGluR5 in the regulation of synaptic plasticity and cognition very little is known about its surface dynamics at the synapse and how it can modulate interactions between synaptic partners.

The dynamic movement of synaptic components has emerged in the last decades

as the main mechanism for dynamically organizing the synaptic membrane and as a key feature of synaptic transmission and plasticity (reviewed by (Anggono & Huganir, 2012; Triller & Choquet, 2005, 2008)). Indeed, receptors on the neuronal surface constantly switch between mobile and immobile states, driven by thermal agitation and reversible binding to stable elements such as scaffolding proteins, cytoskeletal anchoring slots or extracellular anchors (reviewed by (Choquet & Triller, 2013)). In neurons, neurotransmitter receptors are concentrated in the postsynaptic density (PSD), a protein-rich subdomain lining the inner surface of the postsynaptic membrane and located in front of neurotransmitter release sites. The local enrichment of receptors at the PSD is thought to result from receptor immobilization by stable elements that interact in a biochemical and structural network. Importantly, it has been shown that mGluR5 interacts with Homer proteins at the postsynaptic site. Homer proteins are a family of PSD scaffolding proteins that cross-talk mGluR5 to other PSD proteins (Tu et al., 1998, 1999), and are classified as long (Homer1b/c, Homer2, and Homer3) and short (Homer1a) isoforms. The long isoforms of Homer multimerize, localize mGluR5 to the PSD, and scaffold mGluR5 to signalling pathways (Park et al., 2008; Shiraishi-Yamaguchi & Furuichi, 2007). Homer 1a, on the other hand, disrupts the crosstalk of mGluR5 and long Homer proteins, thereby altering mGluR5 signalling and causing constitutive, agonist-independent activity of mGluR5 (Ango et al., 2001).

Interestingly, mGluR5 and NMDA receptor (NMDAR) are linked together in the PSD by a long Homer protein–containing complex (Brakeman et al., 1997; Perroy et al., 2008; Scannevin and Huganir, 2000). This interaction precludes the direct mGluR5/NMDAR association and can be disrupted by the activity of the immediate early gene Homer 1a (Bertaso et al., 2010; Moutin et al., 2012). The disassembly of the synaptic multimeric mGluR5-Homer complex by Homer 1a allows physical and functional interactions between NMDAR and mGluR5 (Moutin et al., 2012). Indeed, The physical interaction between mGluR5 and NMDAR results in mGluR5 mediated inhibition of NMDA currents (Moutin et al., 2012).

Previously, we have shown that the absence of FMRP leads to perturbation of the interaction between mGluR5 and Homer 1 isoforms, resulting in a decreased association with the long isoforms (Giuffrida et al., 2005). This finding has been confirmed and is believed to underlie altered mGluR5 signaling, neocortical circuit dysfunction and behavior in *Fmr1* KO mice (Ronesi et al., 2008, 2012). An extensive literature describes the association of long Homer isoforms and mGluR5 and the importance of this

interaction for the regulation of their activation, surface expression and lateral diffusion (Ango et al., 2002; Coutinho et al., 2001; Kammermeier et al., 2000; Kammermeier & Worley, 2007; Ronesi & Huber, 2008; Sergè et al., 2002). However, very little is known about the consequences of a potential disrupted interaction in FXS for the surface expression and dynamics of mGluR5 at synapses and consequently for the function and plasticity of NMDAR.

In this study we asked how the disrupted mGluR5-Homer interaction affects the membrane surface expression and dynamics of mGluR5 at synapses in *Fmr1* KO mice. We addressed this question using a powerful combination of high-resolution single molecule tracking in real time together with electrophysiological and confocal imaging methods in hippocampal neurons from wild type (WT) and *Fmr1* KO mice. We found that the lateral mobility of mGluR5 was increased specifically at the synaptic, but not at the extrasynaptic, sites of *Fmr1* KO cultured hippocampal neurons. These findings support our hypothesis that the disruption of the crossltalk with the PSD partners affects the lateral mobility of the receptor. In agreement with our prediction, we found that the disruption of the mGluR5/Homer association with a specific TAT-mGluR5ct peptide in WT cultures mimicked the *Fmr1* KO phenotype by inducing a similar increase in synaptic mGluR5 mobility. The altered mGluR5 mobility increased the probability of transient physical interactions between mGluR5 and NMDAR in the PSD of *Fmr1* KO neurons resulting in the altered modulation of NMDAR currents. Indeed, patch clamp recording of NMDAR mediated excitatory postsynaptic currents (EPSCs) in the CA3-CA1 synapse showed that synaptic NMDAR-EPSCs display lower amplitudes in *Fmr1* KO neurons. Moreover, the postsynaptic expression of mGluR1/5 mediated LTD of NMDAR-EPSCs was reduced in *Fmr1* KO neurons. Furthermore, these defects in NMDAR function and plasticity can be mimicked in WT neurons by selectively disrupting the mGluR5/Homer association, strongly supporting our hypothesis that altered mGluR5 dynamics mediate abnormal NMDAR function and plasticity in FXS.

In conclusion, our findings demonstrate an alteration of the mGluR5 dynamics at synapses of *Fmr1* KO neurons due to the altered mGluR5/Homer interaction and provide a new cellular mechanism by which mGluR5 dysfunction disrupts normal NMDAR function and synaptic plasticity.

MATERIALS AND METHODS

Animals

All experiments were conducted in strict compliance with the European Directive (2010/63/EU) and French law governing the use of laboratory animals and approved by the Bordeaux Ethics Committee (C2EA50, authorization #5012023-A). Mice were housed in a SPF animal facility prior to experiments, kept on a 12 h–12 h light–dark cycle and had *ad libitum* access to food and water at all times. Second generation *Fmr1* KO mice (Mijenties et al., 2006) were used in our study. These mice are distinct from the original *Fmr1* KO mouse line, because they are deficient for both *Fmr1* RNA and FMRP protein. Mice were backcrossed six generations into a C57BL/6J (Charles River, L'Abresle, France) background and maintained in this mixed background for all experiments. *Fmr1* KO and WT embryos for dissociated neuron cultures were generated by crossing homozygous ($Fmr1^{+/+}$ X $Fmr1^{+/y}$ or $Fmr1^{-/-}$ X $Fmr1^{-/y}$) progenitor mice. For electrophysiology, male WT and *Fmr1* KO littermates were generated by crossing a heterozygous ($Fmr1^{+/-}$) female mouse with a wild-type ($Fmr1^{+/y}$) male mouse as described previously (Zhang, Bonnan, Bony et al., 2014). Mice were P16 at the time of sacrifice. Mice were subsequently re-genotyped after the experiment by tail PCR as described by Mientjes et al. (2006).

Primary cell cultures

Cultures of hippocampal neurons and glial cells were prepared from E18 WT and *Fmr1* KO embryos. Pregnant mice were sacrificed by cervical dislocation after deep anesthesia with isoflurane and the uterine horn dissected. Hippocampi were subsequently dissected from the embryos in ice-cold dissection solution and then dissociated in (0,25%) trypsin. Briefly, cells were plated at a density of 100 to 200 x 10^3 cells per milliliter on poly-L-lysine precoated coverslips and kept at 37°C in 5% CO_2. After two days *in vitro* (DIV), the original plating neurobasal culture medium (Invitrogen) complemented with 5% fetal bovine serum was replaced with a serum free medium. AraC (5 µM) was added on the 4th DIV. All the experiments were performed at 12/15 DIV.

Pharmacological treatments

A cell-permeable (TAT-fused) peptide containing the proline-rich motif (PPXXF) of the mGluR5 C-terminal tail that binds the EVH1 domain of Homer, TAT-mGluR5ct (YGRKKRRQRRR-ALTPPSPFR) and a control peptide with a mutated Homer binding

motif, mGluR5mu (YGRKKRRQRRR-ALTPLSPRR), were synthesized at the UT Southwestern Protein Chemistry Technology Center. (Tu et al., 1998; Mao et al., 2005; Ronesi et al., 2008). The peptides were dissolved in H_20 at a concentration of 5 mM, and aliquots of this stock concentration were stored at –20°C. Frozen aliquots of both TAT-fused peptides were used within 10 days and diluted to the desired final concentration. Hippocampal culteres were treated with TAT-mGluR5ct or TAT-mGluR5mu within 1 hour with a final concentration of 5 μM in serum free culture medium at 37°C. Slices were incubated during 4 hours with TAT-mGluR5ct or TAT-mGluR5mu 5–10 μM in oxygenated ACSF at room temperature (21–22° C).

Single-Particle (Quantum Dot) Tracking and Surface Diffusion Calculation

For single-molecule tracking experiments, neurons were first exposed for 10 min to mouse monoclonal anti-NH_2 mGluR5 antibody (1:20, gift of Prof. Shigemoto, Nat. Inst. of Physiol. Sciences, Okazaki, Japan) or mouse monoclonal anti-GluA2 (1:200; Millipore) antibody or rabbit monoclonal anti-NR1 antibody (1:200; Alomone Labs) at 37°C. Neurons were then incubated for 10 min in a solution containing quantum dots (QD) 655 coupled to goat anti-mouse IgG (Invitrogen) or coupled to goat anti-rabbit IgG (Invitrogen) (final dilution 1:5000/1:10.000) at 37°C. To label synaptic sites, neurons were incubated for 40 seconds at RT (~22°C) in a solution containing the orange mitochondria marker MitoTracker (20 nM; Invitrogen). A fraction of coverslips was also incubated during 1 h with TAT-mGluR5ct or TAT-mGluR5mu 5 μM in culture medium at 37°C before the incubation with the primary antibodies.

For QD 655 fluorescence imaging we used an EMCCD camera (Evolve 512, Photometrics) with a 512 x 512 imaging array together with an HXP-120 light source (Zeiss) and the appropriate filters for excitation and emission. Images were acquired at an integration time of 50 ms for up to 500 consecutive frames (24 sec). QD movements were followed on randomly selected healthy dendritic regions for up to 20 min, and analyzed using Metamorph software (Universal Imaging Corporation, PA, USA). Briefly, the instantaneous diffusion coefficient, D, was calculated for each trajectory, from linear fits of the first 4 points of the mean-square-displacement versus time function using: MSD (t) = < r2 > (t) = 4Dt. To assign synaptic localization, trajectories were sorted into extrasynaptic (i.e. MitoTracker-negative pixels) and synaptic regions (MitoTracker-positive pixels).

Immunocytochemistry and confocal analysis

The surface expression of mGluR5 was studied using an antibody against the NH_2 terminal of the mGluR5 in non-permeabilized neurons. After removing the medium, cell cultures were incubated with the mouse monoclonal anti-NH_2 mGluR5 antibody (1:10, gift of Prof. Shigemoto, Nat. Inst. of Physiol. Sciences, Okazaki, Japan) for 30 min at 37°C. Subsequently, cultures were fixed with a solution containing 4% paraformaldeyde (PFA) and 4% sucrose for 10 min at RT, permeabilized in PBS containing 0.1% Triton-X for 10 min, incubated with blocking solution containing 4% BSA for 45 min at RT, followed by incubation with the rabbit monoclonal anti-NR1 antibody (1:200; Alomone Labs) and the Guinea pig polyclonal anti-Homer 1 antibody (1:500; Synaptic Sistems) for 1 h at RT. After washing, cultures were incubated for 45 min at RT with the appropriate secondary fluorescent antibodies (Alexa 647 anti-mouse, 1:750, Invitrogen; Alexa 555 anti-rabbit, 1:750, Invitrogen; Alexa 488 anti-Guinea pig, 1:750, Invitrogen). A fraction of coverslips was also incubated during 1 hour with TAT-mGluR5ct or TAT-mGluR5mu 5 μM in culture medium at 37°C before the incubation with primary antibodies.

Images were acquired to measure co-localization of mGluR5, NR1 and Homer 1, using a commercial Leica DMI6000 TCS SP5 confocal microscope with identical settings for all conditions. Ten individual confocal images per coverslip were acquired at 12 bit-depth with a pixelsize of 96.2 nm x 96.2 nm per voxel (63x objective, 1.4 NA, 2.5 digital zoom, 1024 x 1024 pixel per image, 50 Hz scanning speed, 98.41 μm x 98.41 μm field of view). Images were processed with AutoquantX software (MediaCybernetics) and ImageJ software (Molecular Devices). A minimum of eight randomly chosen cells per condition was acquired and analysed. A 2D blind deconvolution algorithm was first applied to each image in order to retrieve better data from our images. Then, analysis of the co-localization of mGluR5, NR1 and Homer 1 was performed using the "Co-localization" module of ImageJ (version 1.49; Scion Image, Frederick, MD). A custom-made macro was used to analyze the dendritic part of each image by measuring the intensity of each label using fixed threshold intensities.

Electrophysiological recordings of NMDA currents in acute hippocampal slices

Hippocampal slices were prepared from male *Fmr1* KO and WT mice (post natal age 12-15 days). The brains were removed and placed in oxygenated ice-cold artificial cerebrospinal fluid (ACSF; in mM NaCl 124; KCl 3.0; NaH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$

2.0; $NaHCO_3$ 26; D-glucose 10, pH 7.3). The two brain hemispheres were separated and transverse slices of dorsal hippocampus (300 μm thick) were cut using a vibratome (Leica VT1200). Slices recovered during at least 3 hours at RT continually perfused with oxygenated ACSF. Some of the slices were incubated for 4 hours with 5–10 μM TAT-mGluR5ct or TAT-mGluR5mu in oxygenated ACSF at RT.

Hippocampal slices were transferred to the recording chamber and visualized using a Leica DMLFS microscope equipped with 20X/0.30 and 40X/0.80 infrared filter x/nA objective and differential interference contrast (DIC). A tungsten monopolar electrode (WPI) was placed in the *stratum radiatum* to stimulate Schaffer collaterals with negative current pulses (duration 0.3 ms), delivered every 30 s by a stimulus generator (A310 Accupulser with A360 stimulus isolator unit, WPI, USA). Evoked EPSCs were recorded at RT from CA1 pyramidal neurons in the whole-cell patch-clamp configuration at a holding potential of –60 mV using an EPC7-plus amplifier (HEKA, Germany). Current traces were filtered at 3 kHz and digitized at 10 kHz. The patch pipettes (open-tip resistance of 1.5-3 MΩ) were filled with intracellular solution containing (in mM): K-gluconate 140; HEPES 10; NaCl 10; $MgCl_2$ 2; EGTA 0.2; QX-314 1; Mg-ATP 3.5; Na-GTP 1; pH 7.3). Slices were continuously superfused with ACSF at a flow rate of 1.0 ml/min. Following whole-cell access, the slice was perfused with Mg^{++}-free ACSF containing CNQX (10 μM), glycine (10 μM) and bicuculline (5 μM) to isolate NMDAR-EPSCs. DHPG (100 μM) was dissolved in the same ACSF and bath-applied for 5 min. Data were acquired and analyzed with Signal software (Cambridge Electronic Design, England). NMDAR-EPSC amplitude values were measured as the difference between peak current and baseline, averaged over 1 min and expressed as percentage of control (calculated from EPSCs recorded during at least 15 min prior to DHPG application).

Data representation and statistical analysis

Group values are expressed as mean ± s.e.m. For co-localization experiments box plots show the median, interquartiles, range, mean and individual values. Comparisons between groups for instantaneous diffusion coefficients and percentage of co-localization were performed using Mann–Whitney test (pair comparison), or Kruskal–Wallis Test (group comparison). Comparisons between groups for cumulative distribution of instantaneous diffusion coefficients were performed using Kolmogorov-Smirnov tests. The comparison of the percentage of immobile and mobile receptors was

performed using Chi-square test. Comparison for the relative synaptic fraction was perfomed using Student t test (pair comparison), or one-way ANOVA test (group comparison). For electrophysiology experiments percent EPSC values from groups of neurons were pooled (mean ± s.e.m.) and graphically represented as a function of time. The amount of mGluR-LTD was calculated over a 5-minute epoch (between 40 and 45 min) after DHPG washout and was expressed as percentage of baseline (% EPSC amplitude). Evoked excitatory postsynaptic current amplitude values from two groups of neurons were compared using the unpaired Student's t-test. Significance levels were defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

RESULTS

Increased lateral diffusion of mGluR5 at synapses in *Fmr1* KO neurons

In a previous study we found that in *Fmr1* KO mouse mGluR5 is less associated to long isoforms of Homer1 proteins at synapses (Giuffrida et al., 2005). We therefore hypothesized that the abnormal interaction of mGluR5 with structural elements of the PSD might affect the surface dynamics of mGluR5 within the synaptic site. Thus, we studied the dynamic properties of mGluR5 in the dendritic membrane of hippocampal neurons using a quantum dots based single-molecule tracking approach (Figure**.** 1A, B ad C). To distinguish trajectories at synaptic from those at extrasynaptic sites, we labeled synapses with the active mitochondria marker, Mitotracker (rhodamine derivative), which is enriched at synaptic sites (Groc et al., 2007) (Figure 1D). We found that the diffusion coefficient (i.e. a measure for the membrane mobility) of mGluR5 in the synaptic compartment was significantly enhanced for *Fmr1* KO as compared to WT neurons (Figure 2A and B; $P < 0.001$). Accordingly, the fraction of mobile mGluR5 (diffusion coefficients > 0.005 $\mu m^2/s$) at the synapse was increased (Figure 2B; $P < 0.05$). In contrast, the diffusion coefficient (Figure 2A and C; $P > 0.9999$) and the mobile fraction (Figure 2C; $P = 0.7765$) of mGluR5 at exstrasynaptic sites were comparable between WT and *Fmr1* KO neurons. These results are in line with our hypothesis that mGluR5 dynamics are altered as a consequence of its loose association with the PSD partners in *Fmr1* KO neurons.

