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Modulation of Stress Granules formation: Role of mGlu5 receptor and FMRP and implications for pathophysiology of Fragile X Syndrome

Ph.D. Thesis

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ABBREVIATIONS

82-FIP	82 kDa-FMRP Interacting Protein
AGO1	Argonoute 1
AGS	Audiogenic seizures
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BC1	Brain Cytoplasmic RNA 1
BRF1	Butyrate Response Factor 1
BTF	Bcl-2-associated transcription factor 1
CaMKII	Calcium Calmodulin-dependent Kinase II
cAMP	amp cyclic
CC	Coiled Coil
CC	coiled coil
CNS	Central Nervous System
CPEB	Cytoplasmic Polyadenylation Element □Binding□ Protein
CREB	cAMP response element binding protein
CYFIP1	Cytoplasmic FMRP Interacting Protein
CYFIP2	Cytoplasmic FMRP Interacting Protein 2
DAG	Diacylglycerol
DIS1	Disrupted In Schizophrenia
eIF2α	Eukaryotic Translation Initiation Factor 2 α
eIF4E	Eukaryotic Initiation Factor 4E
ERK	Extracellular Signal-Regulated Kinase
FAST	TIA1 Interacting Protein
FMR1	Fragile X Mental Retardation 1
FMRP	Fragile X Mental Retardation Protein
FTD	Front□temporal Dementia
FUS	Fused in Sarcoma
FXR	Fragile X Related

FXR1P	Fragile X Related Protein 1
FXR2P	Fragile X Related Protein 2
FXS	Fragile X Syndrome
FXTAS	Fragile X Associated Tremor/Ataxia Syndrome
G3BP	GTPase Activating Protein Binding Protein
GCN2	General Control Nonderepressible 2
Gp1 mGluRs	Group I mGlu Receptors
GPCR	C G-Protein-Coupled Receptor
HITS CLIP	High-Throughput Sequencing–Cross-Linking Immunoprecipitation
HPA	Hypothalamic-Pituitary-Adrenal
HRI	Heme-Regulated Initiation F□ctor 2α Kinase
Htt	huntingtin
ID	Intellectual Disability
iGlu	Ionotropic Glutamate
IP_3	Inositol-1,3,4-Trisphosphate
IQ	Intelligence □Quotien
KA	Kaina□e
KH	K Homology 2
KH_1	K Homology 1
KH_2	K Homology 2
LTD	Long Term Depression
LTP	Long Term□Potentiation
MAP1B	Microtubule-Associated Protein-1B
MAPK	Microtubule Associated Protein Kinase
mGlu	Metabotropic Glutamate
MMP9	Matrix Metallopeptidase 9
MPEP	2-Methyl-6-(Phenylethynyl)-Pyridine
mRNPs	Messenger Ribonucleoprotein Particles
mTOR	Mammalian Target of Rapamycin
NES	Nuclear Exportation Signal

NLS	Nuclear Localization Signal
NMDA	N-Methyl-D-Aspartate
NoS	Nucleolar Localization Signal
NRF1	nuclear respiratory factor 1
NRF2	nuclear respiratory factor 2
NTMs	Normal Transmitting Males
NUFIP1	Nuclear FMRP Interacting Protein 1
PABP-1	Poly(A) Binding Protein 1
PERK	PKR Like Endoplasmic Reticulum Kinase
PI3K	Phosphoinositide 3 Kinase
PIP_2	Phosphatidylinositol-4,5-Bisphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PP2A	Protein Phosphatase 2A
PSD	Postsynaptic Density
Rac1	Ras related C3 botulinum toxin substrate 1
RGG	Arg-Gly-Gly
RISC	RNA-Induced Silencing Complex
SAPAP4	SAP90/PSD-95-associated protein 4
SGs	Stress Granules
SMA	Spinal Muscular Atrophy
SMN	Survival of Motor Neurons
SOD1	Superoxide Dismutase 1
Sp1	specificity protein 1
TDP-43	TAR DNA-Binding Protein of 43 kDa
TIA-1	T Cell Internal Antigen 1
TIAR	TIA-1 Related Protein
TRAF2	Tumor Necrosis Factor Receptor Associated Factor 2
TRAL	Trailer-Hitch Protein
TTP	Tristetraprolin

USF1	Stimulatory Factor 1
USF2	Stimulatory Factor 2

ABSTRACT

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and autism. The genetic defect in FXS is a CGG trinucleotide repeat expansion (>200) in the promoter region of the *FMR1* (fragile X mental retardation 1) gene; this amplification causes the absence of the encoded protein FMRP (Fragile X Mental Retardation Protein). FMRP is an RNA-binding protein involved in mRNA transport and translation. Despite numerous studies, the available treatments are only symptomatic. There is no cure to replace FMRP expression, yet. FMRP can interact with RNA-binding proteins such as FXR1P, FXR2P, NUFIP and 82-FIP, and with proteins that do not bind RNA, like CYFIP1 and CYFIP2. The interaction with these different proteins may modulate FMRP functions and its RNA affinity. A new role of FMRP in mRNA metabolism as component of stress granules (SGs) has been identified. FMRP seems to lead mRNAs in SGs upon cellular stress, during which protein synthesis is blocked. SGs are ribonucleoproteic aggregates containing translation initiation components and RNA binding proteins, like eIF2α and FMRP. Several data also indicate that some of the FXS symptoms are a consequence of a defect in group-I metabotropic glutamate receptor, namely mGlu5; pharmacological blockade of mGlu5 receptors provide a therapeutic target in FXS. mGlu5 receptor, like FMRP, regulates protein synthesis but in a functionally opponent manner: mGlu5 receptor activates protein synthesis, FMRP suppresses it. In the absence of FMRP, mGlu5-dependent protein synthesis is unchecked, with consequent excessive translation. Activation of mGlu5 receptors stimulates FMRP-mediated mRNA transport and protein synthesis, but its role in SGs formation is unknown.

The aim of this PhD thesis was to better investigate FMRP function studying the relationship of FMRP with its interacting proteins and the role of FMRP in stress response under activation of mGlu5 receptor. In **Paper I,** we analyzed the expression pattern of FMRP and its interacting proteins in different brain areas, at different ages in wild type (WT) mice to better define the interplay between FMRP

and its interacting proteins during development. FMRP was strongly expressed at P3, peaked at P7-P14 and gradually decreases thereafter. The analysis of expression pattern of several proteins carried out, indicate that FMRP and its interacting proteins have distinct developmental patterns of expression and suggest that FMRP may be preferentially associated to certain proteins in early and late developmental stages. We found that the RNA binding and cytoskeleton remodeling functions of FMRP may be differently modulated during development. **In Paper II** we studied FMRP under stress condition using WT and *Fmr1* knockout (KO) astrocytes. We have demonstrated that the lack of FMRP impairs SGs formation and furthermore that activation of mGlu5 receptor affects SGs formation through a FMRP-mediated mechanism in WT. Interestingly, the mGlu5 receptor blockade restores SGs formation in *Fmr1* KO. Also, mGlu5 receptor activation before stress reduced FMRP recruitment in SGs and phosphorylation of eIF2α and FMRP. In contrast, mGlu5 receptor activation did not affect SGs formation in *Fmr1* KO astrocytes. Since phosphorylation of eIF2α and FMRP are two crucial key events in SGs formation and modulation of protein synthesis, mGlu5 receptors may act by shifting the balance from inhibition to activation of protein synthesis during stress. These findings suggest a potential novel role for mGlu receptors in SGs formation. We suggest that FMRP may have a positive role in stress response, facilitating and enhancing SGs formation to prevent stress damages. This process is useful to understand what happens in FXS, in which can occur abnormal modulation of different proteins during development with consequent abnormal response during adversal conditions, like oxidative stress that represent a frequent component in FXS and neurodegenerative disorders.

INTRODUCTION

1. FRAGILE X SYNDROME (FXS)

Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability (ID) and a leading cause of autism, affecting 1/4000 males and 1/8000 females (Rousseau et al., 1995; O'Donnel & Warren, 2002). FXS, also called Martin-Bell syndrome in the past, belongs to a large group of human X-linked mental retardation syndromes (Chiurazzi et al., 2004).

FXS is mainly caused by the amplification of CGG trinucleotide repeat in the promoter region of the *FMR1* gene. This abnormal triplet expansion leads to transcriptional silencing of the *FMR1* gene and absence of the *FMR1* encoded protein FMRP (**F**ragile X **M**ental **R**etardation **P**rotein) (Devys et al., 1993; O'Donnel and Warren, 2002; Penagarikano et al., 2007). FMRP is an RNA binding protein involved in the regulation of target mRNA metabolism. FMRP mainly acts as a negative regulator of translation in association with polyribosomes and it plays an important role in synaptic plasticity (Li et al., 2001; Qin et al., 2005; Dolen et al., 2007). FMRP is also a component of RNA granules, the mRNPs (messenger ribonucleoprotein particles) that escort mRNAs in repressed conditions from soma to synapses. The absence of FMRP causes abnormal distribution of its mRNAs cargos and is the basis for the FXS phenotype (Miyashiro et al., 2003).

1.1 Phenotype of FXS

The clinics of FXS is characterized by moderate to severe cognitive impairment (IQ<70), which particularly affects short-term memory for complex information, visuospatial skills, and language acquisition (reviewed by Panagarikano et al., 2007). Moreover, FXS is a frequent cause of autism, indeed 15-50% of FXS individuals show autistic behavior such as poor visual contact, tactile

defensiveness, and repetitive behaviors (Cornish et al., 2004). FXS patients also show epilepsy, hyperactivity, hypersensitivity to sensorial stimuli, attention deficit, sleep disorders; they also manifest physical characteristics such as dysmorphic long face with prominent mandibular and large ears, arched palate, mitral valve prolapse, hypotonia, increased joint laxity and macroorchidism (The Dutch-Belgian Fragile X Consortium, 1994; Hagerman, 2002). Autopsy of brains from FXS patients showed no significant abnormalities (Bakker et al., 1994; Reyniers et al., 1999), although in some brain areas such as cortex and hippocampus, dendritic spines appear immature, like filopodia (Rudelli et al., 1985), longer and thinner than healthy, suggesting a misregulation in dendritic development (The Dutch-Belgian Fragile X Consortium, 1994; Irwin et al., 2001; Nimchinsky et al., 2001; O'Donnel et al., 2002; Penagarikano etal., 2007). During development, an exceeded number of dendritic spines occurs but eventually, a lot of them undergo to elimination by maturation and pruning mechanisms that establish the final synaptic phenotype. An abnormal dendritic spines development has been reported in *Fmr1* knockout (KO) mice, the animal model for FXS (Nimchinsky et al., 2001). Abnormalities in dendritic and spines are associated with neurological problems in FXS such as epileptic seizures (Incorpora et al., 2002). Abnormal dendritic spines phenotype has been evident in FXS since the first autopsy and spine density appeared increased compared to control patients supporting the hypothesis that defects in spine maturation and elimination may underlie ID (Rudelli et al., 1985). Spine abnormalities have long been associated with mental retardation as well as with Down and Rett syndromes (Kaufmann and Moser, 2000) and the spine phenotype observed in FXS patients and *Fmr1* KO mice suggests that FMRP is involved in synaptogenesis early in development.