To test whether the observed alterations are specific for mGluR5s, we also measured the aforementioned parameters for AMPA-type glutamate receptors (AMPAR). We found no significant differences in the lateral diffusion (Figure 3A and

B; $P > 0.9999$) and the mobile fraction (Figure 3A; $P = 1$) of AMPAR in the synaptic or the extrasynaptic compartment of *Fmr1* KO neurons (Figure 3A and C; diffusion coefficient, $P > 0.9999$; mobile fraction, $P = 0.7458$). This data suggest that the changes in membrane dynamics in hippocampal *Fmr1* KO neurons are specific for mGluR5 and do not affect AMPAR type glutamate receptors.

Defects in mGluR5/Homer crosstalk cause increased lateral diffusion of mGluR5

Next we asked whether the increased mGluR5 lateral diffusion in *Fmr1* KO neurons can be caused by the reduced mGluR5/Homer interaction reported previously (Giuffrida et al., 2005; Ronesi et al., 2012). To address this question, we disrupted the mGluR5/Homer link in WT neurons with a cell-permeable specific peptide that mimics the sequence of mGluR5 that binds Homer (TAT-mGluR5ct; Tu et al., 1998; Mao et al 2005; Ronesi et al., 2008). As a control we used a peptide with a mutated Homer binding motif (TAT-mGluR5mu; Mao et al 2005; Ronesi et al., 2008; Tu et al., 1998). The lateral diffusion and the percentage of mobile fraction mGluR5 in the synaptic compartment was higher in WT neurons pre-incubated with TAT-mGluR5ct (Figure 4A and B; diffusion coefficient, $P < 0.001$, mobile fraction $P < 0.01$). Importantly, both parameters were now comparable to those of *Fmr1* KO neurons in control (diffusion coefficient, $P > 0.9999$; mobile fraction, $P = 0.4615$). As expected, pre-incubation with TAT-mGluR5mu had no effect on the lateral diffusion of mGluR5 (Figure 4A and B; diffusion coefficient, $P > 0.9999$, mobile fraction $P = 0.1736$). Moreover, neither TAT-mGluR5ct nor TAT-mGluR5mu treatment had any effect on the mGluR5 mobility in *Fmr1* KO neurons (Figure 4C and D; TAT-mGluR5ct, diffusion coefficient, $P > 0.9999$; mobile fraction, $P = 0.5940$; TAT-mGluR5mu, diffusion coefficient, $P > 0.9999$; mobile fraction, $P = 0.5118$). These experiments confirm our hypothesis that the changes in the lateral diffusion of mGluR5 are indeed due to the disruption of the link between the Homer scaffold and mGluR5.

Altered mGluR5/Homer crosstalk causes increased synaptic mGluR5/NMDAR interaction in *Fmr1* KO neurons

mGluR5 and NMDAR are physically linked together in the PSD by a long Homer protein–containing complex (Brakeman et al., 1997; Scannevin and Huganir, 2000) and this interaction can be disrupted by the immediate early gene Homer 1a (Xiao et al., 1998). Recent work suggests that disruption of this glutamate receptor complex might

have consequences for both the physical association of mGluR5 with NMDAR and the mGlu5 receptor modulation of NMDA receptor activity (Moutin et al., 2012). Therefore, we probed whether the altered mGluR5/Homer link in *Fmr1* KO neurons influences the membrane dynamics of NMDAR, using a similar quantum dot based approach. NMDAR did not display any differences within the lateral diffusion in the synaptic domain of WT neurons (Figure 5A and B; $P = 0.0959$).

However, taking advantage of the accuracy of single nanoparticle detection properties (reviewed by (Groc et al, 2007; Triller & Choquet, 2008)) we elaborated a map of the successive locations of both mGluR5-QD and NR1-NMDAR-QD on the neuronal surface which revealed an increased fraction of co-localized mGluR5-QD and NR1-NMDAR-QD within the synaptic compartment of *Fmr1* KO neurons (Figure 5C; mGluR5-QD $P < 0.01$; NR1-NMDAR-QD $P < 0.01$). In contrast, we found no significant differences in the synaptic fraction of GluA2-AMPAR-QD (Figure 5C; $P = 0.4537$). These data provide a direct evidence that mGluR5 and NMDAR are more confined within the synapse in *Fmr1* KO neurons.

The increased synaptic confinement of mGluR5 and NMDAR may cause a higher co-localization between these receptors in the synapse domain of *Fmr1* KO neurons and therefore increase the probability that these two receptors physically interact within a certain time window. To examine this possibility we performed a triple immunofluorescence labeling experiment for mGluR5, NMDAR and Homer1 (used as synaptic marker) (Figure 5D). Using confocal microscopy imaging we found an increase in the percentage of mGluR5 and NMDAR expression at the synapse (Figure 5E and F; mGluR5 $P < 0.001$; NMDAR $P < 0.01$). Consequently, we observed an increase in the percentage of mGluR5/NMDAR co-clusters in the synaptic sites of *Fmr1* KO neurons (Figure 5D; $P < 0.05$), suggesting a tight association of these receptors. We hypothesized that the mGluR5/Homer crosstalk disruption might be the mechanism causing the increased co-clustering of mGlu5 and NMDA receptors in *Fmr1* KO neurons. Indeed, we observed an increased mGluR5/NMDAR co-clustering in the synaptic compartment of WT neurons pre-incubated with TAT-mGluR5ct, (Figure 5H; $P < 0.01$; Supplementary figure 1A-D). As expected, TAT-mGluR5mu had no effect on the co-localization of the two receptors (Figure 5H; $P = 0.3426$; Supplementary figure 1A-D). Taken together, these data suggest that the disruption of the mGluR5/Homer crosstalk causes a tighter physical association between mGluR5 and NMDAR in the PSD of *Fmr1* KO neurons.

The increased mGluR5/NMDAR interaction alters NMDAR function and plasticity in Fmr1 KO neurons

Our finding that mGluR5 and NMDA are more closely associated at the synaptic sites in *Fmr1* KO, as a consequence of mGlu5/Homer disruption prompted us to to probe deficit in NMDA receptor function in *Fmr1* KO neuros. Synaptic NMDAR-mediated excitatory postsynaptic currents (NMDA-EPSCs) were studied using whole-cell patch-clamp recordings of CA1 pyramidal neurons in acute hippocampal slices and were evoked by stimulation of *Schaffer* collaterals. Previously, NMDA-EPSCs displayed lower amplitudes in *Fmr1* KO neurons when compared to wild-type neurons (Figure 6A; $P < 0.001$). These defects in NMDAR function were mimicked in WT neurons by our peptide mimicking approach as described above (Figure 6A; $P < 0.01$), strongly supporting our hypothesis that alterations in the membrane dynamics of mGluR5 and their tighter coupling with NMDAR mediate abnormal NMDAR function in *Fmr1* KO neurons.

In hippocampal neuron, activation of Group-I mGluR induces long term depression (LTD) of AMPAR-EPSCs that is specifically increased in *Fmr1* KO neurons (Huber et al., 2002). Similarly to AMPAR also synaptically elicited NMDA-EPSCs undergo depression in response to Group-I mGluR activation (Baskys and Malenka 1991; Snyder et al. 2001; Watabe et al. 2002). Indeed we observed that in WT neurons after DHPG stimulation, NMDA-EPSCs undergo depression (Figure 6B). However we found that postsynaptic expression of Group-I mGluR-mediated LTD of NMDA-EPSCs were reduced in *Fmr1* KO neurons (Figure 6B; $P < 0.001$). These defects in NMDAR plasticity were mimicked in WT neurons by the selective disruption of the mGluR5/Homer interaction (Figure 6B; $P < 0.01$). These result suggest that the mGluR5/Homer crosstalk disruption compromises the NMDAR currents and plasticity in *Fmr1* KO neurons.

DISCUSSION

A substantial number of evidences have shown that altred mGluR5 function is causally associated with the pathophysiology of FXS. mGluR5 dysfunction is well established in the animal models of FXS, and genetic or pharmacological reduction of mGluR5 activity reduces or rescues many disease phenotypes in the mouse model (Bear

et al., 2004; De Vrij et al., 2008; Dolen et al., 2007, 2010; Michalon et al., 2012, 2014). Based on these promising results, mGluR5 antagonists were introduced in clinical trials in FXS patients. However several of these clinical trials employing novel mGluR5 antagonists have recently been cancelled because of lack of efficacy of the treatment when compared with the placebo. The reasons why these clinical trials failed may be an inadequate dose range for Fragile X patients and an unexpected development of tolerance (www.fraxa.org). Dosage problems are relatively easy to correct, but tolerance may be something intrinsic to mGluR5 physiology. Thus, an improved understanding of the cellular and subcellular nature of mGluR5 dysfunction in FXS is needed for the identification of new therapeuthic targets. The cellular mechanisms of mGluR5 dysfunction in FXS have been elusive and most of the studies have been focused just on the altered translational processes at the synapse initiated by mGluR5 activation. Recent evidence indicates that some relevant FXS phenotypes are dependent also on mGluR5/Homer crosstalk disruption suggesting that altered mGluR5 function is upstream of protein translation (Ronesi et al., 2012; Spatuzza et al., submitted). Indeed it has been shown that the altered balance in mGluR5 interactions with Homer isoforms leads to some of the pathological phenotypes of FXS (Ronesi et al., 2012; Spatuzza et al., submitted).

Here we demonstrate a new causative role for the reduced mGluR5/Homer crosstalk dysfunction in FXS. We investigated the consequences of the mGlu5/Homer crosstalk disruption for the mGluR5 surface dynamics and mGluR5-dependent modulation of NMDA currents in *Fmr1* KO neurons. mGluR5 is less associated to long isoforms of Homer1 proteins at synapses of *Fmr1* KO neurons although total mGluR5 levels are normal (Giuffrida et al., 2005). We therefore hypothesized that the abnormal interaction of mGluR5 with structural elements of the PSD might affect the surface dynamics of mGluR5 within the synaptic site. We demonstrated that the disruption of the mGluR5/Homer crosstalk specifically increased the lateral diffusion of mGluR5 at the synaptic but not at the extrasynaptic sites of *Fmr1* KO. The fact that we did not find any differences in the dynamics of AMPAR is a valid proof that the observed alterations are specific for mGluR5s and not a more general phenomenon. Indeed, AMPAR is a good canditate control because, as for mGluR5, AMPAR subunits (GluR1, GluR2/3, GluR4) are similarly expressed in *Fmr1* KO and WT mice. However, the link between AMPAR and the PSD structure is not altered in *Fmr1* KO mice (Giuffrida et al., 2005). These findings support our initial hypothesis that the disruption of the crosslink with the Homer

proteins at the synaptic contact affects the lateral mobility of the receptor. In agreement with our prediction, we found that the disruption of the mGluR5/Homer association with a specific TAT-fused peptide in WT neurons mimicked the *Fmr1* KO phenotype by inducing a similar increase in synaptic mGluR5 mobility.

The dynamic movement of synaptic components has emerged as the main mechanism for dynamically organizing the synaptic membrane and as a key feature of synaptic transmission and plasticity (reviewed by (Anggono & Huganir, 2012; Triller & Choquet, 2005, 2008)). Indeed, receptors on the neuronal surface constantly switch between mobile and immobile states, driven by thermal agitation and reversible binding to stable elements such as scaffolding proteins, cytoskeletal anchoring slots or extracellular anchors (reviewed by (Choquet & Triller, 2013)). The restricted motion at synapse results not only from transient interactions of receptors with scaffolding molecules directly or indirectly bound to the cytoskeleton, but also the presence of obstacles in the synapse may reduce the mobility of receptors. Obstacles to diffusion are created by a crowd of transmembrane proteins immobilized at the synapse through binding to the cytoskeleton and these proteins may include the receptors themselves (reviewed by (Triller & Choquet 2008)). The disruption of mGluR5/Homer crosstalk in *Fmr1* KO neuros alters the dynamic switch between mobile and immobile states of the receptor. The higher mobility of mGluR5 in *Fmr1* KO neurons might increase the probability of transient interactions with other transmembrane proteins immobilized at the synapse. Interestingly, a lot of evidences suggest that mGluR5 can physically interact with NMDAR. mGluR5 and NMDAR are linked together in the PSD by a long Homer protein–containing complex (Bertaso et al., 2010; Brakeman et al., 1997; Perroy et al., 2008; Scannevin and Huganir, 2000). The constraint that results from the link between the C-terminus of mGluR5 and NMDAR with the long Homer protein–containing complex preclude the direct mGluR5/NMDAR association (Bertaso et al., 2010; Perroy et al., 2008). This interaction can be disrupted by the immediate early gene Homer 1a allowing physical and functional interactions between NMDAR and mGluR5 (Moutin et al., 2012). The physical interaction between mGluR5 and NMDAR results in mGluR5 mediated inhibition of NMDA currents (Moutin et al., 2012).

We demonstrated that in *Fmr1* KO neurons the disruption of mGluR5/Homer interaction altered mGluR5 dynamics that resulted in an increased probability of transient physical interactions between mGluR5 and NMDAR within the synapse (Figure 7). This remodelling mediated by the postsynaptic partners resultsed in a

modulation of the NMDA currents. We found that in *Fmr1* KO neuros NMDAR-EPSCs evoked by *Schaffer* collateral stimulation showed lower amplitudes. Moreover, we found that the postsynaptic expression of mGluR5 mediated LTD of NMDAR-EPSCs was reduced in *Fmr1* KO neurons. Although *Fmr1* KO mice have modification in synaptic plasticity, this is not likely attributable to an altered expression of the total and synaptic amount of mGluR5 and Homer proteins (Giuffrida et al., 2005). Thus, the altered organization of receptors and proteins at synapses might underlie altered functional responses in these mice. Indeed, we demonstrated that these defects in NMDAR currents were critically dependent on the altered mGluR5/Homer association. These findings strongly support our initial hypothesis that altered mGluR5 dynamics mediate abnormal NMDAR function and plasticity in FXS.

NMDAR have largely been ignored in the study of FXS, perhaps due to early studies reporting normal NMDAR-dependent LTP and LTD in the CA1 region of *Fmr1* KO mice (Godfraind et al., 1996; Huber et al., 2002). In contrast to these results conducted in adult mice, recent studies investigating NMDAR-dependent LTP and LTD in young mice has revealed deficits (Hu et al., 2008; Pilpel et al., 2009) Activation of Group-I mGluRs is involved in the induction of NMDAR-dependent synaptic plasticity and it can induce LTP or LTD depending on whether the currents are elicited by exogenous NMDA application or synaptic stimulation. Exogenous NMDAR mediated currents are transiently potentiated by Group-I mGluR activation (Benquet et al., 2002; Grishin et al., 2004; Heidinger et al., 2002; Mannaioni et al., 2001; Skeberdis et al., 2001; Snyder et al. 2001). Conversely, synaptically elicited NMDAR-EPSCs undergo depression in response to Group-I mGluR activation (Baskys and Malenka 1991; Snyder et al., 2001; Watabe et al. 2002). The reasons for this disparity and the mechanisms underlying the mGluR-induced depression of the NMDAR-EPSCs are poorly understood. Here we suggest that the dynamic remodelling of the link between mGluR5 and Homer proteins plays a key role in the in the induction of NMDAR-EPSCs LTD in response to Group-I mGluR activation.

Our findings demonstrate for the first time an alteration of the mGluR5 dynamics at synapses of *Fmr1* KO neurons and provide a new cellular mechanism by which mGluR5 dysfunction disrupts normal NMDAR synaptic plasticity. This study is not only the first to show that the surface dynamics of mGluR5 at synapses are altered, but also establishes evidence of how this alteration could contribute to the pathophysiology of FXS. This work highlights the importance of the association/dissociation dynamics of

multiprotein complexes in receptor functions and cell physiology. Neurotransmitter receptors can no longer be seen as cell surface–isolated entities. Regulations of receptor signaling by dynamic changes in scaffolding-receptor-associated complex can be considered a core mechanism for synaptic function.

However, how the loss of FMRP leads to alter mGluR5/Homer crosstalk is still not clear. Protein levels of long Homers and Homer 1a are unchanged in total homogenates of *Fmr1* KO hippocampi (Giuffrida et al., 2005), and FMRP is not reported to interact with mRNA for any Homer isoforms (Darnell et al., 2011). Previous work reported a decrease in tyrosine phosphorylation of long Homer in *Fmr1* KO forebrain (Giuffrida et al., 2005), but it is unknown whether or how this affects interactions with mGluR5. Phosphorylation of mGluR5 at the C-terminal Homer interaction domain reduces the affinity of mGluR5 for Homer (Orlando et al., 2009). Therefore, post-translational modification of mGluR5 and/or Homer in *Fmr1* KO mice may underlie the decreased interactions. It will be very interesting to reveal the mechanisms that lead to a reduced tyrosine phosphorylation of Homer proteins in *Fmr1* KO animals and to verify how it can affect the pathophysiology of FXS.

The discovery that altered mGluR5/Homer interaction account for much of the complex dysfunction of mGluR5 in FXS will help to develop alternative, targeted therapies for the disease and provide mechanistic links to other genetic causes of autism.