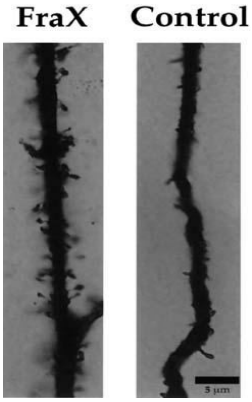

Figure 1. Dendritic spines of fragile X neurons.
Adapted from Irwin et al., 2001

1.2 Synaptic plasticity in FXS

FXS is considered a synaptic disease in which FMRP and several proteins/biochemical pathways associated with FMRP and with spine morphogenesis are involved. FMRP may influence synthesis of proteins involved in dendritic spine morphology; among them Rho GTPase, Rac1, microtubule-associated protein-1B (MAP1B), calcium calmodulin-dependent kinase II (CaMKII), calbindin, cadherins (Grossman et al., 2006). Spines are dynamic structures with an electrondense region, the postsynaptic density (PSD), that consists of dense area containing receptors, channels, scaffolding proteins and enzyme involved in synaptic transmission (Tada and Sheng, 2006). During development, dendritic spines morphology changes from small, immature like filopodia dendritic spines, which are more frequent in early age, to mature mashroom-shape dendritic spines that are characteristic postdevelopmentally (Hinton et al., 1991; Ivanco and Greenough, 2002). The spine structural changes are related to synaptic plasticity. Synaptic plasticity is a phenomenon during which a change, persistent or transient, of morphology, composition, or signal transduction efficiency occurs at a neuronal synapse in response to intrinsic or extrinsic signals (Mosbacher, 2014). The discovery of altered spine morphology

supports a defective synaptic plasticity in FXS but does not elucidate the cause of mental retardation. The most direct evidence about this aspect that underlines FMRP's role in synaptic plasticity comes from studies on long-term depression (LTD) and long-term potentiation (LTP) in wild-type (WT) and *Fmr1* KO mice. LTD and LTP are two forms of synaptic plasticity essential for cognitive functions, memory and learning processes; LTP is associated with synapse creation of new spines and enlargements of existing spines associated with the strengthening of the connection between a presynaptic and post-synaptic neuron, whereas LTD is associated with elimination, shrinkage of spines (reviewed by Panagarikano et al., 2007). One form of LTD is dependent on metabotropic glutamate receptor and requires activation of new protein synthesis (Huber et al., 2000). This form of LTD is enhanced in *Fmr1* KO mice (Bear et al., 2004), and this finding represents a direct evidence that the absence of FMRP alters synaptic plasticity. Indeed FMRP is possibly involved in the assembly and functioning of neuronal circuits and in the regulation of the dendritic spines turnover.

1.3 Causes of FXS

The gene implicated in the pathology of FXS is *FMR1* (fragile X mental retardation 1) gene. It is located on the long arm of the X chromosome at position 27.3. This region cytogenetically displays a fragile site from which the name of the syndrome takes origin. The most common genetic defect in FXS is a CGG trinucleotide repeat expansion in the 5' untranslated region of the *FMR1* gene (Verkerk et al., 1991). This triplet amplification is associated with 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). In the normal population, this CGG repeat is polymorphic, with a repeat length ranging from 6 to 53 units (Fu et al., 1991). During meiosis, expansion of CGG repeats is instable and an increase in lenght from one generation to next may

occur. Carriers show a repeat length of 55-200 CGGs repeats, condition defined premutation, and are asymptomatic; in fact, up to 200 CGG repeats there isn't *FMR1* methylation and reduction in FMRP expression, but there is a higher predisposition to the development of other pathologies such as fragile-X associated tremor/ataxia syndrome (FXTAS) and premature ovarian failure, in males and females respectively (Hagerman and Hagerman, 2003). The full mutation is characterized by a large repeat of > 200 CGGs in the 5' untranslated region of *FMR1* gene. As a result of this repeat amplification, the *FMR1* promoter region and the CGG expansion become methylated, leading to silencing of transcription and translation of the *FMR1* gene (Pieretti et al., 1991; Verheij et al., 1993). Males with a full mutation are affected, and 50-70% of females with a full mutation shows mild to moderate mental impairment (Rousseau et al., 1991). The transition from premutation to full mutation occurs only by transmission through a female carrier, and the probability of triplet expansion increases with the size of the premutation and then with the passing of generations, phenomenon called "Sherman paradox" (Devys et al., 1993). FXS inheritance is more than a simple X-linked recessive model, because data reported in a several number of pedegrees shows different irregularities. The main conflicting datum was the existence of nonaffected male carriers and affected female carriers (Nielsen et al., 1981). Sherman et al. defined nonaffected male carriers "normal transmitting males" (NTMs); in addition, 30% of carrier females showed some form of mental impairment (Sherman et al., 1984, 1985). They also noticed that the risk of inheriting the syndrome depended on the position of an individual within the pedigree: the mothers of NTM showed three times less risk of having affected sons than their daughters. The Sherman paradox was only resolved in the 1991, when the gene responsible for the syndrome was identified and, at the same time, a new mutational mechanism revealing the particular inheritance model: trinucleotide repeat expansion.

CGG repeat expansion cause over 95% of FXS cases, but it is not the only cause for FXS (O'Donnell and Warren, 2002). It has been reported that several deletions and point mutations affecting the *FMR1* gene also cause FXS (De Boulle et al., 1993; Lugenbeel et al., 1995; Hammond et al., 1997). In fact a point mutation may

lead to the expression of non-functional protein and an example of missense point mutation was reported at amino acid site 304 of FMRP, in the nuclear ribonucleoprotein K Homology 2 (KH2) domain. Esactly, a substitution of isoleucine (I) to asparagine (N) (I304N) was reported at this site (De Boulle et al., 1993; Siomi et al., 1993). Thus, the absence or loss-of-function mutation of FMRP causes the FXS phenotype.

1.4 *FMR1* gene

The *FMR1* gene is 38 kb in length and contains 17 exons encoding 4.4 kb transcripts. *FMR1* gene encodes fragile X mental retardation protein (FMRP) composed of 632 amino acids and a molecular mass of 80 kDa, although alternative splicing of exons can produce different isoforms (O'Donnel and Warren, 2002). *FMR1* has two autosomal paralogs, *FXR1* and *FXR2* (Siomi et al., 1995, Zhang et al., 1995) and *FMR1* orthologs are highly conserved in mammals, mouse, chicken, and *Drosophila melanogaster* (Ashley et al., 1993, Price et al., 1996, Wan et al., 2000; Bardoni et al., 2001). *FMR1* mRNA and protein are highly expressed in neurons and testis of fetal and adult brain (Abitbol et al., 1993; Devys et al., 1993).

The transcription start point is located 69 base pairs (bp) downstream the repetitive region (CGG)n that was mapped in the 5'untranslated region (UTR) of *FMR1* (Ashley et al. 1993a). As results of CGG repeat expansion (full mutation), the CpG island and the surrounding sequence become hypermethylated with consequent gene silencing and absence of FMRP encoded protein (Pieretti et al., 1991). *FMR1* promoter activity is regulated by several transcription factors. The *FMR1* gene promoter shows four sites of binding for transcription factors, including a palindrome, two GC like boxes, and an overlapping E-box-cAMP response element (CRE) site. Among transcription factors that regulated *FMR1* promoter activity, stimulatory factor 1/2 (USF1 and USF2), nuclear respiratory factor 1/2 (NRF1 and NRF2), specificity protein 1 (Sp1) and cAMP response element binding protein (CREB) have been identified (Kumari et al., 2005; Smith et al., 2006). *FMR1* gene

methylation acts at two levels by inhibiting the binding of transcription factors, and by inducing chromatin condensation. The *FMR1* 5' region is normally associated with histone proteins H3 and H4 in acetylated form; this acetylation is reduced in FXS cells (Coffe et al., 1999). Furthermore changes in histone methylation have also been described. Generally histone 3 showing methylated lysine 4 and unmethylated lysine 9, but in FXS an opposite methylation pattern has been found (Coffe et al., 2002). In addition, the transition from active to inactive state of *FMR1* gene display different broader chromatin conformations. Overall these data suggest a strong implication of translation modification on *FMR1* to cause FXS. However, the cause-and-effect relationship among promoter activation, local histone modifications, and broader changes in chromatin remains to be determined.