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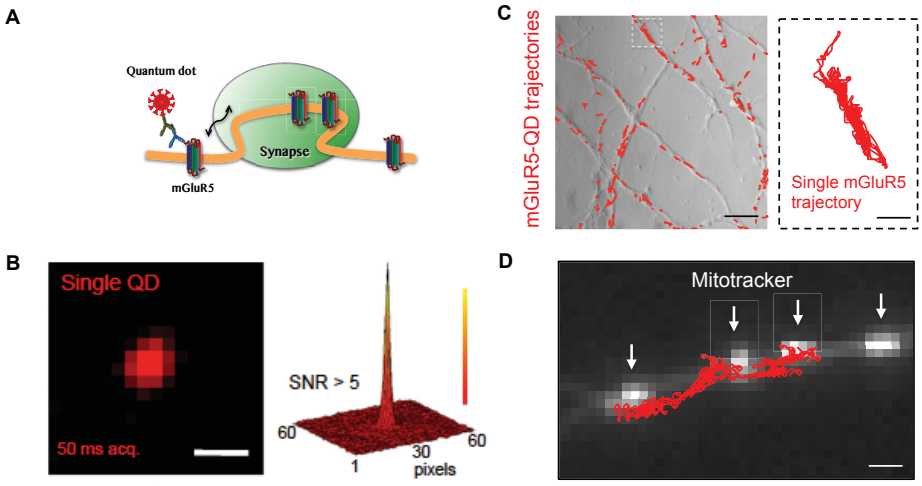


Figure 1. Single mGluR5 molecule tracking. (A) Experimental setup: Endogenous mGluR5 in the dendritic membrane (synaptic and extrasynaptic sites) were labeled with a QD-antibody complex, and the movement of these mGluR5-QD complexes was recorded with a highly sensitive EM-CCD camera. (B) The high signal-to-noise ratio (SNR > 5) enables the detection and location of individual QDs with high spatial accuracy (~30 nm). The fluorescence intensity is quantified on a pseudocolor scale (low: red; high: yellow). Scale bar = 800 nm. (C) Representative trajectories of surface mGluR5 in the dendritic membrane of hippocampal neurons (500 frames at 20 Hz acquisition rate 24 –s duration); scale bar = 5 µm (*left*). Enlarged view of a single mGluR5-QD trajectory; scale bar = 1 µm (*right*). (D) Overlay of a representative mGluR5-QD trajectory and the neuronal dendrite. The synaptic sites are labeled with Mitotracker. Scale bar = 1 µm.

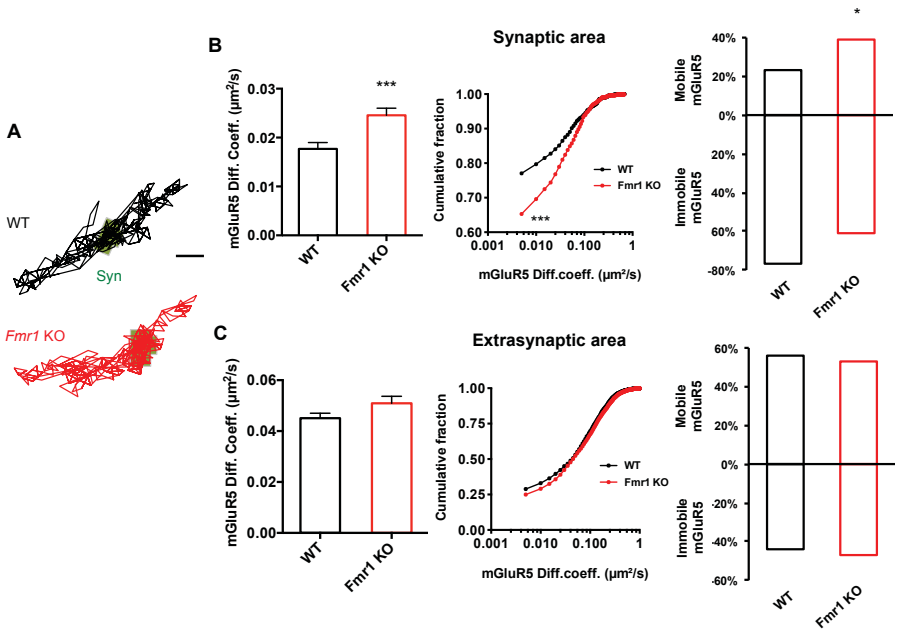


Figure 2. mGluR5 displays a higher lateral diffusion in the synaptic compartment of hippocampal *Fmr1* KO neurons. (A) Representative trajectories (500 frames, 20-Hz acquisition, 24-s duration) of single surface mGluR5-QD in a WT and an *Fmr1* KO neuron.The synaptic sites are represented by the green shapes. Scale bar = 1 µm. (B) Mean distribution (*left panel*) and cumulative distribution (*center panel*) of the instantaneous diffusion coefficient of mGluR5-QDs in the synaptic compartment of WT and *Fmr1* KO neurons. The lateral diffusion of mGluR5-QD is significantly higher in *Fmr1* KO neurons (WT, n = 1633 trajectories, 0.01769 ± 0.001300; *Fmr1* KO, n = 1451 trajectories, 0.02457 ± 0.001453; $P < 0.001$ by Mann-Whitney test on plot distribution; $P < 0.001$ by Kolmogorov-Smirnov test on cumulative distribution). *Right panel*. Percentage of immobile and mobile (i.e. diff. coeff > 0.005 $\mu m^2/s$) mGluR5-QDs in the synaptic area of WT and *Fmr1* KO neurons. The percentage of mobile mGluR5-QDs is increased in *Fmr1* KO neurons in comparison to WT neurons (WT, n = 8 dendritic fields; *Fmr1* KO, n = 7 dendritic fields; $P < 0.05$ by Chi-square test). (C) Mean distribution (*left*) and cumulative distribution (*center*) of the instantaneous diffusion coefficient of mGluR5-QDs in the extrasynaptic area of WT and *Fmr1* KO neurons (WT, n = 1907 trajectories, 0,04507 ± 0.001941; *Fmr1* KO, n = 1350 trajectories, 0.05090 ± 0.002752; $P > 0.9999$ by Mann-Whitney test on plot distribution; $P = 0.0567$ by Kolmogorov-Smirnov test on cumulative distribution). *Right panel*. Percentage of immobile and mobile mGluR5-QDs in the extrasynaptic compartment of WT and *Fmr1* KO neurons (WT, n = 6 dendritic fields; *Fmr1* KO, n = 5 dendritic fields; $P = 0.7765$ by Chi-square test). Data are shown as mean ± s.e.m. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ (*Fmr1* KO versus WT).

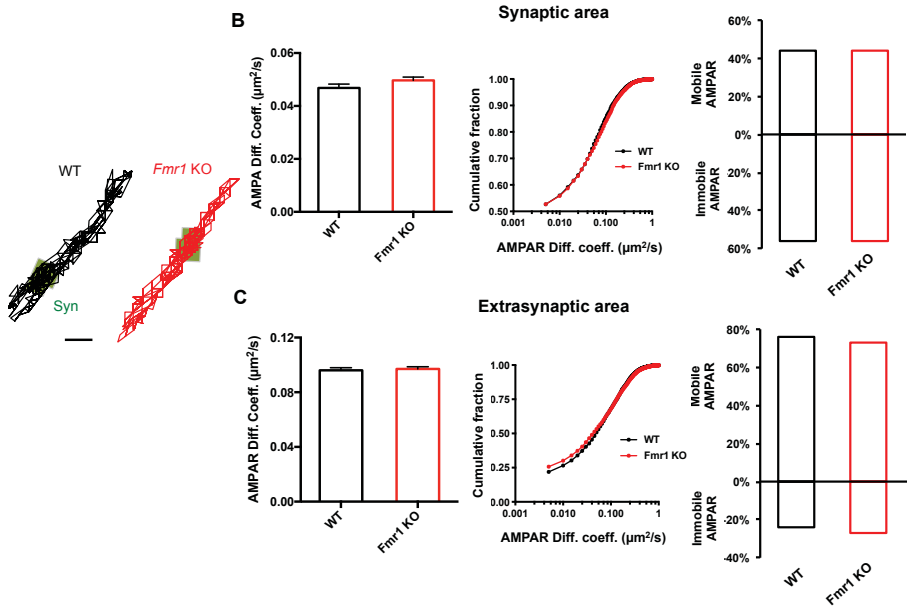


Figure 3, The lateral diffusion of endogenous AMPA receptors is unaltered in hippocampal *Fmr1* KO neurons. (A) Representative trajectories (500 frames, 20-Hz acquisition, 24-s duration) of single surface GluA2-AMPAR-QD in a WT and an *Fmr1* KO neurons.The synaptic sites are represented by the green shapes. Scale bar = 1 µm. (B) Mean distribution (*left panel*) and cumulative distribution (*center panel*) of the instantaneous diffusion coefficient of GluA2-AMPAR-QDs in the synaptic compartment of WT and *Fmr1* KO neurons. The lateral diffusion of GluA2-AMPAR-QD is not altered in *Fmr1* KO neurons (WT, n = 3921 trajectories, 0.04680 ± 0.001485; *Fmr1* KO, n = 6004 trajectories, 0.04871 ± 0.001228; $P > 0{,}9999$ by Mann-Whitney test on plot distribution; $P > 0.05$ by Kolmogorov-Smirnov test on cumulative distribution). *Right panel*. Percentage of immobile and mobile (i.e. diff. coeff > 0.005 $\mu m^2/s$) GluA2-AMPAR-QDs in the synaptic area of WT and *Fmr1* KO neurons. No differences were observed for the percentage of mobile GluA2-AMPAR-QDs in *Fmr1* KO neurons in comparison to WT neurons (WT, n = 15 dendritic fields; *Fmr1* KO, n = 14 dendritic fields; $P = 1$ by Chi-square test). (C) Mean distribution (*left panel*) and cumulative distribution (*center panel*) of the instantaneous diffusion coefficient of GluA2-AMPAR-QDs in the extrasynaptic area of WT and *Fmr1* KO neurons. The lateral diffusion of GluA2-AMPAR-QD is not different in *Fmr1* KO neurons (WT, n = 4651 trajectories, 0.09501 ± 0.001791; *Fmr1* KO, n = 7234 trajectories, 0,09101 ± 0,001468; $P >$ 0.9999 by Mann-Whitney test on plot distribution; $P > 0.05$ by Kolmogorov-Smirnov test on cumulative distribution). *Right panel*. Percentage of immobile and mobile (i.e. diff. coeff > 0,005 $\mu m^2/s$) GluA2-AMPAR-QDs in the extrasynaptic area of WT and *Fmr1* KO neurons. The percentage of mobile receptors is not different in *Fmr1* KO neurons in comparison to WT neurons (WT, n = 12 dendritic fields; *Fmr1* KO, n = 17 dendritic fields; $P = 0.7458$ by Chi-square test). Data are shown as mean ± s.e.m. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ (*Fmr1* KO versus WT).

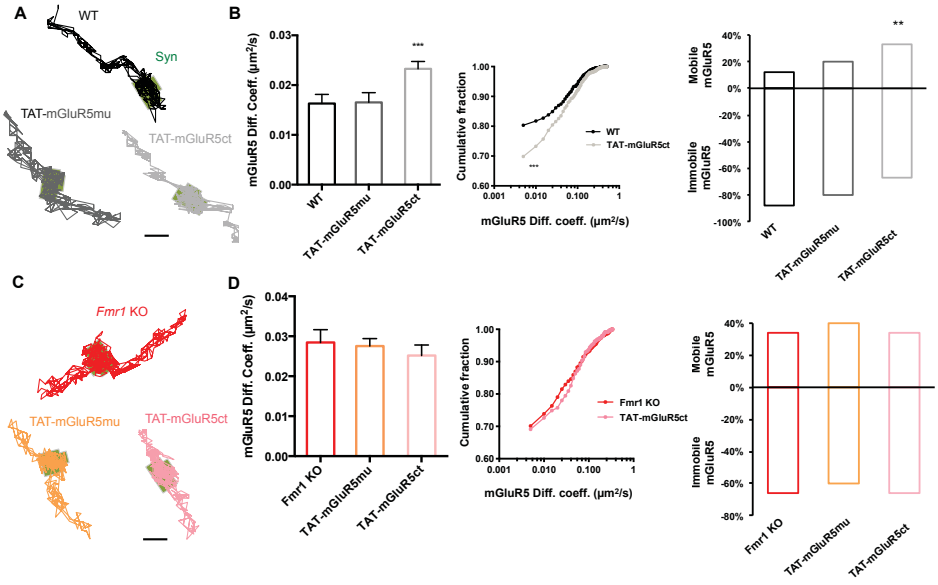
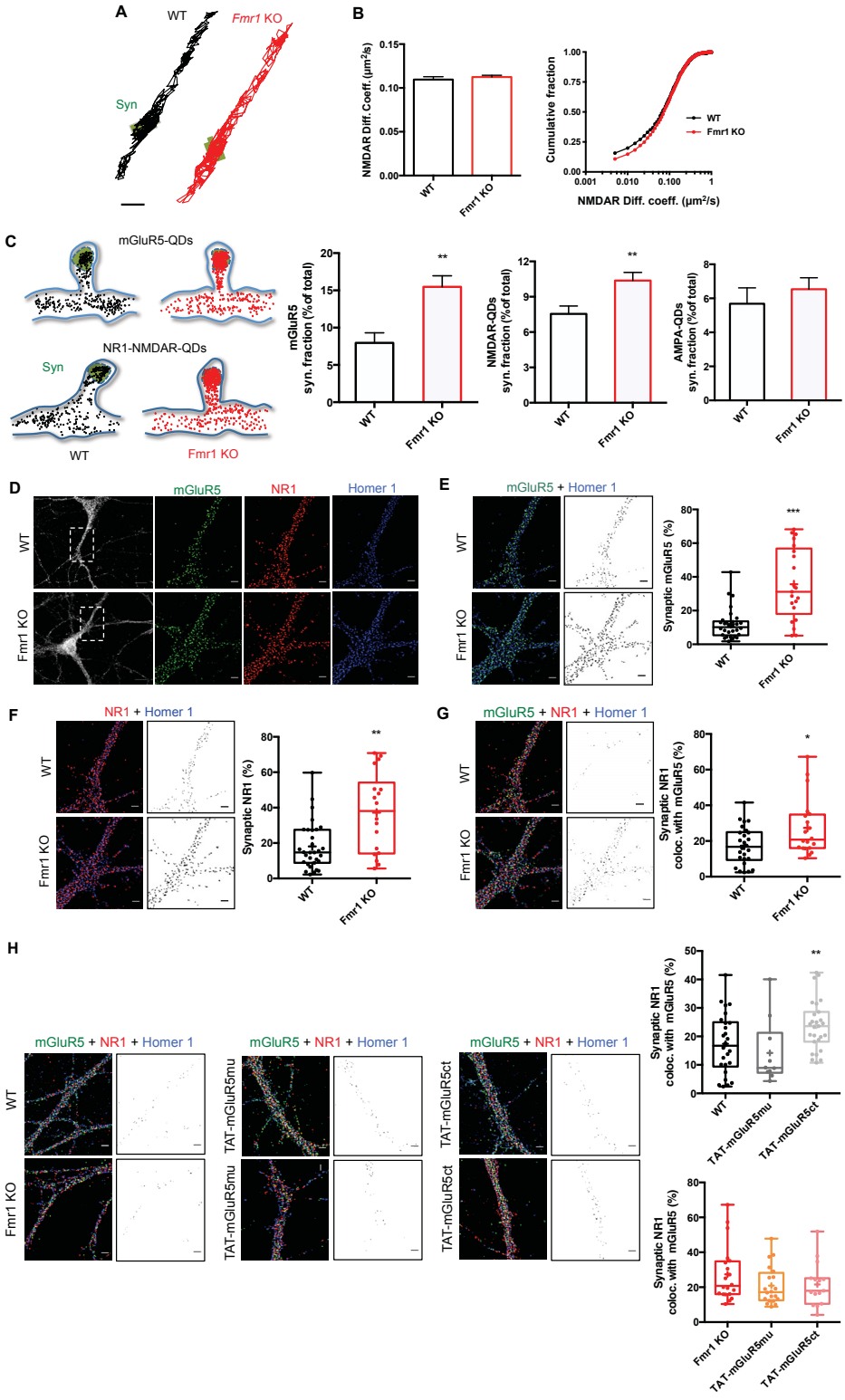


Figure 4. Disruption of the link between mGluR5 and Homer proteins in WT neurons mimicks the *Fmr1* KO phenotype. (A) Representative trajectories of single surface mGluR5-QD in WT neurons and WT neurons treated with TAT-mGluR5mu or TAT-mGluR5ct (both peptides 1h, 5 μM). The synaptic sites are represented by the green shapes. Scale bar = 1 µm. (B) Mean distribution (*left panel*) and cumulative distribution (*center panel*) of the instantaneous diffusion coefficient of mGluR5-QDs in the synaptic area of WT neurons and WT neurons treated with TAT-mGluR5mu or TAT-mGluR5ct (WT, n = 636 trajectories, 0,01630 ± 0,001827; TAT-mGluR5mu, n = 1798 trajectories, 0.01653 ± 0.001965; TAT-mGluR5ct, n = 482, 0.02327 ± 0.001478; $P < 0.001$ by Kruskal Wallis test on plot distribution; $P < 0.001$ by Kolmogorov-Smirnov test on cumulative distribution). *Right panel*. Percentage of immobile and mobile (i.e. diff. coeff > 0,005 $\mu m^2/s$) mGluR5-QDs in the synaptic area of WT neurons and WT neurons treated with TAT-mGluR5mu or TAT-mGluR5ct (WT, n = 3 dendritic fields; TAT-mGluR5 mu, n = 6 dendritic fields; TAT-mGluR5ct, n= 5 dendritic fields; $P < 0.01$ by Chi-square test). (C) Representative trajectories of single surface mGluR5-QD in *Fmr1* KO neurons and *Fmr1* KO neurons treated with TAT-mGluR5mu or TAT-mGluR5ct. The postsynaptic densities are represented by the green shapes. Scale bar = 1 µm. (D) Mean distribution (*left panel*) and cumulative distribution (*center panel*) of the instantaneous diffusion coefficient of mGluR5-QDs in the synaptic area of *Fmr1* KO neurons and *Fmr1* KO neurons treated with TAT-mGluR5mu or TAT-mGluR5ct (*Fmr1* KO, n = 773, 0.02139 ± 0.001835; TAT-mGluR5mu, n = 1413, 0.01973 ± 0.001076; TAT-mGluR5ct, n = 482, 0.02169 ± 0.002203; $P = 0.0934$ by Kruskal Wallis test on plot distribution; $P > 0,05$ by Kolmogorov-Smirnov test on cumulative distribution). *Right panel*. Percentage of immobile and mobile mGluR5-QDs in the synaptic area of *Fmr1* KO neurons and *Fmr1* KO neurons treated with TAT-mGluR5mu or TAT-mGluR5ct (*Fmr1* KO, n = 3 dendritic fields; TAT-mGluR5mu, n = 3 dendritic fields; TAT-mGluR5ct, n = 3 dendritic fields; $P = 0.5940$ by Chi-square test). Data are shown as mean ± s.e.m. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$.