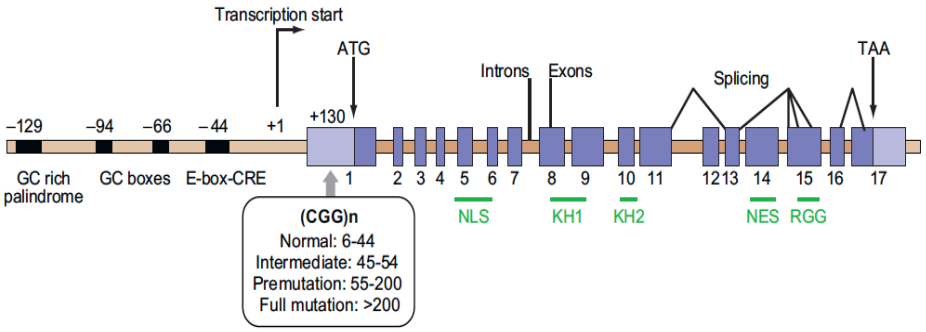

Figure2. *FMR1* gene. KH1/2, nuclear ribonucleoprotein K Homology 1/2; NES, nuclear export signal; NLS, nuclear localization signal; RGG, an Arginine-Glycine-Glycine box. From Penagarikano et al., 2007

1.5 FMRP: expression, structure and functions

Fragile X Mental Retardation protein (FMRP), encoded by *FMR1* gene, is an RNA binding protein involved in the regulation of target mRNA translation, transport and stability. FMRP with its paralogs, FXR1P and FXR2P (Fragile X Related Protein 1/2), 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 it is particular abundant in the brain and testis. FMRP major expression is in neurons, but it has also been detected in non-neuronal cells (Devys et al., 1993). It is associated with translating polyribosomes and mRNPs in the cytoplasm, in dendrites and dendritic spines where it regulates mRNA translation, indeed, FMRP is found to selectively bind ~4% of the mRNA in the mammalian brain (De Diego Otero et al., 2002; Bassel and Warren, 2008; Darnell et al., 2011). Alternative splicing of *FMR1* gene can generate 12 different FMRP isoforms between 67–80 kDa (Devys et al., 1993; Siomi et al., 1993), and they show the same expression pattern in different tissues (Verkerk et al., 1993). The most common isoform lacks exon 12, instead the least expressed isoform lacks exon 14 (Sittler et al., 1996). In general, FMRP is mainly localized in the cytoplasm, but the isoforms lacking exon 14 were localized to the nucleus, being absent the exon encoding for the nuclear exportation signal (NES). FMRP also contains a nuclear localization signal (NLS), indicating that FMRP shuttles between nucleus and cytoplasm (Devys et al., 1993; Feng et al., 1997a). The analysis of the structure of FMRP has revealed the presence of several functional motifs, useful to elucidate FMRP functions. FMRP contains three different RNA binding domains: two heterogeneous nuclear ribonucleoprotein K Homology (KH1, KH2) domains, 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, and an RNA-binding domain in the amino-terminal region of the protein (Darnell et al., 2001, 2005). The identification of three RNA-binding domains in the sequence of FMRP strongly suggest a direct interaction between FMRP and RNAs (Asley et al., 1993; Siomi et al., 1993). KH domains are thought to recognize “kissing-complex” tertiary motifs in RNA (Darnell et al., 2005), indeed a missense mutation in the second hnRNP KH binding domain (I304N) abolishes FMRP association with polyribosomes and causes FXS. The RGG box binds RNA G-quartet loops (Blackwell et al., 2010), whereas another motif called SoSLIP, found in Sod1 mRNA, is able to bind the C-terminal RGG region (Bechara et al., 2009). In addition, U-rich sequences have been isolated as potential RNA binding

motifs, although the precise binding domain within FMRP is unknown (Bhakar et al., 2012).

FMRP presents also two coiled coil (CC) domains involved in protein-protein interactions. Using 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), NUFIP1 (Nuclear FMRP Interacting Protein 1), 82-FIP (82 kDa-FMRP Interacting Protein) and the two closely related proteins CYFIP1 and CYFIP2 (Cytoplasmic FMRP Interacting Protein 1/2). 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 (Bardoni et al., 2006).

Figure 3. FMRP. Nuclear localization signal (NLS), two K-homology domains (KH1 and KH2), an RGG (arginine-glycine-glycine) box and a nuclear export sequence (NES). From Bhakar et al., 2012

1.6 FMRP and protein synthesis

There is a general consensus that FMRP acts mainly as a negative regulator of translation. Many symptoms of FXS are correlated with a modest increase in synaptic protein synthesis, therefore, how FMRP interacts with mRNAs to regulate synaptic protein synthesis is a major interest in the field. Several *in vitro* studies have suggested that FMRP is implicated in the mRNA transport being a component of RNA granules, dynamic escorts mRNAs aggregates that traffic from the soma to dendrites and axons. In the absence of FMRP some of its RNAs cargoes, as well as their encoded proteins, show differential subcellular distribution (dendritic spines vs soma). In the nucleus, FMRP binds RNAs and proteins to form the mRNP

complexes and in this form FMRP transports mRNAs to the cytoplasm (Eberhart et al., 1996; Khandjian et al., 1996; Corbin et al., 1997; Feng et al., 1997b). The mRNP complex can stay in the neuronal cell body or it can move to the dendritic spines through microtubule structures. In this way, FMRP can control the local protein synthesis at the synapses, influencing synaptic function, structure and plasticity (Feng et al., 1997b; Miyashiro et al., 2003; Bardoni et al., 2006; Zukin et al., 2009).

The identification of FMRP mRNA targets has been achieved, using a variety of *in vitro* assays. Darnell et al. has been identified 842 transcripts cross-linked to FMRP in mouse brain polysome using a stringent high-throughput sequencing-cross-linking immunoprecipitation (HITS CLIP) method (Darnell et al., 2011). *In vivo* ligands that are translationally altered by FMRP include a number of transcripts involved in synaptic function (Brown et al., 2001). For example, translation of both UNC-13 and SAPAP4 is downregulated in patient cell lines; UNC-13 is involved in presynaptic vesicle fusion, whereas SAPAP4 is associated with PSD-95 at the postsynaptic density (O'Donnel and Warren, 2002).

In addition to mRNPs there are other different complexes in which FMRP is involved; FMRP is a component of stress granules, the cytoplasmic structures where mRNA is recruited and protected under stress condition, during which cap-dependent translation is blocked (Anderson and Kedersha, 2006). FMRP is also a component of processing bodies, in which RNA is silenced or stocked. Furthermore, FMRP leads its RNAs in different compartment and once localized to the synapse, mRNAs are released from the granules and subsequently translated in response to stimuli (Krichevsky and Kosik, 2001).

Over the years a number of studies have tried to explain the function of FMRP like a repressor of translation (Laggerbauer et al., 2001; Li et al., 2001) and *in vivo* and *in vitro* measurements of protein synthesis performed in *Fmr1* KO mouse, where FMRP is absent, show a global increase in brain protein synthesis (Qinet al., 2005; Dolen et al., 2007; Osterweil et al., 2010). Although a repressor translation role of FMRP has been demonstrated, the underlying mechanism remains controversial and different possibilities through which FMRP could inhibit translation it has been

proposed. FMRP could repress translation by blocking elongation (Khandjian et al., 1996; Ceman et al., 2003; Bardoni et al., 2006). The presence of FMRP in stress granules suggest that FMRP represses translation throughout blocking translation initiation (see more ahead). Studies of co-sedimentation have found an association of FMRP with polyribosomes, with BC1 (brain cytoplasmic RNA 1), with CYFIP1 (cytoplasmic FMRP interacting protein) and with translation initiation factors (Centonze et al., 2008; Gabus et al.. 2004; Johnson et al., 2006; Lacoux et al., 2012; Laggerbauer et al., 2001; Napoli et al., 2008; Zalfa et al., 2007); in this model FMRP represses translation by inhibiting cap-dependent initiation (Napoli et al., 2008). Other data have suggested that association with the microRNA (miRNA) machinery may be involved too. miRNAs are small noncoding RNAs that inhibit translation by association with an RNA-induced silencing complex (RISC). FMRP associates with miRNAs, with RISC proteins and with the mammalian ortholog of Argonoute 1 (AGO1) that work together to silence mRNAs, either by direct cleavage of transcripts or by translational repression (Jin et al., 2004; Didiot et al., 2009).

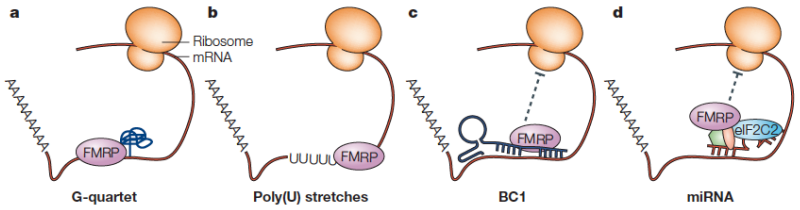

Figure 4. FMRP recognizes different RNA sequences. G-quartet structure (a), or poly(U) stretch (b) to bind directly mRNA; BC1 (c) or miRNAs (d) to bind indirectly mRNA. From Claudia Bagni and William T. Greenough, 2005

Although the exact mechanism by which FMRP stalls ribosomes remains elusive, several 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). Interestingly, FMRP may promote and not only inhibit translation of target

mRNAs, such as Trailer-Hitch (protein TRAL) and Superoxide Dismutase 1 (SOD1) transcripts (Bechara et al., 2009; Monzo et al., 2006). 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 the regulation of FMRP function several post-translational modifications give also a contribute. FMRP is normally methylated in RGG box, mainly on 544 arginine residues and this methylation seems to regulate its protein-protein and protein-RNA interactions (Dolzhanskaya et al., 2006; Stetler et al., 2006). FMRP can also be phosphorylated on a series of serine on the N-terminal side, mainly at serin 499. It has been suggested that the phosphorylated form of FMRP is associated to stalled polyribosome and in this state the association of FMRP with its mRNA is preserved; whereas the unphosphorylated form of FMRP has been observed in association with actively translating polysomes (Ceman et al., 2003; Coffee et al., 2011; Muddashettyet al., 2011). Thus, also phosphorylation of FMRP is implicated in FMRP-mediated protein synthesis.