A
WT
Fmr1 KO
Syn
B
NMDAR Diff. Coeff. (μm²/s)
0.15
0.10
0.05
0.00
WT
Fmr1 KO
Cumulative fraction
1.00
0.75
0.50
0.25
0.00
0.001
0.010
0.100
1
WT
Fmr1 KO
NMDAR Diff. coeff. (μm²/s)
C
mGluR5-QDs
NR1-NMDAR-QDs
Syn
WT
Fmr1 KO
mGluR5 syn. fraction (% of total)
NMDAR-QDs syn. fraction (% of total)
AMPA-QDs syn. fraction (% of total)
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D
mGluR5
NR1
Homer 1
WT
Fmr1 KO
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mGluR5 + Homer 1
Synaptic mGluR5 (%)
***
F
NR1 + Homer 1
Synaptic NR1 (%)
**
G
mGluR5 + NR1 + Homer 1
Synaptic NR1 coloc. with mGluR5 (%)
*
H
mGluR5 + NR1 + Homer 1
TAT-mGluR5mu
TAT-mGluR5ct
Synaptic NR1 coloc. with mGluR5 (%)
**
Fmr1 KO
TAT-mGluR5mu
TAT-mGluR5ct

Figure 5. mGluR5 and NR1 are more strongly co-localized in synapses of *Fmr1* KO neurons. (A) Representative trajectories of single NR1-NMDAR-QD in WT and *Fmr1* KO neurons. The synaptic sites are represented by the green shapes. Scale bar = 1 μm. (B) Mean distribution (*left panel*) and cumulative distribution (*left panel*) of the instantaneous diffusion coefficient of NR1-NMDAR-QDs in the synaptic area of WT and *Fmr1* KO neurons. The lateral diffusion of NR1-NMDAR-QDs is not altered in *Fmr1* KO neurons (WT, n = 452 trajectories, 0.09882 ± 0.005005; *Fmr1* KO, n = 1178 trajectories, 0.01070 ± 0.003215; P = 0.0959 by Mann-Whitney test on plot distribution; P = 0.2583 by Kolmogorov-Smirnov test on cumulative distribution). (C) *Left panel*. Representative surface distribution of a single mGluR5-QD (*upper panel*) and NR1-NMDAR-QD (*lower panel*) complexes detection (500 frames, 20-Hz acquisition rate; each dot represents the detection of a single receptor during a frame), revealing a trapping zone of the single surface mGluR5-QD and NR1-NMDAR-QD complexes in the PSD. *Right panel*. Relative fraction of synaptic mGluR5-QD, NR1-NMDAR-QD, and GluA2-AMPAR-QD particles. The relative fractions of synaptic mGluR5-QD and NR1-NMDAR-QD particles are increased in *Fmr1* KO neurons (mGluR5-QD, WT, n= 4 dendritic fields, 7.954 ± 1.350; *Fmr1* KO, n = 4 dendritic fields, 15.470 ± 1.490; $P < 0.01$ by unpaired Student's t-test; NR1-NMDAR-QD, WT, n = 18 dendritic fields, 7.542 ± 0.6760; *Fmr1* KO, n = 18 dendritic fields, 10.380 ± 0.6855; $P < 0.01$ by unpaired Student's t-test). The relative fraction of synaptic GluA2-AMPAR-QD particles is not altered in *Fmr1* KO neurons (GluA2-AMPAR-QD, WT, n = 7 dendritic fields, 5.681 ± 0.9368; *Fmr1* KO, n = 10 dendritic fields, 6.540 ± 1.350; P = 0.4537 by unpaired Student's t-test). (D) Hippocampal neurons from WT and *Fmr1* KO mice stained with the primary antibodies anti-mGlu5-NH_2, anti-NR1-NH_2 and anti-Homer 1. Scale bar = 2 μm. (E and F). Group data shows a significant increase in mGluR5 and NR1 expression in the synaptic area of *Fmr1* KO neurons (mGluR5, WT, n = 30 dendritic fields, median = 10.19; *Fmr1* KO, n = 21 dendritic fields, median = 31.17; $P < 0.001$ by Mann-Whitney test; NR1, WT n = 31 dendritic fields, median = 14.68; *Fmr1* KO n = 18 dendritic fields, median = 38.09; $P < 0.01$ by Mann-Whitney test). (G) Group data shows a significant increase in mGluR5 and NR1 co-localitation in the synaptic area of *Fmr1* KO neurons (WT, n = 26 dendritic fields, median = 16.74; *Fmr1* KO, n = 20 dendritic fields, median = 20.76; $P < 0.05$ by Mann-Whitney test). (H) Pretreatment with TAT-mGluR5ct peptide (1h; 5 μM) shows a significant increase in mGluR5 and NR1 co-localization in the synaptic area of WT neurons (WT, n = 26 dendritic fields; TAT-mGluR5mu, n = 10 dendritic fields; TAT-mGluR5ct, n = 27 dendritic fields; $P < 0.01$ by Kruskal-Wallis test). No differences were observed for mGluR5 and NR1 co-localization in *Fmr1* KO neurons treated with either TAT-mGluR5mu or TAT-mGluR5ct (*Fmr1* KO, n = 20 dendritic fields; TAT-mGluR5mu, n = 20 dendritic fields; TAT-mGluR5ct, n = 15 dendritic fields; P = 0.3426 by Kruskal-Wallis test). Data are shown as mean ± s.e.m. for the diffusion coefficient. Box plots show the median, interquartiles, range, mean and individual values. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. Scale bar = 2 μm (except panel D for low magnification Scale bar = 10 μm).

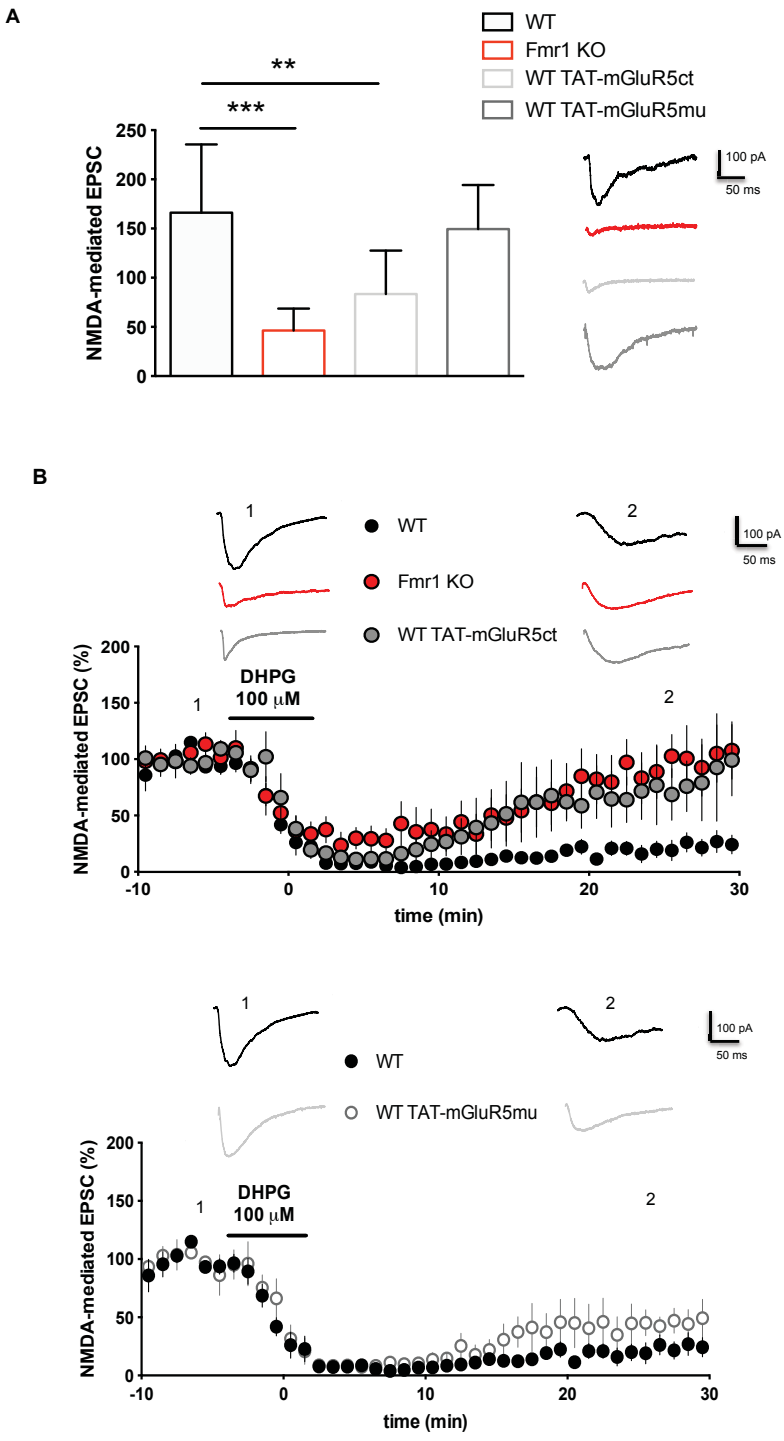

A
WT
Fmr1 KO
WT TAT-mGluR5ct
WT TAT-mGluR5mu
**
***
NMDA-mediated EPSC
250
200
150
100
50
0
100 pA
50 ms
B
1
2
WT
Fmr1 KO
WT TAT-mGluR5ct
100 pA
50 ms
DHPG
100 μM
NMDA-mediated EPSC (%)
200
150
100
50
0
-10
0
10
20
30
time (min)
WT
WT TAT-mGluR5mu

Figure 6. The increased mGluR5/NMDAR physical interaction alters NMDAR function and plasticity in hippocampal CA1 neurons. (A) NMDAR-mediated excitatory post-synaptic currents (NMDAR-EPSCs) were recorded from CA1 pyramidal neurons in acute hippocampal slices following stimulation of *Schaffer* collaterals in the presence of AMPAR blockers. The amplitude of NMDAR-EPSCs was significantly smaller in *Fmr1* KO neurons (WT, n = 8, 166.2 ± 24.4 pA; *Fmr1* KO, n = 7, 46.4 ± 8.4 pA; $P < 0{,}001$ by unpaired Student's t-test). Similarly, in WT slices treated with TAT-mGluR5ct (4h, 5 μM), the amplitude of NMDA-EPSCs was significantly lower than in control conditions (WT, n = 8, 166.2 ± 24.4 pA; TAT-mGluR5ct, n = 10, 83.4 ± 14 pA; $P = 0.01$ by unpaired Student's t-test). TAT-mGluR5mu (4h, 5 μM) had no significant effect on the amplitude of NMDAR-EPSCs (WT, n = 8, 166.2 ± 24.4 pA; TAT-mGluR5mu, n = 5, 149.5 ± 19.9 pA; $P = 0.7$ by unpaired Student's t-test). Traces show average of several currents from representative neurons. (B) mGluR5-mediated LTD of NMDAR-EPSCs is reduced in *Fmr1* KO neurons. Application of DHPG (100 μM for 5 min) induces LTD of NMDAR-EPSCs in WT neurons but not in *Fmr1* KO neurons (time point 2, $P < 0.001$ by unpaired Student's t-test). These defects in NMDAR plasticity can be mimicked in WT after pretreatment of the hippocampal slices with TAT-mGluR5ct peptide (4h, 5 μM) (time point 2, $P < 0.01$ by unpaired Student's t-test) (*upper panel*) but not with its control peptide TAT-mGluR5mu (4h; 5 μM) time point 2, $P > 0.05$ by unpaired Student's t-test (*lower panel*). Traces show average of several currents from representative neurons. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

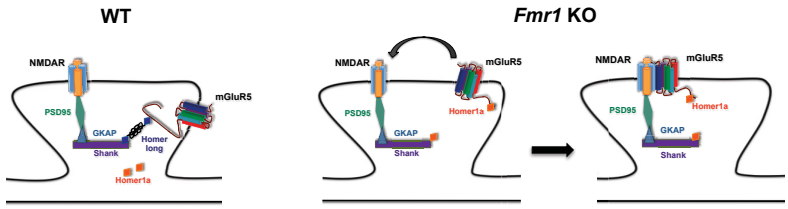
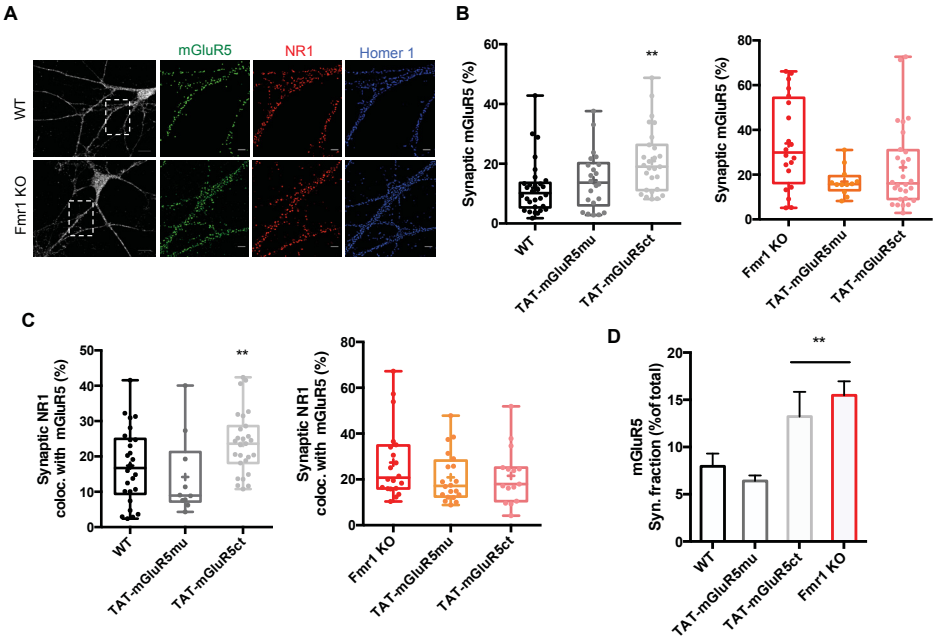


Figure 7. Model for dysfunction of the NMDAR/mGluR5 crosstalk in *Fmr1* KO neurons. In WT neurons, a long Homer protein–containing complex cross-talks mGluR5 to NMDAR in the synapse and prevents mGluR5/NMDAR interaction. In this condition, mGluR5 and NMDAR do not cluster. In *Fmr1* KO neurons, mGluR5 is less associated with the long Homer proteins and more associated with the short isoform, Homer 1a. Homer 1a triggers disengagement of mGluR5 from the long Homer protein–containing complex, thus increasing the lateral diffusion of mGluR5 and allowing the interaction with NMDAR in the synapse. In this condition, mGluR5 co-clusters with NMDAR preventing its boosting under control condition and LTD of NMDA currents following mGluR5 stimulation.



Supplementary figure 1. Disruption of the link between mGluR5 and Homer proteins increases the synaptic expression in WT neurons. (A) Hippocampal neurons from WT mice stained with the primary antibodies anti-mGlu5-NH_2 or anti-NR1-NH_2 with anti-Homer 1. (B and C) Group data show a significant increase in mGluR5 and NR1 expression in the synaptic area of WT neurons after pretreatment with TAT-mGluR5ct peptide (1h; 5 μM) (*left panel*), (mGluR5, WT, n = 31 dendritic fields; TAT-mGluR5mu, n = 22 dendritic fields; TAT-mGluR5ct, n = 27 dendritic fields, $P < 0.01$ by Kruskal-Wallis test; NR1, WT, n = 31 dendritic fields; TAT-mGluR5mu, n,= 20 dendritic fields; TAT-mGluR5ct, n = 28 dendritic fields, $P < 0.01$ by Kruskal-Wallis test). No differences were observed for the expression of mGluR5 and NR1 in the synaptic area of *Fmr1* KO neurons treated with either TAT-mGluR5mu or TAT-mGluR5ct (mGluR5, *Fmr1* KO n= 22 dendritic fields; TAT-mGluR5mu n= 12 dendritic fields; TAT-mGluR5ct n= 29 dendritic fields, by Kruskal-Wallis test; NR1, *Fmr1* KO, n = 18 dendritic fields; TAT-mGluR5mu, n = 17 dendritic fields; TAT-mGluR5ct, n = 27 dendritic fields, by Kruskal-Wallis test). (D) Relative fraction of synaptic mGluR5-QD in WT and *Fmr1* KO neurons neurons and after pretreatment with TAT-mGluR5ct peptide (1h, 5 μM) and its control peptide TAT-mGluR5mu (1h, 5 μM). Note the significant increase in the relative synaptic content in mGluR5-QD in *Fmr1* KO neurons and after pretreatment with TAT-mGluR5ct peptide in WT neurons (WT, n = 31 dendritic fields; TAT-mGluR5mu, n = 22 dendritic fields; TAT-mGluR5ct, n = 27 dendritic fields, *Fmr1* KO, n = 30 $P <$ 0.01 by One way Anova). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Scale bar = 2 µm (except panel D for low magnification Scale bar = 10 µm).