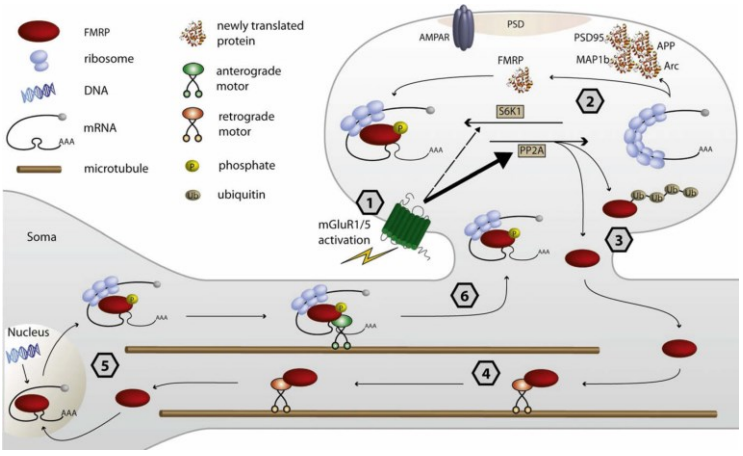

Figure 5. FMRP distribution in neuron. From Bassel and Warren, 2008

1.7 FXR1P and FXR2P

Fragile X related protein 1 and 2 (FXR1P and FXR2P), like their autosomal homolog FMRP, are RNA binding proteins which belong to "FXR family". They are encoded by autosomal genes, named FXR1 and FXR2, which are located in the chromosomal regions 3q28 and 17p13.1, respectively (Coy et al., 1995). FXR1P and FXR2P show a 86% of similarity with FMRP and a similar FMRP structure, being characterized by the presence of two KH, one RGG box RNA binding domains and nuclear localization and export signals (NLS and NES), suggesting that these two proteins may play a similar role than FMRP (Siomi et al., 1995; Zhang et al., 1995; Eberhart et al., 1996; Corbin et al., 1997; Feng et al., 1997). FXR proteins are also present in brain, testis and skeletal muscle tissue of mice (Bakker et al., 2000). The brain and testis isoforms of FXR1P have a molecular mass of 70 and 78 kDa, 80-84 kDa in skeletal muscle, instead FXR2P is of 95 kDa (Bakker et al., 2000; Khandjian et al., 1998; Zhang et al., 1995). In the absence of FMRP, like in *Fmr1* KO mice, no change in FXR1P and FXR2P distribution was observed; it is possible that both proteins compensate for the absence of FMRP, which would suggest functional redundancy (Bakker et al., 2000), but this aspect is controversial (Coffe et al., 2010). FXR1P, FXR2P with FMRP are highly expressed in the neurons of adult human brain (Tamanini et al., 1997). They are mainly localized to the cytoplasm associated with polyribosomes, but they are also present in the nucleus; for example, they have been observed in the nucleus of hippocampal neurons (Bakker et al., 2000). They also shuttle between the nucleus and cytoplasm, and FXR2P and certain isoforms of FXR1P show a nucleolar localization signal (NoS) and shuttles between the cytoplasm and the nucleolus (Tamanini et al., 1999, 2000). These data suggest that while the FXR proteins are associated in the cytoplasm with FMRP in an mRNP, they may be playing different functional roles in the nucleus.

FMRP interacts with the two paralogs FXR1P and FXR2P, although the significance of this interaction is not clear. It has been proposed that the interaction with these proteins might modulate the RNA binding function of FMRP, for

istance FXRP1 can modulate the FMRP affinity for the G-quartet RNA structure (Bechara et al., 2007). Using an *in vitro* RNA binding assay, Bechara and collaborators have indeed showed that the brain isoforms of FXR1P negatively regulate the affinity of FMRP for target RNAs, suggesting that FMRP works in the context of its multimolecular complex (Bechara et al., 2007).

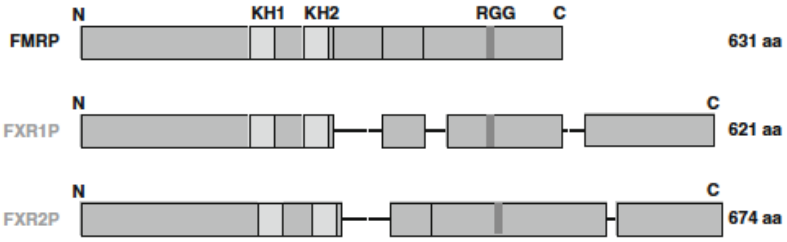

Figure 6. FXR family. From Pop et al., 2013

1.8 FXS mouse model

One of the most important advancement for the investigation and a better understanding of the molecular mechanisms implicated in FXS is represented by the development of FXS animal models. For these purposes, mouse and *Drosophila melanogaster* genetic models organisms have been generated. Both the fly and the mouse models exhibit a phenotype with similarities to humans (O'Donnel and Warren, 2002). *FMR1* gene is highly conserved between human and mouse: the murine *Fmr1* gene shows nucleotide sequence homology of 95% to human gene, the CGG repeat in the promoter region (Ashley et al., 1993) and a similar temporal tissue expression. Also protein sequence show a 97% of similarity between mouse and human (Hinds et al., 1993; Hergersberg, 1995). The mouse model is not a perfect representation of the human desease because it lakcs the trinucleotide repeat expansion mutation in *FMR1* gene, without which cannot mimic the timing of methylation and inactivation of the *FMR1* gene seen in

humans; however, it is characterized by a total loss of FMRP that is sufficient to cause the FXS phenotype. In the 1994, a consortium of different labs created an accurate animal model for the human condition: by inserting a neomycin cassette, that causes gene inactivation, into exon 5 of the murine gene by homologous recombination (The Dutch-Belgian fragile X consortium, 1994; O'Donnel and Warren, 2002). The *Fmr1* KO mouse lacks normal *Fmr1* RNA and FMRP in any of the tissues. Adult mutant mice show symptoms similar to those found in the human syndrome: significant macroorchidism, hyperactivity, spatial learning defect, altered sensorimotor integration (The Dutch-Belgian fragile X consortium, 1994; Van Dam et al., 2000; Chen and Toth 2001). Furthermore, *Fmr1* KO mice brains, like those of FXS patients, show an increased density of long and tortuous dendritic spines suggesting a delay in spine maturation (Dolen et al., 2010). To evaluate aspects of cognition and behavior in mice is particular difficult, but to overcome this issue a number of paradigms have been designed like approved sostitutes of IQ test (Crawley and Paylor, 1997). For istance, Morris water maze and radial arm maze are both test to measure spatial learning in which *Fmr1* KO mouse shows a defect (The Dutch-Belgian fragile X consortium, 1994; D'Hooge et al., 1997; Peier et al., 2000; Van Dam et al., 2000). *Fmr1* KO mice also exhibit 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). *Fmr1* KO mice is currently considered one of the leading animal models of autism (Bernardet and Crusio, 2006).

To study the function of FXR2P and FXR1P and their possible implication in FXS, *Fxr1* and *Fxr2* KO mouse models have been also generated. *Fxr1* KO mice display a phenotype completely different to those observed in *Fmr1* KO mice because they show abnormalities in muscle development. *Fxr1* KO neonates die early by cardiac or respiratory failure; this mouse model expresses very low levels of FXR1P, reduced limb musculature and has a reduced life span, suggesting a role for *Fxr1* gene in muscle mRNA transport/translation control similar to the function of *Fmr1* gene in neuronal cells but in muscle instead of neurons (Mientjes et al., 2004). In contrast, *Fxr2* KO mouse shows no pathological defects in brain or testes, and a lot

of similarities with *Fmr1* KO mouse (Bontekoe et al., 2002); these mice are hyperactive in the open-field test, show learning defect in the Morris water maze task, have reduced levels of prepulse inhibition, display less contextual conditioned fear and are less sensitive to a heat stimulus (Bontekoe et al., 2002). These features suggest a similar but not identical function for the *Fmr1* gene and the *Fxr2* gene.

A double *Fmr1/Fxr2* KO has also been created. These double *Fmr1/Fxr2* KO mouse exhibits an exaggerated behavioural phenotype 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 these homologous genes play a similar role in the regulation of locomotor activity, sensorimotor gating and cognitive processes (Zhang et al., 2009).

Also *Drosophila melanogaster* is often used like a valuable FXS model. In the fruit fly the *dFMR1* gene, a structurally and functionally well-conserved ortholog for the human *FMR1* gene is present (Wan et al., 2000). Several studies on *Drosophila melanogaster* have provided insight into the molecular biology of FXS (Zhang et al., 2001; Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002).

To summarize, many animal models have been created to study FXS, which have proved really useful and without which it would be impossible obtain a clear dissection of the molecular, physiological, cognitive, and behavioral phenotypes of FXS.

2. METABOTROPIC GLUTAMATE RECEPTORS

Most evidence over the past 15 years supports a role of group I metabotropic glutamate (mGlu) receptors in the pathophysiology of FXS. Metabotropic glutamate (mGlu) receptors are key players in excitatory transmission and important regulators of synaptic plasticity. Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system (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 (Monaghan et al., 1985; Cincotta et al., 1989). mGlu receptors are members of a C G-protein-coupled receptor (GPCR) superfamily that consists of eight receptor subtypes (mGlu1-mGlu8) categorized into three groups, Group 1, 2 and 3, on the basis of their sequence homology, G-protein coupling specificity similarities and different pharmacological response (Abe et al., 1992; Nakanishi, 1992; Tanabe et al., 1992; Conn and Pin, 1997; Nicoletti et al., 2011).

Group I mGlu receptors includes mGlu1 and mGlu5 receptor subtypes which are coupled to Gq/G11 proteins and are mainly localized at postsynaptic level. Their stimulation activates phospholipase C (PLC) that hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) with consequent production of inositol-1,3,4-trisphosphate (IP_3) and diacylglycerol (DAG), responsible for the release of intracellular Ca^{2+} and activation of protein kinase C (PKC) respectively (Kawabata et al., 1996; reviewed by Hermans and Challiss, 2001).

Group II and group III include mGlu 2, 3 and mGlu 4, 6, 7, 8 receptor subtypes respectively, they are coupled to Gi/Go proteins and are mostly presynaptic. Their activation negatively regulates adenylyl cyclase activity and voltage-sensitive Ca^{2+} channels. Because of their distribution, mGlu1 and mGlu5 receptors generally modulate postsynaptic efficacy, instead mGlu 2, 3, 4, 7, and 8 receptors regulate neurotransmitter release (Luján et al., 1997; Schoepp, 2001).

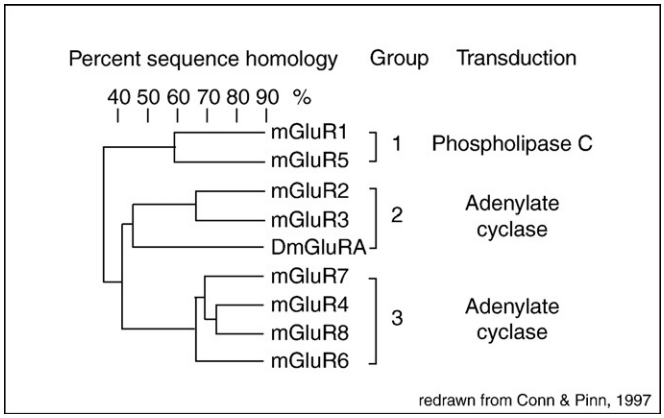

Figure 7. Classification of mammalian mGlu1-8 receptors.
From Dolen et al., 2010

Although hydrolysis of PIP_2 represent the canonical trasduction pathway coupled to mGlu5 receptor by PLC, actually mGlu5 receptor signalling occurs also throught other two cascades: ERK cascade and PI3K/Akt/mTOR cascade.