CHAPTER V

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

A substantial body of evidence points to a central role of the mGluR5 in the pathophysiology of FXS. In particular, an exaggerated signaling response following activation of mGluR5 underlies the synaptic dysfunction in this disorder. The first indication for a link between mGluR5 and FXS was the evidence that activation of Group-I mGluR in rat and mouse brain synaptoneurosomes stimulates the rapid translation of pre-existing mRNA, including the mRNA for FMRP (Weiler et al., 1997). Since then, a growing number of studies support a crucial role of Group-I mGluR in the pathophysiology of FXS. These studies, together with the finding that mGlu5 protein synthesis-dependent LTD is increased in the mouse model of FXS, led Mark Bear and collaborators to formulate the so-called "mGlu theory" of FXS (Bear et al., 2004). This theory postulates that in the absence of FMRP, which acts by reducing the mGlu5-activated mRNA translation at the synapse, levels of FMRP-regulated proteins are increased. It also postulates that FXS symptoms might therefore be corrected by suppressing mGluR5 signalling (Bear et al., 2004). Several pharmacological studies have supported the "mGlu theory", by demonstrating that phenotypic features of FXS can be corrected with the use of mGlu5 antagonists. In addition, genetic deleteion of mGluR5 by 50% in *Fmr1* KO mice corrected many FXS phenotypes such as excessive protein synthesis, altered mGluR-LTD and other types of neuronal plasticity, abnormal spines morphology, and audiogenic seizures (Dölen et al., 2007). Overall, the prospect for the development of mGluR5 NAMs as a therapeutic rescue approach seems promising. The positive results of preclinical animal model studies have led to the development of drugs for testing in clinical trials in FXS patients. To date, however, these clinical trials have not had the expected outome. As a consequence of these failed trials, Novartis and Hoffmann La Roche have discontinued their drug develpment programs for mGluR5 NAMs (www.fraxa.org, reported in 2014). The reasons why these clinical trials failed may be an inadequate dose range for Fragile X patients and an unexpected development of tolerance (www.fraxa.org). Dosage problems are relatively easy to correct, but tolerance may be something intrinsic to the physiology of mGluR5, requiring a better understanding of these processes.

Thus, an improved understanding of the mechanisms underlying mGluR dependent deficits of FXS are needed to define better targets for pharmacological intervention. Indeed, the cellular mechanisms of mGluR5 dysfunction in FXS have been elusive and most of the studies have been focused just on the altered translational processes at the synapse initiated by mGluR5 activation. However, recent evidence

indicates that some relevant phenotypes might be dependent also on other mechanisms namely mGluR5/Homer crosstalk disruption suggesting that altered mGluR5 function is upstream of protein translation in FXS (Giuffrida et al., 2005; Ronesi et al., 2012). Indeed, recent data have shown that disrupted mGluR5/Homer scaffolds may cause several phenotypes of *Fmr1* KO mice, including altered mGlu5 receptor signaling, neocortical circuit dysfunction and aberrant behavior (Ronesi et al., 2012). This data lend strong support to the concept that mGlu5/Homer disruption may contribute substantially to the pathophysiology of FXS (Ronesi et al., 2012). The goal of my thesis was therefore to examine the consequences of this mGlu5/Homer crosstalk disruption for the behavior of mGluR5 at hippocampal synapses and for the subsequent regulation of NMDA receptors. Here, I provide new evidence for defects in the synaptic mobility of mGlu5. These changes alter NMDA currents during normal synaptic activity and following mGlu5 dependent induction of NMDA receptor plasticity.

In the first set of experiments we looked at the expression of mGluR5 in hippocampal synaptosomes of mice compared to WT at different ages. We found that mGluR5 expression was higher in *Fmr1* KO mice with a major effect in the juvenile epoch (P21). We also confirmed that mGlu5 mRNA is an FMRP target using UV-crosslinking and immunoprecipitation mRNA interaction assay as previously reported by Darnell and collaborators (2011). Thus, we believe that the increased expression of mGluR5 is caused by the lack of inhibitory control of FMRP on mGluR5 dependent protein synthesis. The increased expression of mGluR5 is more evident in early developmental when both FMRP and mRNA encoding mGluR5 are highly expressed (Catania et al., 1994; Bonaccorso et al., submitted). Accordingly, we observed an increased expression of mGluR5 also in hippocampal cell cultures at early developmental stages, when FMRP is highly expressed (Bonaccorso et al., submitted). Overall these data support the view that mGluR5 protein is more expressed in FXS, in particular during early postnatal development.

These findings provide for the first time a systematic analysis of the expression of mGluR5 in the hippocampus of *Fmr1* KO mice and at different developmental stages. Indeed, reports of mGluR5 expression levels in brain samples from animal models of FXS are limited. A previous study from our lab showed that total mGluR5 levels were similar in the forebrain of WT and *Fmr1* KO mice (Giuffrida et al., 2005), but it is likely that mixing different forebrain regions masked brain region specific differences. For example, differences between the neocortex and hippocampus of *Fmr1* KO mice have

been reported for mGluR signaling or synaptic plasticity defects (Krueger & Bear, 2011). A recent study in human brain tissue has shown that mGluR5 expression was significantly increased in the postmortem prefrontal cortex of Fragile X patients (Lohith et al., 2013). In the *Fmr1* KO hippocampus, however, Western blot analysis revealed no difference in mGluR5 protein expression (Dölen et al., 2007). Our results indicated significantly elevated expression of mGluR5 in the hippocampus of juvenile *Fmr1* KO mice compared with control mice. Altogether, the discrepancy of our findings with some of the aforementioned studies might be due to several key factors, including differences between species, experimental procedures, brain regions studied and developmental stages.

Our findings led us to hypothesize that mGluR5 surface expression might be also increased in *Fmr1* KO mice as a consequence of the mGluR5/Homer crosstalk disruption. Indeed, it has been shown that mGluR5/Homer interaction facilitates mGluR5 intracellular retention (Ango et al., 2001; Coutinho et al., 2001). The Homer 1a isoform, on the other hand, disrupts mGluR5-long Homer complex by altering mGluR5 localization and surface expression (Ango et al., 2001; Coutinho et al., 2001). As predicted, we found that mGluR5 expression was increased on the surface of cultured hippocampal neurons from *Fmr1* KO mice. Increased mGluR5 surface expression was detected at any developmental stages in and was critically dependent on the disruption of mGlu5/Homer crosstalk. Overall these data suggest that, in addition to a reduced inhibitory control by FMRP on mGlu5 protein synthesis operating mainly at early stages, an additional mechanism may be responsible for the increased targeting or maintenance of surface mGlu5 receptors at later developmental stages. Moreover, we observed that *Fmr1* KO neurons mGluR5 was expressed not only in the surface of soma and dendrites, but also along the axons. Interestingly, agonist-induced internalization of mGlu5 receptors was absent in *Fmr1* KO neurons, but independently on mGlu5/Homer interaction. Here we suggest that mechanisms for mGluR5 dysfunction in FXS might be also upstream of protein translation and dependent on mGlu5/Homer crosstalk disruption.

Based on these results we predicted that the disruption of mGluR5/Homer crosstalk might affect the surface dynamics of mGluR5 within the synaptic site. In agreement with this prediction, we demonstrated that the mGluR5/Homer crosstalk disruption increased the lateral diffusion of mGluR5 specifically at the synapses of *Fmr1* KO hippocampal neurons. The disruption of mGluR5/Homer crosstalk alters the

dynamic switch between mobile and immobile states of the receptor. This higher mobility of mGluR5 likely increases the probability of transient interactions with other transmembrane proteins at the synapse. These proteins may include the mGluR5 themselves (Choquet & Triller, 2003), but also other types of glutamate receptors. Interestingly, there is strong evidence suggesting that mGluR5 can physically interact with NMDAR. mGluR5 and NMDAR are physically linked together in the postsynaptic density by a long Homer protein–containing complex (Brakeman et al., 1997; Scannevin & Huganir, 2000). The constraint that results from the link between the C-terminus of mGluR5 and NMDAR with the long Homer protein–containing complex preclude the direct mGluR5/NMDAR association (Moutin et al., 2012). Homer 1a disassembles the synaptic multimeric mGluR5/Homer complex allowing physical and functional interactions between NMDAR and mGluR5 (Moutin et al., 2012). The physical interaction between mGluR5 and NMDAR results in mGluR5 mediated inhibition of NMDA currents (Moutin et al., 2012).

We demonstrated that in *Fmr1* KO neurons the disruption of mGluR5/Homer crosstalk and the consequent higher mGluR5 lateral mobility increased the probability that during the diffusion on the neuronal membrane mGluR5 might interact with NMDAR in the PSD. The increased interaction induced the mGluR5-mediated inhibition of NMDAR synaptic current. We found that NMDAR-EPSCs evoked by *Schaffer* collateral stimulation showed lower amplitudes in *Fmr1* KO neurons. Moreover, we found that the postsynaptic expression of mGluR5 mediated LTD of NMDAR-EPSCs was reduced in *Fmr1* KO neurons Exogenous NMDAR mediated currents are transiently potentiated by Group-I mGluR activation (Benquet et al., 2002; Grishin et al. 2004; Heidinger et al., 2002; Mannaioni et al., 2001; Skeberdis et al., 2001; Snyder et al. 2001). Conversely, synaptically elicited NMDA-EPSCs undergo depression in response to Group-I mGluR activation (Baskys & Malenka, 1991; Watabe et al., 2002). reasons for this disparity and the mechanisms underlying the mGluR-induced depression of the NMDA-EPSCs are poorly understood. Although the mechanism for the transient acute depression is most likely pre-synaptic in origin (Watabe et al., 2002), Group-I mGluR-mediated rapid depression and LTD of the synaptically evoked NMDAR currents appears to be mediated post-synaptically by lateral movement of synaptic NMDARs via actin depolymerization (Ireland & Abraham, 2009). It has been shown that Group-I mGluR-mediated LTD of NMDA-EPSCs is not dependent on protein synthesis, or tyrosine kinase or phosphatase activity, or an increase in intracellular calcium. We

demonstrate that the defects in NMDAR plasticity were critically dependent on the altered mGluR5/Homer association. These results show how altered mGluR5 dynamics mediate abnormal NMDAR currents in FXS.

This work highlights the importance of the dynamic association/dissociation of multiprotein complexes in receptor functions and cell physiology. Neurotransmitter receptors can no longer be seen as cell surface-isolated entities. Regulations of receptor signaling by dynamic changes in scaffolding-receptor-associated complex can be considered a core mechanism for synaptic function. The dynamic movement of synaptic components has emerged as the main mechanism for dynamically organizing the synaptic membrane and as a key feature of synaptic transmission and plasticity (Triller & Choquet, 2005; 2008).

Here we provide new evidence that the dynamic remodelling within the synapse mediated by mGluR5/Homer crosstalk triggers an altered modulation of NMDAR by mGluR5 in *Fmr1* KO neurons. NMDAR have largely been ignored in the study of FXS, perhaps due to an early study reporting normal NMDAR-dependent LTP in the CA1 region of *Fmr1* KO mice (Godfraind et al., 1996). Consistent with this observation was the report that NMDAR-LTD was normal in the CA1 region of the hippocampus (Huber et al., 2002). In contrast to these results conducted in adult mice, recent studies investigating NMDAR-dependent LTP and LTD in young mice have revealed deficits. Hu et al. (2008) show impaired NMDAR-dependent LTP in the CA1 region of 2-week old *Fmr1* KO mice (H. Hu et al., 2008). In a separate study, Pilpel et al. (2009) noted abnormally enhanced NMDAR-dependent LTP in the CA1 subfield of 2-week old, but not 6 to 7-week old *Fmr1* KO mice (Pilpel et al., 2009). Activation of Group-I mGluRs is involved in the induction of NMDAR-dependent synaptic plasticity. Activation of Group-I mGluRs can induce LTP or LTD depending on whether the currents are elicited by exogenous NMDA application or synaptic stimulation. Here we suggest that the dynamic remodelling of the link between mGluR5 and Homer proteins might play a key role in the in the induction of NMDAR-EPSCs LTD in response to Group-I mGluR. We show that the physical interaction between mGluR5 and NMDAR caused by the disruption of mGluR5/Homer crosstalk alters synaptic function and LTD in *Fmr1* KO neurons. This study provides new directions to understand the role of mGluR5/Homer crosstalk in the regulation of synaptic plasticity and how its disruption contributes to the pathopysiology of FXS.

How does the loss of FMRP lead to altered mGluR5/Homer crosstalk? Protein

levels of long Homers and Homer 1a are unchanged in total homogenates of *Fmr1* KO hippocampi (Giuffrida et al., 2005), and FMRP is not reported to interact with mRNA for any Homer isoforms (Darnell et al., 2011). Previous work reported a decrease in tyrosine phosphorylation of long Homer in *Fmr1* KO forebrain (Giuffrida et al., 2005), but it is unknown whether or how this affects interactions with mGluR5. Phosphorylation of mGluR5 at the C-terminal Homer interaction domain reduces the affinity of mGluR5 for Homer (Orlando et al., 2009). Therefore, post-translational modification of mGluR5 and/or Homer in *Fmr1* KO mice may underlie the decreased interactions. It will be interesting to investigate the mechanisms that lead to a reduced tyrosine phosphorylation of Homer proteins in *Fmr1* KO animals and to verify how it can affect the pathophysiology of FXS. The discovery that altered mGluR5/Homer interaction account for much of the complex dysfunction of mGluR5 in FXS will help to develop alternative, targeted therapies for the disease and provide mechanistic links to other genetic causes of autism.

The modulation and restoration of mGluR5/Homer interaction by reducing the expression of Homer 1a in hippocampal neurons may correct the mGluR5 dysfunction that alters mGluR5 surface dynamics and NMDAR currents in *Fmr1* KO neurons. To address this question we have begun to exploit adeno-associated virus (AAV) vectors expressing either a small interfering hairpin RNA (shRNA) targeted against the 3'-untranslated region of Homer 1a mRNA (H1a shRNA), which is unique to the Homer 1a splice variant, or scrambled shRNA control (Scr shRNA). We are currently testing whether Homer 1a knockdown rescues the NMDAR currents in hippocampal neurons. To address this question we are stereotaxically injecting these AAV vectors into the CA1 area of the hippocampus of *Fmr1* KO mice. After 3-4 weeks we perform patch-clamp recording of NMDAR-EPSCs in the CA3-CA1 synapse in acute slices derived from injected animals.

This may represent a novel strategy for the rescue of altered synaptic function that might underlie cognitive impairments in FXS. Indeed, altered synaptic plasticity might be associated with cognitive impairment in the *Fmr1* KO mouse. Mechanisms of LTP and LTD are believed to be the underlying cellular basis of learning and memory formations and altered mGluR dependent NMDAR LTD might impact memory formation and learning in FXS. The hippocampus has a pivotal role in memory processing, recognition, acquisition, and storage of the contextual and temporal details (reviewed by (Eichenbaum, 2004; Kesner & Hunsaker, 2010; Rolls & Kesner, 2006;

Squire, 2004)) The hippocampus receives inputs from the perirhinal cortex, which is itself the site of several information entrances as visual, olfactory, and somatosensory stimulus, all of them involved in different memory tasks (Clarke, Cammarota, Gruart, Izquierdo, & Delgado-García, 2010). The hippocampus is an essential neural structure in developing contextual memory in a situation in which rapid development of associative learning should occur (J. J. Kim, Rison, & Fanselow, 1993; Phillips & LeDoux, 1992). Lesions in the hippocampus impair the ability to learn and remember a spatial context where a noxious stimulus (e.g., electric shock) occurs (C. Chen, Kim, Thompson, & Tonegawa, 1996; Moses, Winocur, Ryan, & Moscovitch, 2007; Wiltgen, Sanders, Anagnostaras, Sage, & Fanselow, 2006). In conditional fear, a non-threatening conditioned stimulus (CS) occurs in association with an aversive unconditioned stimulus and, as a result of this pairing, the CS acquires the aversive properties of the unconditioned stimulus (C. Chen et al., 1996; Moses et al., 2007; Wiltgen et al., 2006). When presented alone, the CS will evoke defensive behaviors such as freezing (Cornish et al., 2008).

The *Fmr1* KO mice display significantly decreased levels of freezing 24 h after training for both contextual and cued fear conditioning, in comparison to the WT group (Paradee et al., 1999) To determine whether the cognitive defects in contextual memory of *Fmr1* KO mice may be related to mGluR/Homer crosstalk disruption, we are currently testing a fear conditioning paradigm in WT, *Fmr1* KO, and injected *Fmr1* KO mice with H1a shRNA-AAV and Scr shRNA-AAV.

In summary, in this study I provide new evidence for the role of the mGluR5/Homer crosstalk disruption in the pathophysiology of FXS by using a powerful combination of innovative techniques. These experiments improve our understanding of the pathophysiology of FXS in particular, and intellectual disability disorders in general A more detailed understanding of the complex dysfunction of mGluR5 in FXS will help to develop alternative, targeted therapies for the disease and provide mechanistic links to other genetic causes of autism.