In the first case, the tyrosine kinase Src phosphorylates and activates MEK kinase, which in turn phosphorylates and activates ERK (also known as microtubule associated protein kinase, MAPK) (Ferraguti et al., 1999; Garcia et al., 2008; Dolen et al., 2010). In the second case, phosphorylation of the phosphoinositide 3-kinase (PI3K) activates Akt (also known as protein kinase B, PKB), which turns on the mammalian target of rapamycin (mTOR) (Ronesi and Huber, 2008; Dolen et al., 2010). ERK1/2 MAP kinase and/or PI-3-K pathways are involved in cell proliferation, differentiation, survival, and in synaptic plasticity (Ferraguti et al., 1999; Rong et al., 2003). The activation of these pathways directly or indirectly regulates protein synthesis; for istance, activation of mTOR phosphorylates 40S ribosomal protein S6 kinase (Hou and Klann, 2004; Klann and Dever, 2004; Banko et al., 2006; Antion et al., 2008), ERK is responsible for the phosphorylation of the eukaryotic initiation factor 4E (eIF4E) (Banko et al., 2006). Both activated mTOR and ERK phosphorylate eIF4E binding proteins (4E-BPs) (Klann and Dever, 2004). The binding of glutamate to mGlu5 receptor stimulates translation of several mRNAs with consequent increase in protein synthesis throught the activation of

these pathways (Proud, 2007; Klann and Dever, 2004;). The activation of ERK and PI3K requires the interaction of group-I mGlu receptors with Homer proteins, a class of scaffolding proteins that cross-link group-I mGlu receptors to inositol triphosphate (IP3) receptors and to other proteins of the post synaptic density such as SHANK (Tu et al., 1998, 1999; Rong et al., 2003; Mao et al., 2005). Homer proteins are also involved in the regulation of several properties of group-I mGlu receptors functions such as constitutive activity (Ango et al., 2001), cell surface expression and trafficking (Coutinho et al., 2001; Ango et al., 2002;), lateral mobility (Sergé et al., 2002) and coupling to ion channels of the cytoplasmic membrane (Kammermeier et al., 2000).

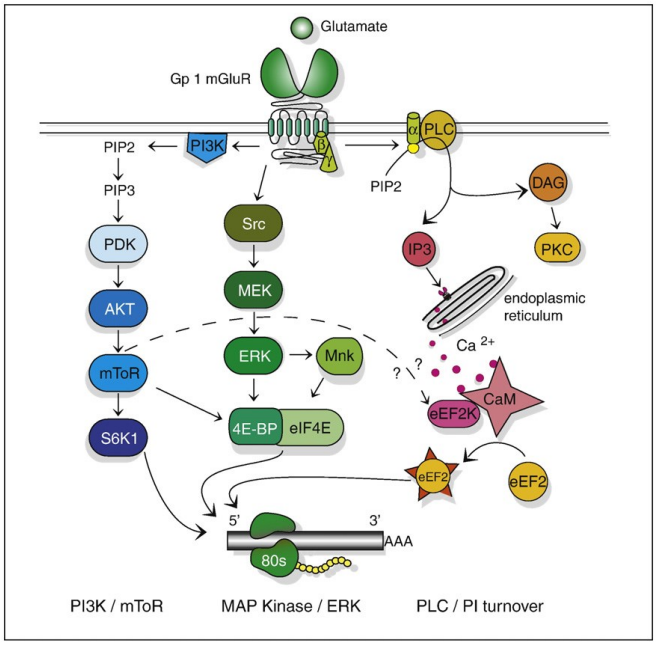

Figure 8. Signaling cascades coupling to group-I mGlu receptors.
From Dolen et al., 2010

mGlu1 and mGlu5 receptors show different regional and developmental expression profiles; mGlu5 receptor expression is elevated in hippocampus, neocortex and striatum (Shigemoto, 2000) and its expression is elevated during the first three

postnatal weeks while declines afterwards; mGlu1 receptors is maximally expressed in the cerebellum (Catania et al., 1994; Catania et al., 2007, reviewed in D'Antoni et al., 2014), it is also expressed in olfactory bulb, thalamus, and pars compacta of the substantia nigra. Its expression is higher in adulthood, instead it is barely expressed during early development (Lopez-Bendito et al., 2002). Both mGlu1 and mGlu5 receptors are present in cortical and hippocampal interneurons (van Hooft et al., 2000), where they partecipate to regulation of brain connectivity. mGlu5 receptors are also found in astrocytes under physiological and pathological conditions (D'Antoni et al., 2008), but they are also present in oligodendrocytes, microglia, stem progenitor cells, and a variety of peripheral cells (Nicoletti et al., 2011). In dendritic spines, mGlu1 and mGlu5 receptors are localized in the perisynaptic region (Baude et al., 1993; Nusser et al., 1994), but are also present at extrasynaptic sites with a higher frequency for mGlu5 than mGlu1 receptors (Lujan et al., 1997). Expression studies of mGlu receptors suggest that mGlu5 receptors have a crucial role in plastic remodelling during post-natal development (Catania et al., 2007).

2.1 Metabotropic glutamate receptors and FXS

In the last years, several evidence suggested a strong implication of mGlu5 receptor in the pathogenesis of FXS. An interplay between FMRP and mGlu5 receptor has emerged, particularly the role of mGlu5 receptor in several FMRP-mediated function. (Huber et al., 2002; Bear et al., 2004). The first indication for a link between mGlu5 receptor and FXS was the evidence that activation of group-I mGlu receptors in synaptoneurosomes stimulates the rapid translation of pre-existing mRNA, including the mRNA encoding FMRP (Weiler et al., 1997). Furthermore the activation of group-I mGlu receptors is necessary for FMRP trafficking from the cell body into dendrites (Antar, 2004) and enhances the dendritic transport of several FMRP target mRNAs, such as Map1b, CaMKII and also FMRP mRNA, in hippocampal cultured neurons (Antar et al., 2004;

Dictenberg et al., 2008; Ferrari et al., 2007). Dictenberg showed that, upon stimulation with the selective agonist of group-I mGlu receptors (S)-3,5-Dihydroxyphenylglycine (DHPG), FMRP interacts more efficiently with kinesin and that this group-I mGlu receptor mediated transport is substantially attenuated in the absence of FMRP. These data suggest that, as a consequence of the lack of FMRP, levels and distribution of several synaptic and non synaptic proteins are altered and also that key biochemical pathways might be dysregulated in FXS.

Subsequent studies revealed the finding that group-I mGlu receptors has an influence on LTD and LTP. In particular, group-I mGlu receptor-dependent long term depression (LTD), that requires mGlu5 receptor activation and local protein synthesis (Huber et al., 2000), is increased in *Fmr1* KO hippocampus, whereas NMDA receptor-dependent LTD is not (Huber et al., 2002). Curiously, mice lacking mGlu5 receptor show impaired learning and reduced LTP in the hippocampal CA1 region (Lu et al., 1997). Hippocampal epileptogenesis, another form of synaptic plasticity that depends on group-I mGlu receptor activation and protein synthesis, is also altered in *Fmr1* KO mice (Chuang et al., 2005; Dolen et al., 2010). Furthermore, the mGlu receptor-dependent LTD in *Fmr1* KO is insensitive to inhibitors of protein synthesis (Hou et al., 2006; Nosyreva and Huber, 2006), suggesting that the abnormal expression of synaptic proteins alters long-term responses to mGlu5 receptor activation in FXS. The finding that mGluR-LTD is exaggerated in *Fmr1* KO mice suggested that FMRP and mGlu5 receptor might work in functional opposition, where mGlu5 receptor activates protein synthesis and FMRP suppresses it (Dolen and Bear, 2008). Excitement for the proposed mechanistic link between FMRP and mGlu5 receptor in the regulation of protein synthesis, led Bear and collaborators to formulate the “mGlu theory” of FXS, which postulates that mGlu5 receptor and FMRP regulate translation of mRNAs at the synapse in a functionally opponent manner: activation of mGlu5 receptors stimulates protein synthesis and FMRP blocks it. In the absence of FMRP, like in FXS, mGlu5 receptor-dependent protein synthesis proceeds unchecked, and consequent excessive translation leads to development of FXS clinical features. According to ‘the mGlu Theory”, this defect can be corrected

using mGlu5 receptor antagonist, like MPEP, or by genetic reduction of mGlu5 receptor activity (Bear et al., 2004; Dolen and Bear, 2008).

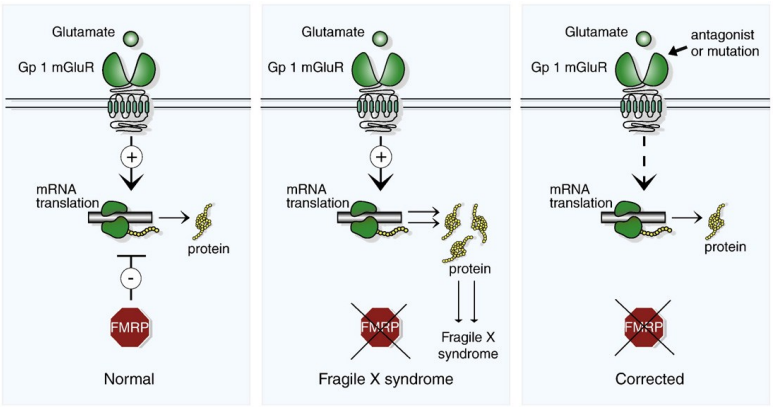

Figure 9. The mGluR theory: Opponent regulation of protein synthesis by group-I mGlu receptor and FMRP is disrupted in the absence of FMRP. Reduction of mGlu receptor signaling restores the balance and corrects FXS phenotype.
From Dolen et al. 2010

To validate "The mGluR Theory", Dolen and Bear generated double mutant mice by crossing *Fmr1* mutant mice with *Grm5* mutant mice, the gene that encodes for mGlu5 receptor. In this study they observed that 50% genetic reduction of mGlu5 receptor is able to rescue *Fmr1* KO phenotypes to levels closer to WT; the mGlu5 receptor genetic reduction in the *Fmr1KO/Grm5* heterozygote rescued altered ocular dominance plasticity, increased density of dendritic spines, increased basal protein synthesis, audiogenic seizure susceptibility, but not macroorchidism. These data confermed the opponent regulatory role for mGlu5 receptor and FMRP (Dolen et al., 2007; Dolen and Bear, 2008; Dolen et al., 2010).

FMRP suppresses translation of several proteins implicated in mGlu-LTD; upon group-I mGlu receptor activation, FMRP is dephosphorylated, ubiquitinated and degraded. It is known that when FMRP is associated with its mRNAs is in the phosphorylated form (Ceman et al., 2003; Narayanan et al., 2007; Bassel and

Warren, 2008). Activation of mGlu5 receptors stimulates FMRP dephosphorylation by activation of protein phosphatase 2A (PP2A), an FMRP phosphatase that can rapidly dephosphorylate FMRP in response to stimulation. Unphosphorylated FMRP loses the affinity for its mRNAs with consequent increase in translation (Narayanan et al., 2007; Bassel and Warren, 2008). This derepresses translation of FMRP mRNA targets contributes to rapid translational activation of proteins necessary for LTD such as Arc (Nalavadi et al., 2012; Niere et al., 2012). Recently, 831 mRNAs directly interacting with FMRP have been identified; among these 1/3 encode synaptic proteins and mGlu5 mRNA is higly represented (Darnell et al., 2011). These data all together illustrate that group-I mGlu receptor dysfunction is a large contributor to the pathophysiology of FXS (reviewed by D'Antoni et al., 2014).

3. THERAPEUTIC STRATEGIES IN FXS

Over the years, several studies have been aimed at ultimately achieving of a good treatment for FXS, which is currently merely symptomatic. However, the knowledge of countless targets involved in FXS suggests that is not simple to find a single therapy. Unfortunately, there is not yet a treatment to compensate the absence of FMRP and the therapy commonly used is designed specifically for each patient and is based on his/her specific behavior symptoms (reviewed by Penagarikano et al., 2007). One potential therapeutic approach in FXS consists in the reactivation of the silenced FMR1 gene to restore the production of FMRP (Chiurazzi et al., 1999; Pietrobono et al., 2002). For this purpose two compounds have been suggested, such as 5-Azadeoxycytidine (Chiurazzi et al., 1999; Tabolacci et al., 2005) and valproic acid (Tabolacci et al., 2008). Unfortunaly, reactivation processes are too general and not specific for *FMR1* gene and also toxicity of these approaches is too high. The possibility of gene therapy is currently not possible due to difficulties in the restoration of the normal gene into neurons

(reviewed by Penagarikano et al., 2007). Thus, common medications include stimulants, antipsychotic, anti-depressant and anticonvulsant. Patients with FXS also seem to benefit from behavioral intervention and special educational programs. As demonstrated in the FXS mouse model, an enriched environment can improve behavior, and thus this therapy might also be beneficial for patients (Restivo et al., 2005).

A Significant progress in the treatment of FXS was obtained by understanding the mGlu5 receptor role in the pathophysiology of FXS. Many studies have been aimed use of drugs to correct the abnormal activity of the mGlu receptor in FXS using specific mGlu5 receptor antagonists and nowadays mGlu5 receptor is considered a valid target to treat FXS. The first potent and selective, noncompetitive antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) was used in several drug discovery programs in industrial and academic research laboratories (Pop et al., 2013). Treatment with MPEP resulted in suppression of audiogenic seizure susceptibility in *Fmr1* KO mice (Chuang et al., 2005) and reduction in repetitive-like behavior (Thomas et al., 2012). These data indicate that the interaction between mGlu receptor signaling and FMRP function is responsible for some of the symptoms associated with FXS (reviewed by Penagarikano et al., 2007). Another drug commonly used is Fenobam; previously investigated as an anxyolitic, was later tested as a negative modulator of mGlu5 receptor (Porter et al., 2005). Beneficial effects included reduced anxiety and improvement of prepulse inhibition (Berry-Kravis et al., 2009). Both MPEP and fenobam restored dendritic spine morphology in hippocampal cell cultures from *Fmr1* KO mice (De Vrij et al., 2008). Recently, another novel mGlu5 receptor antagonistis, CTEP, has proven effective in restoring cognitive defects, auditory seizures, abnormal dendritic spine density; it is also able to stimulate ERK and mTOR signaling and partially to correct macroorchidism (Michalon et al., 2012). mGlu5 receptor antagonists appeared promising during preclinical studies; however preclinical studies did not translate into a broadly effective treatment for FXS. mGlu5 receptor antagonists, such as AFQ056 compound by Novartis, have been tested in clinical studies; however, this trial has been discontinued after Phase II because treatment

did not improve phenotypes or showed side effects. Currently a few preclinical and clinical studies for FXS are still in progress, for example RO4917523, another mGlu5 receptor negative modulator is in trials by Roche in U.S.A. Further experiments should help to understand discrepancies between outcomes obtained in pre-clinical and clinical studies using mGlu5 antagonists.

Another approach aimed to reduce excessive mGlu5 receptor signaling inhibiting glutamate release via the presynaptic activation of $GABA_B$ receptors. (Dolen et al., 2010; Pop et al., 2013). The most frequent $GABA_B$ receptors agonist used are baclofen and its enantiomer arbaclofen. Additional approaches consist of drugs like lithium, that reduces group-I mGlu receptor activity by attenuating GSK3β activity and probably phosphatidyl inositol turnover. Also Minocycline, an antibiotic that inhibits MMP9 (Matrix metallopeptidase 9), normalizes dendritic spine phenotypes and improves anxiety and exploratory behavior in the *Fmr1* KO (Bilousova et al., 2009). Finally, a preclinically intervention in the *Fmr1* KO mouse model, aim to modulate intracellular targets such as PI3K, (Gross et al., 2010), mTOR (Hoeffer et al., 2012), or MEK to investigate the effects of new agents without compromising patient safety (Wang et al., 2012). These agents represent potential target in emerging cellular models to realize the reprogramming of patient tissue samples in inducible pluripotent cells with a subsequent differentiation in neuronal cells (Sheridan et al., 2011).

Preclinical	Early Phase 1/2 Clinical Studies	Large Phase 2/3 Clinical Trials
STEP inhibitor (2)	CX516 – AMPA activator (3)	Arbaclofen – GABA-B agonist (4)
ERK inhibitor (1B)	Minocycline – MMP9 blocker (2)*	AFQ056 – mGluR5 blocker (1A)
PAK inhibitor (1B)	Acamprosate – GABA agonist (4)*	RO4917523 – mGluR5 blocker (1A)
PI3K Inhibitor (1B, 2)	Ganaxolone – GABA-A agonist (4)	
SLACK channel blocker (4)	Lovostatin – ERK inhibitor (1B)*	
Endocannabanoid blocker (4)	Fenobam – mGluR5 blocker (1A)	
GSK3β inhibitor (1B)	Donepezil – anticholinesterase (4)*	
Muscarinic M1, M4 blocker (1A)	Lithium – GSK3 B inhibitor (1B)*	
Antisense RNAs (5)	NNZ-2566 – IGF-1 agonist (4)	
Translation stalling agents (5)	Oxytocin (4)	

Abbreviations:
AMPA = α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ERK = Extracellular signal-regulated kinase
FXS = Fragile X syndrome
GABA = γ-Aminobutyric acid
GSK3β = Glycogen Synthase Kinase-3b
mGluR5 = Group 1 metabotropic glutamate receptor, type 5
IGF-1 = Insulin growth factor-1
MMP9 = Matrix metalloproteinase-9
PAK = p21-activated kinase
PI3K inhibitor = Phosphatidyl inositol 3 kinase
SLACK = Sequence like a calcium-activated K channel
STEP = Striatal-enriched tyrosine phosphatase
Numbers in parentheses indicate category of proposed targeted therapeutic agent based on location of impact on signaling pathway regulated by FMRP (see Fig 2).
* Drugs that are already approved by the Food and Drug Administration for another indication but have been or are being tested for repurposing for FXS.

Figure 10. Candidate drugs in preclinical and clinical trials for the treatment of FXS. Form Berry-Kravis, 2014

4. OXIDATIVE STRESS AND FRAGILE X SYNDROME

Several evidences suggest a role of oxidative stress in FXS. Oxidative stress is defined as damage to cellular tissue caused by free radicals such as reactive oxygen species (Maurin et al., 2014). Oxidative stress is implicated in a wide variety of neurodegenerative disorders and psychiatric diseases such as autism (Chauhan and Chauhan, 2006; James et al., 2006; Rossignol and Frye, 2012). In FXS an increased sensitivity to oxidative stress has been detected, with possible impacts on neuronal and glial function (Davidovic et al., 2011). For istance, 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 (El Bekay et al., 2007). This suggests that the oxidative stress in the brain may play a role in the pathophysiology of FXS. Recently, a study of metabolomic analysis performed on different brain regions of 12-day-old newborn *Fmr1* KO mice, has identified multiple metabolic abnormalities in *Fmr1* KO mice brains. Metabolites implicated in neurotransmission, osmoregulation, energy metabolism and oxidative stress response are altered (Brown et al., 2001; Miyashiro et al., 2003; Davidovic et al., 2011). *Fmr1* KO mice show altered mRNA profiles in glutathione transferase and SOD1; levels of SOD1 protein are reduced in the absence of FMRP suggesting that increased oxidative stress in *Fmr1* KO brain might be due to the altered SOD1 expression (Bechara et al., 2009).

In addition, FXS patients display an increase in adrenocortical activity and an altered hypothalamic-pituitary-adrenal (HPA) axis (Hessl et al., 2004); adrenal hormones is a source of oxidative stress in the brain, causing oxidation of molecules and depletion of antioxidants such as glutathione (Herman & Cullinan, 1997).

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 free radical overproduction, oxidative stress, macroorchidism, and also behaviour and learning

deficits (de Diego-Otero et al., 2009). Chronic administration of melatonin protects *Fmr1* KO mouse from the oxidative stress reverting several behavioural and learning deficits, normalizes free-radical production in macrophage cells and brain slices, and normalizes carbonyl content in proteins and lipid peroxidation (Romero-Zerbo et al., 2009). As mentioned before, a promising clinical trials in FXS is based on minocycline treatment (Leigh et al., 2013). This drug acts like an inhibitor of metalloproteases, the expression of which is increased in FXS cells (Siller and Broadie, 2011).

5. STRESS GRANULES (SGs)

Stress granules (SGs) are multimolecular cytoplasmic aggregates composed of non-translating messenger ribonucleoproteins (mRNPs) that rapidly aggregate when cells are exposed to adverse environmental conditions (Kedersha et al., 2005).