CHAPTER V

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CHAPTER VI

ANNEXES

1. List of Publications and Scientific Contributions

List of Publications

- D'Antoni S., Spatuzza M., Bonaccorso C.M., **Aloisi E.,** Musumeci S., Catania M.V. *Fragile X Syndrome: From Pathophysiology to New Therapeutic Perspectives*. Latest Findings in Intellectual and Developmental Disabilities Research, Prof. Uner Tan (Ed.), ISBN: 978-953-307-865-6, InTech, DOI: 10.5772/31387. Published: February 15, 2012 under CC BY 3.0 license.
- Spatuzza M., D'Antoni S., **Aloisi E.,** Bonaccorso C.M., Molinaro G., Battaglia G., Musumeci S., Maurin T., Bardoni B., Shigemoto R., Nicoletti F., Catania M.V. *Metabotropic Glutamate subtype 5 receptors are increased at synapses and do not undergo agonist-induced internalization in the Fmr1 KO mouse model of Fragile X Syndrome*. To be submitted to The Journal of Neuroscience.
- **Aloisi E.,** Dupuis J.P., Labrousse V., Haberl M.G., Costa L., Drago F., Piazza P.V., Ciranna L., Groc L., Catania M.V. and Frick A. *Altered mGlu5 receptor surface dynamics are linked to abnormal NMDA receptor function and plasticity in Fragile X Syndrome*. To be submitted.

Oral communications

- **Aloisi E.**, Dupuis J.P., Haberl M.G., Drago F., Catania M.V. and Frick A. *"Altered surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome"*. 27th ECNP Congress, 18-21 October 2014, Berlin - Germany.
- **Aloisi E.**, Spatuzza M., D'Antoni S., Drago F., Catania M.V. and Frick A. *"Altered surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome"*. 12th Synapse Day Meeting. 28 March 2014 - Bordeaux, France
- **Aloisi E.**, Spatuzza M., D'Antoni S., Drago F., Catania M.V. and Frick A. *"Altered surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome"*. ECNP Workshop for Junior Scientists in Europe. 6-9 March 2014 - Nice, France.
- **Aloisi E.**, Spatuzza M., D'Antoni S., Drago F., Catania M.V. and Frick A. "*Pharmacological investigation into the mechanisms underlying altered mGlu5*

receptor dynamics in a mouse model of Fragile X Syndrome". 36° Congresso della Società italiana di farmacologia. 23-26 October 2013 - Turin, Italy.

Poster communications

- **Aloisi E.,** Dupuis J.P., Costa L., Haberl M.G., Labrousse V., Ladépêche L., Drago F., Piazza P.V., Ciranna L., Groc L., Catania M.V. and Frick A. *Altered mGlu5 receptor surface dynamics are linked to abnormal NMDA receptor function and plasticity in Fragile X Syndrome*. 8th International Meeting on Metabotropic Glutamate Receptors, 28 September – 3 October 2014, Taormina - Italy
- **Aloisi E.**, Spatuzza M., D'Antoni S., Drago F., Catania M.V. and Frick A. *"Altered surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome"*. 9th FENS forum of Neuroscience, 5-9 July 2014 - Milan, Italy.
- **Aloisi E.**, Spatuzza M., D'Antoni S., Drago F., Catania M.V. and Frick A. *"Altered surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome"*. ECNP Workshop for Junior Scientists in Europe. 6-9 March 2014 - Nice, France.
- **Aloisi E.**, Spatuzza M., D'Antoni S., Drago F., Catania M.V. and Frick A. *"Surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome"*. Society for Neuroscience. 9-13 November 2013 - San Diego (CA), USA.
- **Aloisi E.**, Spatuzza M., D'Antoni S., Drago F., Catania M.V. and Frick A. *"Surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome"*. 4th European Synapse Meeting. 28-30 August 2013 - Bordeaux, France.
- **Aloisi E.**, Spatuzza M., D'Antoni S., Drago F., Catania M.V. and Frick A. *"Surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome"*. 1st European ENCODS Conference. 18-19 April 2013 - Bordeaux, France.
- **Aloisi E.**, Spatuzza M., D'Antoni S., Catania M.V. and Frick A. *"Surface trafficking of mGlu5 receptors in the Fmr1 knockout mouse (Fmr1KO) model of Fragile X Syndrome"*. Conférences Jacques-Monod. Mechanisms of intellectual disability: from genes to treatment. 3-7 October 2012 – Roscoff, France.

Honours and awards

- Travel grant for the 9th FENS Forum (5-9 July 2014 - Milan, Italy) awarded from the French Neuroscience Society (Société des Neurosciences).
- Poster award at ECNP Workshop on Neuropsychopharmacology for Young Scientists in Europe (6-9 March 2014 - Nice, France).
- Prix jeune chercheur – Jérôme Lejeune 2013 with the thesis *"Surface dynamics of mGlu5 receptor in a mouse model of Fragile X Syndrome*".

2. Book chapter

Latest Findings in Intellectual and Developmental Disabilities Research, Prof. Uner Tan (Ed.), ISBN: 978-953-307-865-6, InTech, DOI: 10.5772/31387. Published: February 15, 2012 under CC BY 3.0 license.

Fragile X syndrome: from pathophysiology to new therapeutic perspectives

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1. Introduction

In the present chapter we will provide an overview of recent literature regarding new therapeutic perspectives in Fragile X syndrome (FXS), which are based on a rational approach well-grounded on a deeper understanding of the disease pathophysiology. FXS represents a paradigmatic example of how research can be translated into therapy targeting dysfunctional mechanisms rather than symptoms. Several clinical trials using these new strategies are underway. Here, we will mainly describe the basic mechanisms and the animal studies, which suggest the use of these innovative pharmacological approaches. In addition, an emerging concept is that developmental pathologies with intellectual disability (ID) presenting common features such as autism, behavioural disturbances and epilepsy might share dysregulation of the same biochemical pathways. The identification of common altered pathways in ID might help to develop new therapeutic strategies helpful for apparently diverse pathologies.

2. Fragile X syndrome

2.1 FXS: clinic and genetics

ID, also referred with the term Mental Retardation, is the most common developmental disorder, with a prevalence of 1-3%, and includes a highly diverse group of cognitive disorders. It is defined, according to the American Psychiatric Association,

by an intelligence quotient (IQ) of 70 or below, and deficits in at least two behaviours related to adaptive functioning diagnosed by 18 years of age. Gene defects account for about half of all patients and mutations have been identified in more than 400 genes, of which 97 are positioned in the X chromosome (reviewed in Kaufman et al., 2010).

FXS is an X-linked developmental disorder which represents the most common form of inherited ID, affecting approximately 1 in 2500-6000 males and 1 in 4000-8000 females. ID ranges from severe to mild and may be associated with Attention Deficit and/or Hyperactive Disorder (ADHD), autism, behavioral disturbances, hyperactivity, seizures and hypersensitivity to sensory stimuli. People with FXS may also exhibit facial dysmorphic features including a long face with prominent ears and arched palate, hyperextensible joints, mitral valve prolapse and macroorchidism (R.J. Hagerman, 2002).

The most common genetic defect in FXS is a CGG trinucleotide repeat expansion of >200 repeats in the 5' untranslated region of the *Fmr1* (fragile X mental retardation 1) gene, located on the long arm of the X chromosome at position 27.3 (Verkerk et al., 1991). This triplet amplification is associated to methylation of the *Fmr1* promoter region and transcriptional silencing of the *Fmr1* gene with consequent loss or significant reduction of the *Fmr1* encoded protein FMRP (fragile X mental retardation protein) (Devys et al., 1993; O'Donnel & Warren, 2002). Expansions of CGG repeats are instable during meiosis, increasing in length from one generation to the next. In carriers of the premutation, the expansion is between 55–200 repeats (normal is <45), and do not result in *Fmr1* methylation and loss of FMRP expression, but give rise to two independent pathologies such as fragile X-associated tremor/ataxia syndrome (FXTAS) and premature ovarian failure, primarily in males and females respectively (reviewed in Berry-Kravis et al., 2007; P.J. Hagerman et al., 2008; Toniolo, 2006). It has been hypothesised that these conditions are caused by a gain of function toxic effect of increased levels of CGG repeat-containing *Fmr1* mRNA (Berry-Kravis et al., 2007), although decreased levels of FMRP may also play a role (Qin et al., 2011).

2.2 FXS: alterations of dendritic spine morphology

Microscopic analysis of brain material from both patients with FXS and mouse models of the disease reveals no gross morphological abnormalities (Bakker et al., 1994; Reyniers et al., 1999). However, in certain brain areas such as cortex and hippocampus,

long and thin dendritic spines have been observed, consistent with an immature spine phenotype (Comery et al., 1997; Irwin et al., 2001, 2002; Nimchinsky et al., 2001).
Dendritic spines are protrusions of dendritic membrane and serve as the postsynaptic component for the vast majority of central nervous system (CNS) excitatory synapses. Spines are dynamic structures that can regulate many neurochemical events related to synaptic transmission and modulate synaptic efficacy. The tip of the spine contains an electrondense region, the "postsynaptic density" (PSD), that is a protein dense specialization and consists of receptors, channels, and signaling systems involved in synaptic transmission. Spines are highly motile structures, their density varies across areas of different brain regions but also within individual dendritic trees; spine morphology changes with development and requires actin cytoskeleton remodelling and local protein translation in response to synaptic activity. Notably, spines are equipped with translational machinery and protein synthesis may occur in response to receptor activation. The structural modifications of spines are correlated with synaptic plasticity (see below); in fact Long Term Depression (LTD) is generally associated with a shrinkage of spines, whereas Long Term Potentiation (LTP) causes formation of new spines and enlargements of existing spines (Tada & Sheng, 2006).

Abnormalities in dendrites and spines have been implicated in several psychiatric disorders and have been associated with cognitive impairment and mental retardation disorders (Tuberous Sclerosis Type I, Fetal alcohol syndrome, Down syndrome Rett syndrome, autism and FXS) (Nimchinsky et al., 2002), but, the causes of these malformations are not yet well understood.

2.3 FMRP: expression, structure and interacting proteins

FMRP is an RNA binding protein involved in the regulation of target mRNA translation and transport. It belongs to a small family of highly conserved RNA binding proteins referred to as the fragile X–related (FXR) proteins; it is expressed in several tissues and organs and has been found to be most abundant in the brain and testis. FMRP is highly expressed in neurons, and is associated with translating polyribosomes and ribonucleoprotein complexes (mRNP) in the cytoplasm, in dendrites and dendritic spines where it is believed to regulate mRNA translation (De Diego Otero et al., 2002). Recent data also suggest that FMRP is present in axons and pre-synaptic terminals (Christie et al., 2008).

The analysis of the structure of FMRP has revealed the presence of different functional motifs and has contributed to elucidate the function of the protein. FMRP contains three different RNA binding domains: two hnRNP K-protein homology (KH) domains and an Arg-Gly-Gly (RGG) box (Siomi et al., 1993), which bind sequence – specific elements such as the U-rich sequences called FMRP kissing complex and G-quartet, respectively (Darnell et al., 2001, 2005). Interestingly, a missense mutation in the second hnRNP KH binding domain (I304N) abolishes FMRP association with polyribosomes and causes FXS. The presence within FMRP of a nuclear localization signal (NLS) and a nuclear export signal (NES) suggests that FMRP is a shuttle protein and that it travels between the nucleus and the cytoplasm (Darnell et al., 2001, 2005; Eberhart et al., 1996; Sittler et al., 1996). In the nucleus, FMRP binds to RNAs and proteins to form the mRNP particle and is then exported to the cytoplasm where it could associate with translating ribosomes (Corbin et al., 1997; Eberhart et al., 1996; Feng et al., 1997a; Khandjian et al., 1996). The mRNP complex can stay in the neuronal cell body or it can move to the dendritic spines via the microtubule structures present in the dendrites. In this way, FMRP can control the local protein synthesis at the synapses, influencing synaptic function, structure and plasticity (Bardoni et al., 2006; Feng et al., 1997b; Miyashiro et al., 2003; Zukin et al., 2009).

The structure of FMRP presents also two coiled coil (CC) domains involved in protein:protein interactions. Using immunoprecipitation two-hybrid screens or large mass spectrometry analysis several FMRP interacting proteins have been identified including its two close paralogs, FXR1P and FXR2P (Fragile X Related Protein 1/2) (Y. Zhang et al., 1995), NUFIP1 (Nuclear FMRP Interacting Protein 1) (Bardoni et al., 2003a), 82-FIP (82 kDa-FMRP Interacting Protein) (Bardoni et al., 2003b) and the two closely related proteins CYFIP1 and CYFIP2 (Cytoplasmic FMRP Interacting Protein 1/2) (Schenck et al., 2001). The role and importance of these interacting proteins in the function of FMRP is not clear; it is possible that the interaction with these proteins might modulate the function of FMRP in different cellular compartments.

FXR1P and FXR2P show a similar structure to that of FMRP, being characterized by the presence of two KH and one RGG box RNA binding domains and nuclear localization and export signals (NLS and NES). In the absence of FMRP there is not a compensatory increase in levels of FXR1P and FXR2P, which would suggest functional redundancy. However, the precise role of the two FMRP paralogues and their reciprocal interaction is still under investigation. The interaction of FMRP with these proteins may

modulate the function of FMRP by influencing its affinity for RNA, as shown for FXR1P isoforms (Bechara et al., 2007). In addition, recent data (Darnell et al., 2009) show that the properties of the KH2 domains of the three proteins are similar, whereas only FMRP recognizes G-quadruplex RNA evidencing that the RGG domain may have a non redundant role in FXS.

NUFIP1 is an RNA-binding protein which at subcellular level co-localizes with isoforms of FMRP present in the nucleus; it shows no homology with known proteins. NUFIP1 is also present in the cytoplasm, where it is associated with ribosomes in the cell soma and activated synaptoneurosomes (Bardoni et al. 2003a). 82-FIP is also not an homolog protein of FMRP and its subcellular distribution is cell-cycle dependent, indicating that the composition of some FMRP-containing RNP complexes might be cell-cycle modulated (Bardoni et al., 2003b). CYFIP1 and CYFIP2 are highly homologous to each other; CYFIP2 interacts with all members of the FXR family, while CYFIP1 is specific for FMRP (Schenck et al., 2003). CYFIP1 and 2 are localized at synapses and CYFIP1 also interacts with activated Rac1 (Kobayashi et al., 1998; Schenck et al., 2003), a small RhoGTPase involved in maturation and maintenance of dendritic spines (Govek at al., 2005), suggesting that FMRP might influence cytoskeleton remodelling through Rho/Rac GTPase (Schenck et al., 2003). The interaction between FMRP and CYFIP1 has been proposed to mediate the inhibition of translation initiation by sequestering the cap-binding protein eIF4E (De Rubeins et al., 2011; Napoli et al., 2008;).

2.4 FMRP: regulation of target mRNA translation and transport

There is a general consensus that FMRP act mainly as a negative regulator of translation although the underlying mechanisms are not clear. Several mechanisms have been proposed and they may not be mutually exclusive. The majority of co-sedimentation studies have found an association of FMRP with polyribosomes and suggest that FMRP acts by repressing elongation (reviewed by Bardoni et al., 2002), although other studies suggest that FMRP is associated with BC1 (a non translatable RNA), a complex which will block the initiation step through an interaction with elF-4E-BP and CYFIP1 (Napoli et al., 2008). FMRP has been found also associated to high-density granules, which represent ribonucleic aggregates where mRNA translation is stalled (Aschrafi et al., 2005). A recent work supports a model in which FMRP acts to stall ribosomal translocation during elongation; although the exact mechanism by which

FMRP stalls ribosomes remains to be determined, authors suggest that it is a dynamic and reversible mechanism related with plastic changes occurring both in the cytoplasm and at synapses (Darnell et al., 2011). Another mechanism by which FMRP might control expression levels of proteins is through the regulation of transcript stability, such as that of microRNA-124a (miRNA-124a) and PSD-95 (Xu et al., 2008; Zalfa et al., 2007). A further element of complexity is added by recent data suggesting that FMRP may also promote translation of target mRNAs, such as Trailer-Hitch and Superoxide Dismutase 1 (SOD1) transcripts (Bechara et al., 2009; Monzo et al., 2006). FMRP specifically binds SOD1 mRNA with high affinity through a novel RNA motif, SoSLIP (Sod1 mRNA Stem Loops Interacting with FMRP), which is folded as three independent stem-loop structures and levels of SOD1 protein are lower in *Fmr1* null cells and brain (Bechara et al., 2009). Thus, the translation and expression of FMRP targets can be either positively or negatively affected by FMRP expression, indicating that the potential role of FMRP as a translational regulator is much more complex than it was originally believed.

In addition to its role as a regulator of translation FMRP has been involved in the regulation of RNA transport along dendrites. A number of putative RNA targets have been found to be abundantly expressed in dendrites, although no major changes have been detected in the steady-state distribution and expression levels in the absence of FMRP (Bassel & Warren, 2008). FMRP traffics in the form of motile "RNA granules", structures different in size and composition containing translationally repressed mRNP complexes which travels on microtubules to the dendrites. mRNA, once localized to the appropriate sites, are released from granules and translated in response to appropriate stimuli (reviewed in Bassel & Warren, 2008). FMRP trafficking is regulated in response to activation of group I metabotropic glutamate (mGlu) receptors. Application of DHPG, a selective agonist of group-I mGlu receptors enhances the dendritic transport of several FMRP target mRNAs, including those encoding FMRP, Map1b, CaMKII in hippocampal cultured neurons (Antar et al., 2004; Dictenberg et al., 2008; Ferrari et al., 2007). Dictemberg shows that FMRP, upon DHPG stimulation, interacts more efficiently with the kinesin light chain and this mGlu-receptor mediated transport is markedly attenuated in the absence of FMRP. These data suggest that FMRP is involved in the promoting the activity-dependent localization of bound mRNAs, but not in the constitutive transport of mRNAs in dendrites.

It is clear that, as a consequence of the lack of FMRP, levels of several synaptic and non-synaptic proteins are altered and key biochemical pathways might be dysregulated in FXS. The *in vivo* evidence that an overall increase of protein synthesis in several brain regions occurs in FXS has been provided by quantitative autoradiographic studies using radioactively labelled aminoacid L-[1-14C]leucine, which showed an increase in several regions of *Fmr1* KO mice compared to wild type (WT) (Qin et al., 2005). Accordingly, Dölen and collaborators have shown a 20% increase in hippocampal slices of *Fmr1* KO mice compared to WT using 35S-methionine/cystine labelling (Dölen et al., 2007). These studies corroborate the view that FMRP acts mainly as inhibitor of protein synthesis in the brain, although do not exclude the possibility that certain proteins might be downregulated in a direct or an indirect way as a result of dysregulated pathways.

The identification of target mRNAs has been object of intense research during the last years, using a variety of in vitro assays. A recent work has identified 842 FMRP mRNA targets using a stringent high-throughput sequencing–cross-linking immunoprecipitation (HITS-CLIP) method (Darnell et al., 2011). An overlap has been found with a list of FMRP mRNA targets previously identified with a co-immunoprecipitation method (181 mRNAs) (V. Brown et al., 2001), but a significant number of mRNAs are newly identified. Interestingly, this list includes several well-studied autism candidate genes such as NLGN3, NRXN1, SHANK3, PTEN, TSC2 and NF1 and components of pre- and post-synaptic compartments.

2.5 FXS animal models

A major advancement towards a better understanding of the molecular mechanisms implicated in FXS is represented by the development of FXS animal models, which have been also used for pre-clinical studies aimed at testing potential therapeutic intervention. Mouse and Drosophila melanogaster are the main genetic model organisms used to these purposes. The mouse *Fmr1* gene and its two related genes Fxr1 and Fxr2 are well conserved relative to their human homologs *Fmr1*, FXR1 and FXR2, respectively (Bakker et al., 1994; Bontekoe et al., 2002; Mientjes et al., 2004), whereas the fly model organism has a single *Fmr1* homolog (d*Fmr1*) that is more functionally similar to human FMRP than to human FXR1 or FXR2 (Coffee et al., 2010). Both the fly and the mouse model present phenotypic abnormalities that are similar to those observed in humans such as: behavioural changes, altered axon

morphology and connectivity, social, memory and learning deficits. The *Fmr1* KO mouse shows macroorchidism, hyperactivity, a mild spatial learning impairment in the Morris water maze (Bakker et al., 1994), and abnormalities in dendritic spines (Comery et al., 1997; Nimchinski et al., 2001) and altered synaptic plasticity (see below). *Fmr1* KO mice have also an increased susceptibility to audiogenic seizures (AGS) (Musumeci et al., 2000), which is specifically reverted by the introduction of constructs codifying the human *Fmr1* gene (Musumeci et al., 2007). In addition, *Fmr1* KO mice is currently considered one of the leading animal models of autism (Bernardet & Crusio, 2006).

To study the function of FXR2P and FXR1P and their possible implication in FXS, Fxr1 and *Fxr2* KO mouse models have been generated. Homozygous Fxr1 KO neonates die shortly after birth for cardiac or respiratory failure; whereas a mouse model expressing very low levels of FXR1P displays a strongly reduced limb musculature and has a reduced life span, suggesting a role for FXR1P in muscle mRNA transport/translation control similar to that seen for FMRP in neuronal cells (Mientjes et al., 2004).

Fxr2 KO mice do not show gross abnormalities in brain or testis, but are hyperactive in the open-field test, have reduced levels of prepulse inhibition, display less contextual conditioned fear and are less sensitive to a heat stimulus. Interestingly, *Fxr2* KO mice present some behavioural phenotypes similar to those observed in *Fmr1* KO mice (Bontekoe et al., 2002).

A double *Fmr1*/*Fxr2* KO has also been created. These mice have exaggerated behavioural phenotypes in open-field activity, prepulse inhibition of acoustic startle response and contextual fear conditioning when compared with *Fmr1* KO mice, Fxr2 KO mice or WT (Spencer et al., 2006). This is in line with the hypothesis that *Fmr1* and Fxr2 play a similar role in pathways controlling locomotor activity, sensorimotor gating and cognitive processes. In addition, *Fmr1*/*Fxr2* double KO mice exhibit more severe electrophysiological alterations than either single KO model, which suggests that FMRP and FXR2P regulate synaptic plasticity both together and separately (J. Zhang et al., 2009).

2.6 Role of FMRP in the formation of neuronal network

Although FXS has traditionally been thought of as a disorder of the postsynaptic compartment, several evidences suggest a potential axonal or pre-synaptic role for FMRP. The first evidence that suggests a pre-synaptic role for FMRP was the

observation that FMRP is present in growth cones of developing axons and distal segments of mature axons in hippocampal cell cultures (Antar et al., 2006). More recently, FMRP (but also FXR1P and FXR2P) have been detected in pre-synaptic terminals in discrete small structures defined as granules (Fragile X granules) by light and electron microscopy in brain slices (Christie et al., 2009). The expression of such pre-synaptic FMRP granules is regulated both developmentally and regionally in the brain, being maximal in the frontal cortex and hippocampal area CA3 in two-week-old mice but virtually nonexistent in adult neocortex or in CA1 (Christie et al., 2009). A second line of evidence comes from studies in Drosophila, where mutations in the *Fmr1* gene result in axonal defects. It has been demonstrated that in Drosophila loss of dFMRP causes defects in axonal targeting and arborisation (Y.Q. Zhang et al., 2001), misregulated presynaptic structure (Michel et al., 2004), neuromuscular junction (NMJ) synapse overelaboration (overgrowth, overbranching, excess synaptic boutons), and altered neurotransmission (Gatto & Broadie, 2008). Two recent papers in Drosophila highlights the role of FMRP in activity-dependent axon pruning and in regulation of synaptic structure during development (Gatto & Broadie, 2008; Tessier & Broadie 2008). Using the Drosophila model these authors addressed the question whether FXS is mainly a disease of development, characterized by structural defects, or a disease of plasticity, or both. The establishment of neural circuits proceeds via a two-stage processes: an early, activity-independent wiring to produce a rough map characterized by excessive synaptic connections and subsequent, use-dependent pruning to eliminate inappropriate connections and reinforce maintained synapses. dFMRP expression and function are maximal during late-stage periods of axon pruning, which requires both dFMRP and sensory input activity. dFMRP has a primary role in activity-dependent neural circuit refinement during late brain development (Tessier & Broadie, 2008). Gatto and Broadie (2008) observed that constitutive neuronal dFMRP expression rescues all NMJ synaptic structural defects, demonstrating a strictly pre-synaptic dFMRP requirement. By contrast, targeted presynaptic dFMRP expression does not rescue neurotransmission function in the null mutant, indicating a separable post-synaptic dFMRP requirement. Temporally, transient early-development expression of dFMRP strongly rescues synaptic architecture, demonstrating primarily an early role for dFMRP in establishing synapse morphology. Interestingly, acute dFMRP expression at maturity weakly rescues synaptic structure defects, showing that that late-stage intervention might only partially compensate for structural abnormalities established early during

development. Thus, FMRP may play a double crucial role by regulating the structure of neural circuits during development and by regulating synaptic plasticity during maturity.

Recent data in the mouse model also suggest that FMRP might be involved in the establishment of neuronal connectivity, possibly through mechanisms which involve guidance and stabilization of axons during development (Bureau, 2009). Bureau et al. (2008) investigated the development of excitatory projections in the barrel cortex of *Fmr1* KO mice and they observed that projections are altered both functionally and morphologically, suggesting an important role for FMRP in this process. Dysregulated neuronal connectivity in the barrel cortex causes defective glutamatergic synapse maturation, delayed and aberrant formation of sensory maps, and altered synaptic plasticity during the critical period (Harlow et al, 2010). In general, the absence of FMRP could lead to altered network synchrony and hyperexcitable neuronal networks (Chuang et al, 2005; Gibson et al, 2008).

These data have a very strong implication for the therapeutic approach to FXS, but also to other developmental disorders characterized by altered neuronal connectivity. Interestingly in a list of newly identified FMRP mRNA target several transcripts encode for pre-synaptic proteins and are implicated in autism spectrum disorder (Darnell et al., 2011). It will be important in the future to establish whether a therapeutic intervention is able to rescue these early established abnormalities in neuronal circuitry.

3. Therapeutic strategies in FXS

Current therapeutic approach to patients with FXS is aimed at correcting symptoms or behavioural deficits, including hyperactivity and anxiety. Medications include stimulants, antipsychotic, anti-depressant and anticonvulsant. Patients with FXS also seem to benefit from behavioural intervention and special educational programs. As demonstrated in the FXS mouse model, an enriched environment can improve behaviour, and thus this therapy might also be beneficial for patients (Meredith, et al. 2007; Restivo et al., 2005).

In the last few years the amount of scientific publications in the field of neurobiology of FXS have exponentially increased and these efforts have led to important discoveries, which are now partially translated in therapeutic perspectives. These include the use of drugs to correct the abnormal activity of the metabotropic glutamate (mGlu) receptor- and GABA- pathways. In addition, novel therapeutic targets

will be discussed based on other pathways, which have been found to be dysregulated in mouse models of FXS.

3. 1 mGlu5 receptor: a key protein for synaptic plasticity

Glutamate, the major excitatory neurotransmitter in the mammalian CNS, exerts its action interacting with ionotropic (iGlu) and metabotropic (mGlu) receptors. iGlu receptors are multimeric ion channels responsible for fast synaptic transmission and are subdivided into three distinct subtypes: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate (KA), and NMDA (N-methyl-D-aspartate) receptors. mGlu receptors are members of a G-protein-coupled receptor superfamily that includes $GABA_B$, Ca^{2+} sensing, some taste and pheromone receptors (Bockaert & Pin, 1999).

The family of mGlu receptors comprises eight subtypes (mGlu1-mGlu8) that are divided into three distinct groups on the basis of sequence similarities and different pharmacological response. Group I includes mGlu1 and mGlu5 receptor subtypes which are coupled to Gq/G11 proteins and whose activation stimulates PI hydrolysis and an increase in intracellular Ca^{2+} release as a result of a PKC-mediated receptor phosphorylation (Kawabata et al., 1996). Activation of group-I mGlu receptors also stimulates the ERK1/2 MAP kinase pathway and the phosphatidylinositol-3-kinase (PI-3-K) pathways, which are involved in cell proliferation, differentiation, and survival, as well as in processes of activity-dependent synaptic plasticity (Ferraguti et al., 1999; Peavy & Conn, 1998; Rong et al., 2003). Activation of ERK in striatum and PI3K in hippocampus (Mao et al., 2005; Rong et al., 2003) requires the interaction of group-I mGlu receptors with Homer proteins, a class of scaffolding proteins cross-linking group-I mGlu receptors (mGlu1 and mGlu5) to inositol triphosphate (IP3) receptors and to other proteins of the post synaptic density such as SHANK (Tu et al., 1998, 1999). Homer proteins also controls several functions of group-I mGlu receptors such as constitutive activity (Ango et al., 2001), cell surface expression and trafficking (Ango et al., 2002; Coutinho et al., 2001), lateral mobility (Sergé et al., 2002) and coupling to ion channels of the cytoplasmic membrane (Kammermeier et al., 2000). Group II and group III include mGlu2/3 and mGlu4, 6, 7, 8, respectively and are coupled to Gi/Go proteins. While mGlu1 and mGlu5 receptors are generally found in postsynaptic densities and modulate postsynaptic efficacy, mGlu2, -3, -4, -7, and -8 receptors are mainly (but not exclusively) pre-synaptic and regulate neurotransmitter release (Luján et al., 1997; Schoepp, 2001). The pharmacology of mGlu receptors has expanded in the last years and

ligands for mGlu receptors are now considered the most promising drugs in the treatment of neurological and psychiatric disorders (reviewed by Nicoletti et al., 2011). Here we will focus on group I mGlu receptors, namely mGlu5, for their implication in the pathophysiology of FXS.

mGlu1 and mGlu5 receptors have a different temporal and regional expression pattern. While the transcript of mGlu1 receptors is low at birth and progressively increases during postnatal development, the transcript of mGlu5 receptors is highly expressed early after birth and progressively decreases afterwards (Catania et al., 1994). Expression of mGlu5 receptors is high and widespread in the first two weeks of postnatal life (Casabona et al., 1997; Romano et al., 1996, 2002; Van den Pol et al., 1995), when the polyphosphoinositide (PI) response to group-I mGlu receptor agonists in brain slices is substantial (Casabona et al., 1997; Nicoletti et al., 1986a, 1986b). A much lower receptor response is detected in hippocampal, cortical or striatal slices of adult rats, where only agonists endowed with high intrinsic efficacy can stimulate PI hydrolysis (Casabona et al., 1997; Dudek et al., 1989; Nicoletti et al., 1986a, 1986b). More recent studies have shown that mGlu5 receptors are expressed in the embryonic brain and, particularly, in zones of active neurogenesis (Di Giorgi Gerevini et al., 2004). The mGlu1a receptor protein is highly expressed in discrete regions of the adult brain including the cerebellum, olfactory bulb, thalamus, and pars compacta of the substantia nigra and is barely detectable during early development (Lopez-Bendito et al., 2002). These expression studies suggest that mGlu5 receptors may have an important role in plastic changes occurring early during post-natal development (Catania et al., 2007).

Most of group I mGlu receptors are located in dendritic spines (Baude et al., 1993; Lujan et al., 1996; Nusser et al., 1994; Shigemoto et al., 1996), in an annulus that circumscribes the PSD, but some (probably mGlu5) are also distributed on glutamatergic nerve terminals (Cochilla & Alford, 1998; Gereau & Conn, 1995; Manzoni & Bockaert, 1995; Romano et al., 1995; Rodriguez-Moreno et al., 1998; Sistiaga et al., 1998). mGlu5 receptors are also expressed in non-neuronal cells, including astrocytes, oligodendrocytes, and microglia, stem progenitor cells, and a variety of peripheral cells (Nicoletti et al., 2011).

mGlu5 receptors are involved in the regulation of synaptic plasticity, including the induction of LTP (important for retaining nascent synapses) and LTD (important for activity-guided synapse elimination), two electrophysiological substrates that, working in concert, contribute to learning and memory storage throughout postnatal life (Bear,

1998). LTP is a long term increase in synaptic efficacy and is associated with the strengthening of the connection between a presynaptic and post-synaptic neuron, whereas LTD is defined as the weakening of the synapse, and is mainly reflected by a reduced number of iGlu responsive AMPA receptors at the post-synaptic membrane (Collingridge et al., 2010). Activation of mGlu5 receptors is involved in both LTP and LTD. Mice lacking mGlu5 receptors show impaired learning and reduced LTP in the hippocampal CA1 region (Lu et al., 1997).

There are two forms of LTD: one dependent on activation of post-synaptic NMDA receptors, the other requires activation of post-synaptic group-I mGlu receptors (Oliet et al., 1997) and also can be readily induced by the selective group-I mGlu receptors agonist DHPG (Huber et al., 2001; Palmer et al., 1997). Both types of LTD determine a decrease in the number of post-synaptic AMPA receptors by distinct mechanisms (Bear et al., 2004). One important distinction is that LTD triggered by mGlu receptor activation (mGlu-LTD), but not NMDA-receptor-dependent LTD, requires the activation of mGlu5 receptors and the rapid translation of preexisting mRNA in the postsynaptic dendrites through a mechanism that involves ERK phosphorylation (Gallagher et al., 2004).

3.2 mGlu5 receptor: a pharmacological target in FXS

The first indication for a link between mGlu receptors and FXS was the evidence that activation of group-I mGlu receptors in rat and mouse brain synaptoneurosomes stimulates the rapid translation of pre-existing mRNA, including the FMRP mRNA (Weiler et al., 1997, 2004). Since, a growing number of studies was carried out to support a role of group I mGlu receptors in the pathophysiology of FXS. In particular, the finding that mGlu5-/protein synthesis-dependent forms of synaptic plasticity, namely mGlu5-dependent LTD, are increased in the mouse model of FXS led Bear and collaborators to formulate the so-called “mGlu theory” of FXS, which postulates that in the absence of FMRP, which acts reducing the mGlu5-activated mRNA translation at synapse, levels of FMRP-regulated proteins are increased and, as a consequence, can be reduced by mGlu5 pharmacological antagonism (Bear et al., 2004). Other forms of synaptic plasticity, including the more classical NMDA-receptor dependent LTD, show no abnormalities in the hippocampus of *Fmr1* KO mice. Another important step towards the understanding of FXS physiopathology was represented by the finding that, while in WT mice mGlu5-dependent LTD is blocked by inhibitors of protein synthesis, this is not

the case in *Fmr1* KO mice, suggesting that in the absence of FMRP LTD proteins are constitutively and highly expressed before LTD induction (Waung & Huber, 2009).