The first evidence about SGs dates back to 1989, when they were observed in tomato cell cultures (Nover et al., 1989). Subsequently, reversible aggregates of mRNPs were discovered in yeast (such as Saccharomyces pombe), protozoa (Trypanosoma brucei) and metazoa (such as Homo sapiens and Caenorhabditis elegans. At the beginning, SGs were described as large cytoplasmic mRNA aggregates, microscopically visible only in response to different types of stress. Then, it was discovered that SGs are composed not only by mRNAs, but also by abortive preinitiation translation complexes and RNA binding proteins (Stoecklin and Kedersha, 2013). Different types of stress such as heat shock, oxidative stress, UV irradiation and viral infection cause polysomes disassembly and therefore inhibit translation and promot SGs formation (Anderson and Kedersha, 2002). Translational initiation is regulated through different pathways, but under stress condition the pathways most frequently involved is the phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α). eIF2α phosphorylation is

the trigger for SGs formation after which several RNA binding protein are recruited to contribute to SGs formation (Kedersha et al., 2013). SGs show a dynamic nature that suggests that they are sites of mRNA triage, wherein mRNAs are arranged for storage, degradation, or translation during stress and after during recovery. SGs are formed to protect cells from stress and to favore cell survival by synthesizing stress protective proteins, such as heat shock proteins, and transiently blocking house-keeping proteins translation (Anderson and Kedersha, 2008). It has been established that more than 100 proteins regulate SGs assembly (Ohn et al., 2008), suggesting that SGs are the main sites that under stress condition play in helping cells respond to adverse environmental conditions. Furthermore, the cytoprotective effects of SGs is corroborated by the finding that multiple interventions that prevent SGs assembly are associated with higher cells susceptibility (Arimoto et al., 2008; Buchan and Parker 2009; Kedersha et al., 2013), but specifically how SGs exert a protective role is obscure.

5.1 SGs assembly

SGs formation is a complex mechanism related to stalled translational initiation, polysome disassembly and mRNPs aggregation, which occurs through a number of reversible steps. It is possible to identify different phases in the process of SGs assembly:

1) SGs initiation

Generally, the first step in SGs formation is the phosphorylation of eIF2α. Eukaryotic cells express a family of eIF2α kinases (eg, PKR, PERK-PEK, HRI, GCN2) that are activated in response to distinct types of environmental stress (Anderson and Kedersha, 2002). Of these kinases, PKR (protein kinase R) is a double-stranded RNA-dependent kinase activated by viral infection, heat, UV irradiation and oxidative stress (Williams, 2001), whereas PERK (PKR like endoplasmic reticulum kinase, also called PEK, or pancreatic eIF2α kinase) is activated by endoplasmic reticulum stress, such as unfolded proteins accumulated

in the ER lumen (Harding et al., 2000). HRI (heme-regulated initiation factor 2α kinase) is activated by oxidative stress and regulates changes in the availability of heme during erythrocyte differentiation (Han et al., 2001; Lu et al., 2001; McEwen et al., 2005; Anderson and Kedersha 2008); GCN2 (general control nonderepressible 2) is a protein that controls amino acid levels in the cell and responds to amino acid deprivation (Wek et al., 1995). Activation of one or more of these eIF2α kinases results in the phosphorylation of eIF2α at serine 51, a crucial component of the ternary eIF-2 complex that loads the initiator tRNA (Met-$tRNA^{Met}$) onto 40S ribosomal subunit to initiate protein translation. Phosphorylation of eIF2α results in abortive initiation complexes with consequent arrest in translation initiation (Anderson and Kedersha, 2002). Generally, the ternary complex is composed by eIF2αβγ bound to $tRNA_i^{Met}$ and GTP that loads initiator $tRNA_i^{Met}$ onto the small ribosomal subunit (40S) to assembly the 43S preinitiation compex. The 43S complex, with other initiation factors (eg, eIF4E, eIF4G, and poly(A)-binding protein), recruits a 7-methyl guanosine-capped mRNA to makes up the canonical 48S preinitiation complex. At this point, the 48S complex scans the 5' untranslated region (UTR) of the mRNA transcript, starting by classical AUG codon that is recognized by the $tRNA^{Met}$ anticodon. Recognition of the initiation codon triggers hydrolysis of eIF2-GTP, a reaction catalyzed by eIF5. After this event, eIF2–GDP, eIF3, eIF5, eIF1A dissociate from the 40S subunit, and the 60S ribosomal subunit can take part to form a functional 80S ribosome (see figure 11A) (Anderson and Kedersha, 2008). Under stress condition, phosphorylated eIF2α inhibits eIF2B function, the GTP/GDP exchange factor that converts inactive ternary complex (GDP-associated) to active ternary complex (GTP-associated) (Krishnamoorthy et al., 2001). Thus, eIF2α phosphorylation inhibits protein synthesis by reducing the availability of the active eIF2-GTP-$tRNA_i^{Met}$ ternary complex that is required for cap-dependent translation initiation (Anderson and Kedersha, 2002). When ternary complex levels are reduced, TIA-1 and TIAR (RNA-binding proteins) promote the assembly of a noncanonical preinitiation complex that lacks eIF2-GTP-$tRNA_i^{Met}$ becoming SGs core proteins. The TIA proteins dynamically arrange these incompetent preinitiation complexes

into discrete cytoplasmic aggregates namely stress granules. Inhibition of translation initiation allow ribosomes to 'run off' from translating mRNA, with consequent polysome disassembly and 48S pre-initiation complexes accumulation. Most of the mRNA derived from disassembled polysomes are immediately recruited into SGs (Anderson et al., 2006).

Using arsenite, an inhibitor of the citric acid cycle that induces oxidative stress, it was possible to demonstrate that eIF2α phosphorylation is able to induct translational arrest and SGs assembly (reviewed by Stoecklin and Kedersha, 2013). In Contrast, inhibition of both arsenite-induced translational arrest and SGs assembly it was observed in a nonphosphorylatable eIF2α mutant (S51A) (Kedersha et al 1999). Furthermore, pharmacological treatments performed with different protein synthesis inhibitors, like cycloheximide, that stabilizes polysomes by freezing them on translating mRNA, or puromycin, that disassembles polysomes by destabilizing their coupling with mRNA transcripts, have demonstrated that conditions preventing ribosome elongation, disassembly and run-off (i.e. cycloheximide, emetine) do not permit SG assembly. eIF2α-dependent SGs formation is not the only one; another factor often involved during stress is eIF4A helicase. Chemical agents, such as Hippuristanol or pateamine A, promote SGs assembly in a eIF2α-indipendent manner, by blocking eIF4A helicase that in normal condition recruits ribosome for translation initiation (Bentmann et al., 2013).

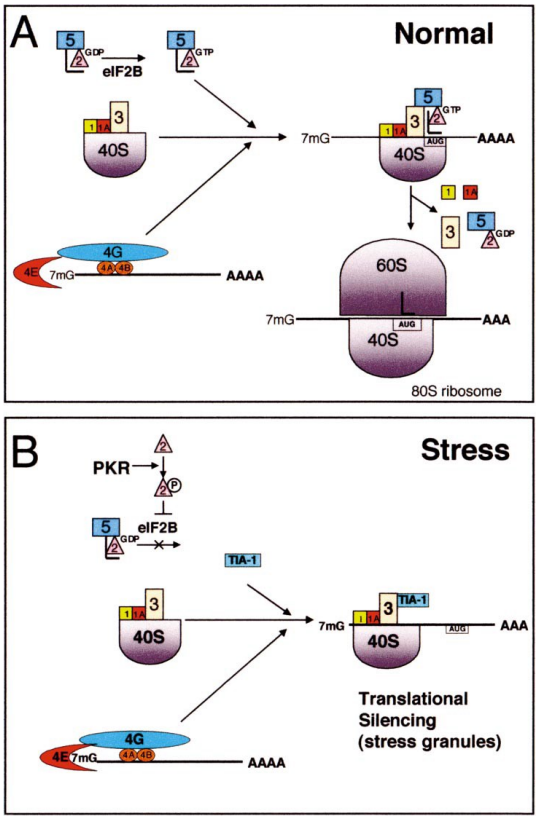

Figure 11. Translation initiation process in the absence or presence of stress. From Anderson and Kedersha, 2002

2) SGs nucleation

The abnormal 48S complex is the essential SGs substrate that includes mRNPs transcripts, eukaryotic initiation factors eIF3, eIF4F (comprising eIF4E, eIF4A and eIF4G), PABP-1 (the poly(A) binding protein 1, involved in mRNAs stability) and small ribosomal subunits (Tourrière et al., 2003; Kedersha et al., 2013).

Immediately after the formation of the 48S complex, several effector proteins are recruited in SGs assembly becoming SGs-associated proteins (see table below). Generally they are RNA binding proteins involved in translational silencing or mRNA stability and among them, TIA-1 (T cell internal antigen 1) and TIAR (TIA-1 related protein) have been identified, as the most important SGs proteins responsible for "SGs core" formation, that inhibit mRNA translation (Kedersha et

al., 1999; Buchan et al., 2008); TTP (Tristetraprolin) and BRF1 (Butyrate response factor 1) that enhance mRNA decay (Stoecklin et al., 2004), G3BP (GTPase activating protein binding protein) phosphorylation-dependent endoribonuclease that interacts with a Ras-GTPase–activating protein and binds free transcripts in the cytoplasm (Tourrière et al., 2003), RCK an helicase that may binds mRNAs released from polysomes and facilitates mRNPs packaging into SGs, CPEB (Cytoplasmic polyadenylation element binding protein) inhibitor of translation by RNAs silencing (Wilczynska et al., 2005), FAST (TIA1-interacting protein) a splicing antiapoptotic and proinflammatory regulator (Kedersha et al., 2005), FMRP (fragile x mental retardation protein) and FXR1P (fragile X mental retardation-related protein 1) that are RNA binding proteins involved in translational control of specific mRNAs (Mazroui et al., 2002; Kim et al., 2006; Didiot et al., 2009), Argonaute-2 ribonuclease involved in miRNA and siRNAs silencing (Leung et al., 2006), SMN (survival of motor neurons) involved in RNPs assembly (Zou et al., 2011; Hua and Zou, 2004), smaug, an RNA binding protein involved in the control of mRNA translation and decay (Baez and Boccaccio, 2005). These proteins initiate mRNPs aggregation and mediate "primary aggregation" thus representing constitute examples of SGs nucleators. SGs nucleation is a crucial step in SGs formation that shifts the equilibrium between mRNPs and polysomes (Anderson and Kedersha, 2008). Curiously, some of these proteins have been observed in polyribosomes, such as FMRP, FXR1P, RCK, whereas others are excluded from polyribosomes, for example TIA-1 and TIAR). These data suggest that TIA-1 and TIAR, like translational silencer, promote SGs formation; when they are associated with mRNA, translation is suppressed and SGs assembly is promoted. Conversely, other proteins that take part in SGs formation, are associated with polysomes in normal condition. Thus, this suggest that they may be under regulatory control depending on environmental conditions; indeed, they are associated in an inactive state with translating polysomes, instead under stress condition they promote translational silencing, polysome disassembly and SGs assembly (Kedersha et al., 2013).