Thus, the absence of FMRP causes an abnormal expression of dendritic proteins leading to the amplification of mGlu-mediated long-term responses. The identification of these proteins, which may be critical for the pathophysiology of synaptic dysfunction in FXS, is crucial. Some proteins encoded by FMRP target mRNAs may play a role. For example, Map1b interacts with the GluR2 interacting protein and scaffold GRIP1 (Davidkova & Carroll, 2007; Seog, 2004). Other proteins which are rapidly synthesized after mGlu5 receptor activation and that are basally elevated in Fmr1 KO mice include CaMKII (Zalfa et al., 2003), amyloid precur protein (APP), Arc/Arg3.1 (Park et al., 2008; Zalfa et al., 2003) which are all involved in mechanisms underlying synaptic plasticity. The list of FMRP mRNA targets has recently grown with the discovery of 842 mRNA by using the high stringent CLIP method (Darnell et al., 2011). Further studies examining the expression levels of the encoded proteins in FXS and their regulation by mGlu receptors may corroborate the link between mGlu5 activation and protein synthesis of FMRP target mRNAs and its role in synaptic plasticity under physiological and pathological conditions. In addition, as a direct or indirect consequence of altered protein synthesis at synapses, several mGlu-mediated signalling pathways might be dysregulated. Interestingly, mGlu5 receptors in *Fmr1* KO mice are less tightly associated to Homer proteins (Giuffrida et al., 2005), which suggest either an increase of mGlu5 constitutive activity or an altered coupling of mGlu5 receptors with downstream signalling pathways. Accordingly, Ronesi and Huber (2008) reported that induction of PI3K-Akt-mTOR signalling by mGlu5 is impaired in *Fmr1* KO mice and, differently than in WT mice, mGlu5 dependent LTD is insensitive to disruption of mGlu5/Homer interaction. Further studies are needed to understand how the lack of FMRP affects mGlu5-mediated responses in FXS.

Several pharmacological studies have supported the "mGlu theory", by demonstrating that phenotypic features of FXS can be corrected with the use of antagonists of mGlu5 such as 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and fenobam. MPEP is a systemically active negative allosteric modulator of mGlu5 receptors and can also inhibit constitutive activity of mGlu5 acting as an inverse agonist (Yan et al., 2005). Fenobam, which had previously been investigated as an anxyolitic, has been identified as a highly potent, selective negative modulator of mGlu5 receptor (Porter et al., 2005). In particular, MPEP blocked audiogenic seizure susceptibility of

Fmr1 KO mice (Chuang et al., 2005) and both MPEP and fenobam restored dendritic spine morphology in hippocampal cell cultures from *Fmr1* KO mice (De Vrij et al., 2008).

A more direct evidence that the "mGluR theory" might be corrected has been provided using genetic interaction experiments (Dölen et al., 2007). In this study, *Fmr1* KO mice were crossed with heterozygous mGlu5 receptor KO mice generating double mutants of *Fmr1* and Grm5 (the gene that encodes mGlu5 receptor) and multiple phenotypes relevant to the pathogenesis of FXS were examined. Reduction of mGlu5 expression by 50% in the *Fmr1*KO/Grm5 heterozygote cross rescued altered ocular dominance plasticity, increased density of dendritic spines, increased basal protein synthesis, exaggeration of avoidance extinction and audiogenic seizure susceptibility, but not macroorchidism (Dölen et al., 2007). Interestingly, no change in protein synthesis was detected in Grm5 heterozygote, suggesting that a therapeutic dose of an mGlu5 receptor antagonist for FXS patients should not have negative side effects in unaffected individuals. These pre-clinical studies support the therapeutic utility in FXS patients. Interestingly, the potential use of mGlu5 antagonists is not restricted to FXS but is considered for a variety of human conditions including anxiety, convulsions, pain, depression, Parkinson's disease and gastroesophageal reflux disease (see Nicoletti et al.., 2011).

An initial small pilot open label, single dose trial with fenobam in adults with FXS did not reveal any adverse effect and produced promising results showing an improvement of prepulse inhibition (Berry-Kravis et al., 2009). More recently, the Novartis compound AFQ056 has been used in a randomized, double-blind study in 30 male FXS patients aged 18-35 years. Although an initial assessment did not show any improvement after treatment, when patients were divided into two groups on the basis of a full or partial methylation of the *Fmr1* promoter a significant improvement on stereotypic behaviour, hyperactivity and inappropriate speech were detected only in the full methylation group (Jacquemont et al., 2011). While this work confirms the clinical efficacy of mGlu5 pharmacological blockade in FXS, there is no clear explanation for the lack of improvement in patients with partial methylated *Fmr1* gene. More clinical studies in a higher number of patients are needed.

3.3 GABA system as target of viable pharmacological treatments in FXS.

In addition to the mGlu receptors, several evidences suggest that gamma-aminobutyric acid (GABA) signalling is another molecular pathway involved in FXS. Expression and functional studies suggest that defects in GABA transmission might be region specific and might involve different components of the GABAergic system in different brain regions.

GABA is the major inhibitory neurotransmitter in the CNS and plays a key role in modulating neuronal activity, by maintaining the inhibitory tone and the physiological balance between inhibition and excitation at synapses. GABA mediates its action via two distinct receptor systems, the ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors.

Ionotropic $GABA_A$ receptors are heteropentameric complexes, formed by the assembly of various classes of at least 19 different subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π and ϱ1–3) (Simon et al., 2004) associated with channel permeable to Cl^- ions. In brain, a high diversity of $GABA_A$ receptor subtypes having a spatio-temporal specific distribution in different regions has been found (Barnard et al., 1998; Kneussel, 2002; Korpi et al., 2002). The subunit combination confers highly different pharmacological and physiological properties to $GABA_A$ receptors (Fritschy et al., 1995).

$GABA_B$ receptors are heterodimeric G protein–linked receptors constituted by two different subunits. They have a pre- and post-synaptic distribution; at pre-synaptic level they can inhibit the release of neurotransmitters through a decrease of calcium entry, whereas, at post-synaptic level they reduce neuronal excitability through an increase of potassium conductance. In general, they mediate a slower and more prolonged inhibitory signal than $GABA_A$ receptors (Bormann, 2000; Chebib et al., 1999). Interestingly, $GABA_B$ receptors agonists inhibit pre-synaptic glutamate release and consequently the post-synaptic glutamate responses (reviewed by Chalifoux & Carter, 2011).

An important indication that the GABAergic system might be involved in FXS was the evidence, obtained using the Antibody Positioned RNA Amplification (APRA) technique, that the mRNA of the d subunit of the $GABA_A$ receptor is directly bound to FMRP (Miyashiro et al., 2003). In *Fmr1* KO mouse, changes in levels of expression of both $GABA_A$ and $GABA_B$ receptors have been found by different authors. Several studies have revealed in different brain regions, all playing an important role in cognitive functions (behaviour, learning, memory and anxiety), as cortex, hippocampus, diencephalon and brainstem an under expression of many distinct $GABA_A$ receptor subunits (α1, α3, α4, a5 β1 and β2 and γ1 and γ2 and δ) at the mRNA (Curia et al.,

2009; D'Hulst et al., 2006; Gantois et al., 2006) and protein level (Adusei et al., 2010; El Idrissi et al., 2005;).

Altered GABA transmission has been reported in different brain regions. An alteration of both GABAergic and cholinergic system, with a lower inhibitory effect mediate by $GABA_A$ receptor in subiculum neurons has been detected by electrophysiology in brain slices of *Fmr1* KO mice (D'Antuono et al., 2003). More recently, other electrophysiological findings in subiculum have shown that tonic $GABA_A$ currents were down regulated in *Fmr1* KO mice, whereas no significant differences were observed in phasic currents (Curia et al., 2009). An increased GABA transmission has been found in the striatum (Centonze et al., 2008), whereas a robust reduction in the inhibitory transmission has been revealed in the amygdala, which results in hyper-excitability of principal neurons and is likely due to presynaptic defects such as decreases in GABA production and release (Olmos-Serrano et al., 2010). Accordingly, a reduction of GABA has been detected in *Fmr1* KO mice using a metabolomic approach (Davidovic et al., in press).

Furthermore, cytoarchitectonic and morphological studies from somatosensory cortex highlighted a significant reorganization of neocortical inhibitory circuits of GABAergic interneurons in the *Fmr1* KO mouse. In fact, this animal model showed a marked reduction of parvalbumin-positive neurons compared to the WT mice, whereas no difference was observed for calbindin- and calretinin-positive neurons (Selby et al., 2007).

Thus, most expression and functional data suggest that increasing GABAergic transmission might result in a beneficial effect, at least in certain regions. Accordingly, experiments from *Fmr1* mutant Drosophila have shown that GABA treatment during development using GABA, nipecotic acid (a known GABA reuptake inhibitor) and creatinine (a potential activator of $GABA_A$ receptor) rescued the lethality induced by glutamate toxicity of d*Fmr1* mutant flies, when they were reared on food containing increased levels of glutamate (Chang et al., 2008) and rescued many *Fmr1* mutant phenotypes, such as *Futsch* overexpression, defects in mushroom bodies structure and altered male courtship behaviour (Chang et al., 2008). In addition, treatment of *Fmr1* KO mice with the $GABA_A$ receptor agonist taurine is reported to increase acquisition of a passive-avoidance task (El Idrissi et al., 2009). More recently, a treatment with the systemically active agonist acting at δ subunit-containing $GABA_A$ receptors, 4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol hydrochloride (THIP hydrochloride), that is able

to determine an augmentation of tonic inhibitory tone (Glykys & Mody, 2007), was shown to rescue neuronal hyperexcitability recorded from principal neurons of BL nucleus of amygdala in *Fmr1* KO mice (Olmos-Serrano et al., 2010).

The involvement of $GABA_B$ receptors is also under investigation in FXS. In fact, it has been observed a reduced expression of the $GABA_B$ R1 subunits in the forebrain of *Fmr1* KO mice, early during the development and in adulthood; whereas no significant differences have been observed in $GABA_B$ R2 expression (Adusei et al., 2010; Pacey et al., 2011). Reduced functioning of $GABA_B$ receptors might explain the increased susceptibility of *Fmr1* KO mice to audiogenic seizures (Musumeci et al., 2000). Accordingly, stimulation of $GABA_B$ receptors with agonist Baclofen, reduces the rate of audiogenic seizures in *Fmr1* KO mice (Pacey et al., 2009). These receptors play also a role in the pathophysiology of anxiety and depression, so $GABA_B$ receptor agonist treatment might be used for reducing anxiety symptoms in patients with FXS (Cryan & Kaupmann., 2005).

3.4 Protein dysregulation and other biochemical pathways as potential targets of intervention

As soon as the list of validated FMRP-targeted RNAs will grow, more pathways will be shown to be affected and more drugs will be proposed for the future therapy of FXS. In the next paragraph we will discuss recent advances concerning relevant pathways which may lead to treatment.

3.4 .1 Oxidative stress and fragile X syndrome

Several evidences suggest a role of oxidative stress in FXS. FXS patients display an increase in adrenocortical activity and an altered hypothalamic–pituitary–adrenal (HPA) axis (Hessl et al., 2004); adrenal hormones have been involved in the induction of brain oxidative stress resulting in oxidation of molecules and depletion of antioxidants such as glutathione (Herman & Cullinan, 1997). In *Fmr1* null flies changes in the expression of proteins involved in redox reactions have been observed, suggesting a possible alteration in the oxidative balance (Y.Q. Zhang et al., 2005). In the brain of *Fmr1* KO mice higher levels of reactive oxygen species, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activation, lipid and protein oxidation have been found, suggesting that a moderate increase in the oxidative stress in the brain may play a role in the pathophysiology of FXS (el Bekay et al., 2007). In addition, microarray

identification has revealed altered mRNA translational profiles in the absence of FMRP, involving proteins which participate in homeostasis of the antioxidant status such as glutathione transferase and SOD1 (M.R. Brown et al., 2001; Miyashiro et al., 2003). Recently, a reduction of protein levels of SOD1 has been found in *Fmr1* null cells and brain (Bechara et al., 2009), suggesting that in the absence of FMRP the increase in brain oxidative stress might be due to the altered SOD1 expression. A comprehensive profiling of the metabolome of the *Fmr1*-deficient brain has revealed an increase in lipid-oxidized species at early age (Davidovic et al., in press), further corroborating the hypothesis that oxidative stress is indeed involved in FXS pathophysiology.

The therapeutic implication of these findings are that anti-oxidant agents may be useful in the treatment of FXS and are supported by recent results obtained in *Fmr1* KO mice after treatment with alpha-tocopherol and melatonin (de Diego-Otero et al., 2009; Romero-Zerbo et al., 2009). Chronic pharmacological treatment with alpha-tocopherol reverses pathophysiological hallmarks including free radical overproduction, oxidative stress, macroorchidism, and also behaviour and learning deficits (de Diego-Otero et al., 2009). Chronic administration of melatonin protects the *Fmr1* KO mouse from the oxidative stress in brain and testes, reverses several behavioural and learning deficits, normalizes several abnormalities observed in the *Fmr1* KO mouse, including biochemical hallmarks, such as free-radical production in macrophage cells and brain slices, as well as carbonyl content in proteins and lipid peroxidation (Romero-Zerbo et al., 2009). Additionally, it also normalizes reduced glutathione levels in the brain and testicles of *Fmr1* KO mice. The treatment controls corticosterone plasma levels, locomotion (hyperactivity), anxiety responses and fear learning deficits.

3.4.2 matrix metallo-proteinase 9 and minocycline

Another example of protein dysregulated in the mouse model of FXS and considered a valuable target of a pharmacological treatment is the matrix metallo-proteinase 9 (MMP9). MMP-9 is an extracellular endopeptidase that cleaves extracellular matrix proteins that impact synaptogenesis and spine morphology (Ethell & Ethell, 2007). MMP-9 could affect dendritic spine morphology by cleaving components of the extracellular matrix and/or cell surface proteins that participate in synaptogenesis and dendritic spine maturation (Ethell & Ethell, 2007). High MMP-9 activity interferes with normal physiological functions and induces dendritic spine remodelling, whereas modest concentrations of MMP-9 regulates non-pathological synaptic functions and

plasticity in mature hippocampus through an integrin dependent mechanism and NMDA receptors activation (Bozdagi et al., 2007). It has been shown that MMPs are elevated in the hippocampus of *Fmr1* KO mice and may be partially responsible for the immature dendritic spine profile of hippocampal neurons and for synaptic instability (Bilousova et al., 2006). A treatment with minocycline, a tetracycline analogue that can inhibit matrix MMP-9 and reduce inflammation in the CNS, promotes the formation of mature dendritic spines and reduces dendritic spine abnormalities respectively in WT and *Fmr1* KO hippocampal neurons. Minocycline effects on dendritic spine morphology may be related to its inhibitory actions on MMP-9 expression and activity, which is up-regulated in FMRP deficient hippocampal neurons. It is possible that high basal levels of MMP-9 activity in the brains of *Fmr1* KO mice may interfere with normal physiological responses and induce dendritic spine remodelling. Indeed, it has been shown that excessive MMP-9 activity disrupts mature dendritic spines in hippocampal neurons. The beneficial effects of this drug on dendritic spine morphology are also accompanied by changes in the behavioural performance of 3-week-old *Fmr1* KO mice (Bilousova et al., 2009).

Clinical trials have been started for patients with FXS and an open-label trial has been recently completed to study the effects of minocycline patients with FXS (Utari et al., 2010). The results show that minocycline provides significant functional benefits to FXS patients, it is well-tolerated, and both adolescents and adults with FXS can benefit from minocyline treatment.

3.4.3 Phosphoinositide 3-kinase and FXS

It has been hypothesized that FMRP controls protein synthesis-dependent regulation of synaptic morphology and function through regulation of PI3K signalling. PI3K regulates different pathways. Deficiency of FMRP results in excess activity of PI3K; loss of FMRP leads to excess mRNA translation and synaptic protein expression of p110beta, a catalytic subunit of PI3K and a putative FMRP-target mRNA (Miyashiro et al., 2003). FMRP regulates the synthesis and synaptic localization of p110beta. In WT, mGlu receptor activation induces p110beta translation, p110beta protein expression, and PI3K activity; in contrast, both p110beta protein synthesis and PI3K activity are elevated and insensitive to mGlu receptor stimulation in *Fmr1* KO mice. Excess of PI3K activity in the absence of FMRP can occur independently of mGlu receptors (Gross et al., 2010). PI3K is a downstream signalling molecule of many cell surface receptors;

aberrant regulation of p110beta could provide a molecular explanation for dysregulation of D1 dopamine receptors (Wang et al., 2008), of Gq-proteins (Volk et al., 2007), and of Ras (Hu et al., 2008) observed in *Fmr1* KO mice. Dysregulated PI3K signalling may also underlie the synaptic impairments in FXS. In support of this hypothesis, it has been observed that a treatment with LY294002 (PI3K antagonist) in *Fmr1* KO neurons can rescue the enhanced AMPA receptor internalization and the increased spine density (Gross et al., 2010). Targeting excessive PI3K activity might thus be another therapeutic strategy for FXS.

4. Conclusion and future direction

A deeper understanding of the function of FMRP and the molecular mechanisms underlying FXS using animal models has recently led to propose new therapeutic approaches, which will prove to be corrected in the next future as soon as several ongoing clinical trials will be completed. As a consequence of altered protein expression both at pre- and post-synaptic levels it is possible that several interconnected biochemical pathways are altered in FXS. It will be important to identify these cascades. System biology approaches and bioinformatic tools may help to identify the metabolic consequences of dysregulated biochemical cascades in FXS and in other neurological disorders associated with intellectual disability and autism. Given the high number of proteins and pathways which are likely to be dysregulated in FXS it will be also very important to establish which of them are involved in determining structural changes during development and which are more involved in plasticity defects.

It is possible that different therapeutic interventions might be used during development and in adult patients.

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