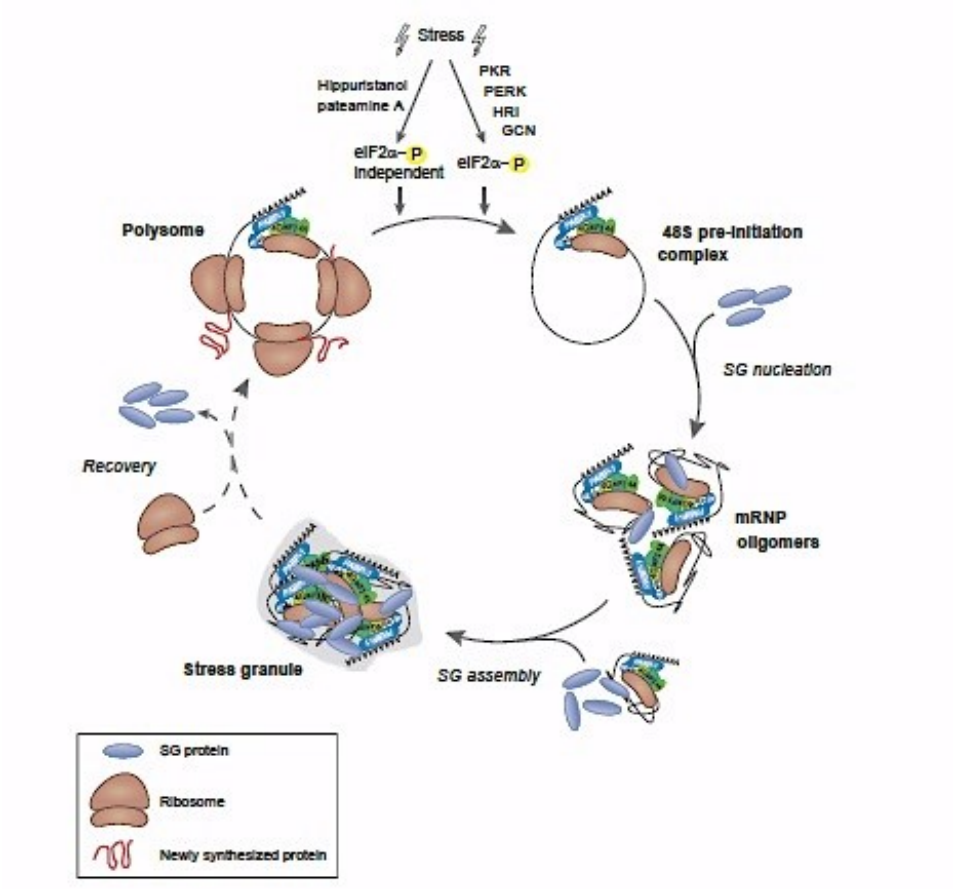

Figure 12. Stress granules formation. From Bentmann et al., 2013

3) SGs secondary aggregation and signaling pathways integration

SGs are initially small and increase in size when recruited mRNAs interact and bind SGs-proteins that in turn interact to each other through the glycine rich protein aggregation domains (Anderson and Kedersha, 2008). This secondary SGs maturation or aggregation allows microscopical visualization of SGs. Time-lapse photomicroscopy studies show SGs assembly: is possible to note numerous small SGs that progressively cluster into larger and fewer granules (Kedersha et al., 2000). After SGs nucleation, several other proteins are recruited in SGs, also proteins that don't exert mRNA-binding properties, but that are involved in

metabolic signaling pathways. They are integrated in SGs formation through protein-protein interactions with the SGs nucleating proteins. For example TIA-1-binding proteins, such as SRC3, FAST, PMR1 and FBP are recruited to SGs in this manner, often defined “piggyback” manner (Rothe et al., 2006; Yang et al., 2006; Yu et al., 2007). Additional examples of piggyback recruitment include TRAF2 (tumor necrosis factor receptor-associated factor 2 bound to eIF4G) a protein that regulates NF-kB-dependent cell survival (Kim et al., 2005), plakophilin 3 (bound to G3BP and PABP-1) a protein that promotes cellular adhesion (Hofman et al., 2006) and DIS1 (Disrupted-in-Schizophrenia bound to eIF3). It has been supposed that these proteins might make an integration between SGs formation and other cellular signaling pathways; SGs proteins can integrate different aspects of cellular metabolism with the translational response to stress. It is known that the translational initiation is under tight regulatory control. Thus, an excess in translational initiation can cause cellular transformation, instead abnormal translational initiation leads to cell death. This close association between translational initiation and cell survival is crucial under stress condition (Anderson and Kedersha, 2008). Interestingly, several studies suggest that SGs mediate cellular protection, but how they regulate this aspect is poorly understood. It has been demonstrated that some of signaling proteins sequestered in SGs, such as eIF4E, TRAF2 and FAST, regulate cell survival upon stress (Fournier et al., 2013; Arimoto et al., 2008; Eisinger-Mathason et al., 2008). Fournier et al. showed that inactivation of mTORC1-eIF4E pathway impairs SGs formation and sensitizes cancer cells, strengthening the antiapoptotic SGs role. Also, the discovery about another important factor in SGs, such as RISC (RNA-induced silencing complexes), suggests that SGs are integrated with microRNA-induced silencing pathways, thus they are able to influence cell fate decisions (Leung et al., 2007).

Protein	Function	Reference
TIA-1/ TIAR	inhibitor of translation	Kedersha et al., 1999; Buchan et al., 2008;
Dhh1p	mRNa decay	Reijns et al., 2008
G3BP	RNA signaling	Tourrière et al., 2003
MNL51	mRNA splicing	Baguet et al., 2007
Pumilio 2	RNA silencing	Vessey et al., 2006
Caprin	mRNA transport and translation	Solomon et al., 2007
TDP43	transcriptional repressor	Johnson et al., 2008; Colombrita et al., 2009
Ago1	Component of RNA-induced silencing complex	Leung et al., 2006
Ago2	Component of RNA-induced silencing complex	Leung et al., 2006
CPEB1	Inhibitor of translation	Wilczynska et al., 2005
eIF4E	Translation initiation	Kedersha et al., 2005
FAST	Antiapoptotic and proinflammatory regulator	Kedersha et al., 2005
FMRP	translational control of specific mRNAs	Mazroui et al., 2002; Didiot et al., 2002
FXR1P	translational control of specific mRNAs	Mazroui et al., 2002; Didiot et al., 2009
HuR	mRNA stability	Gallouzi et al., 2000
Lsm14	endoplasmic reticulum organization	Yang et al., 2006
RCK	mRNA decay	Wilczynska et al., 2005
smaug	mRNA translation and decay	Baez and Boccaccio, 2005
TTP or BRF1	mRNA decay	Stoecklin et al., 2004
DIS1	cell proliferation and differentiation,	Ogawa et al., 2005
eIF4G	translation initiation	Kedersha et al., 2005
PABP-1	mRNAs translation and stability	Tourrière et al., 2003
Plakophilin 3	Cellular adhesion	Hofman et al., 2006
Staufen	mRNA silencing	
TRAF2	Cell survival	Kim et al., 2005
FBP	RNA decay	Rothe et al., 2006
PMR1	RNA decay	Yang et al., 2006
SRC3	Trascription	Yu et al., 2007
SMN	RNA assembly	Hua and Zhou, 2004; Zou et al.; 2011

Figure 13. SGs proteins

Several post-translational modifications play an important role in the regulation of SG assembly. Of course, one example are phosphorylation and dephosphorylation of eIF2α, but also phosphorylation of G3BP regulates SGs assembly/disassembly (Ohn and Anderson, 2010). Another post-translational modification involved in SGs formation is O-GlcNAcylation. O-Glc-NAc-modified proteins tend to accumulate in SGs, immunofluorescence analysis with two different anti-O-GlcNAc antibodies showed that O-Glc-NAcylated proteins are in SGs (Ohn et al., 2008). Immuno-purification analysis of these proteins revealed among them RACK1, prohibitin-2 and several other proteins (Ohn and Anderson, 2010). Furthermore, the lack of enzymes to convert glucose to GlcNAc, abolishes SGs formation, suggesting that O-GlcNAc modifications are important in SGs formation (Ohn et al., 2008). It is supposed that these sugars works as molecular glue in the mRNPs aggregation process and that O-Glc-NAc modifications can promote translational repression interfering with ribosomal subunits (Ohn and Anderson, 2010). Another post-translational modification associated to SGs is methylation of arginine residues by peptidylarginine methyltransferases. Several RNA-binding proteins, such as FMRP, CIRP and FUS, can be methylated in their RGG repeat motifs and localize to SGs in their methylated state (Tradewell et al., 2011; Dolzhanskaya et al., 2006); the inhibition of methylation decreases the recruitment of these proteins into SGs, maybe altering the protein's cellular localization and/or changing its RNA-binding affinity (Dormann et al., 2012; Tradewell et al., 2011). The finding that SGs assembly depends on a number of post-translational modification indicates that this mechanism is subject to complex regulation.

4) mRNA triage

SGs could protect most cellular mRNAs, redirecting them to translation when environmental conditions improved. After stress, mRNA fate is decided in SGs, that work like selective compartments within specific transcripts are destined for decay, whereas other transcripts are addressed for export or storage and still others can be reinitiated and reconverted into polysomes (Anderson and Kedersha, 2008).

mRNA recruitment in SGs is selective but less is known about their specificity; it has been demonstrated that only 50% of cytoplasmic poly(A) RNA and poly(A)-binding protein-1 is recruited to SGs, instead nearly 90% of TIA-1 is recruited to SGs (Anderson and Kedersha, 2002). In general, it seems that endogenous cellular mRNAs (housekeeping) are recruited to SGs, whereas mRNAs encoding heat-shock protein 70 (HSP70) and heat-shock protein 90 (HSP90) are largely excluded (Kedersha and Anderson, 2002; Stohr et al., 2006). This because heat shock proteins are activated for translation during stress simultaneously to SGs formation and both work in parallel to exceed stress condition.

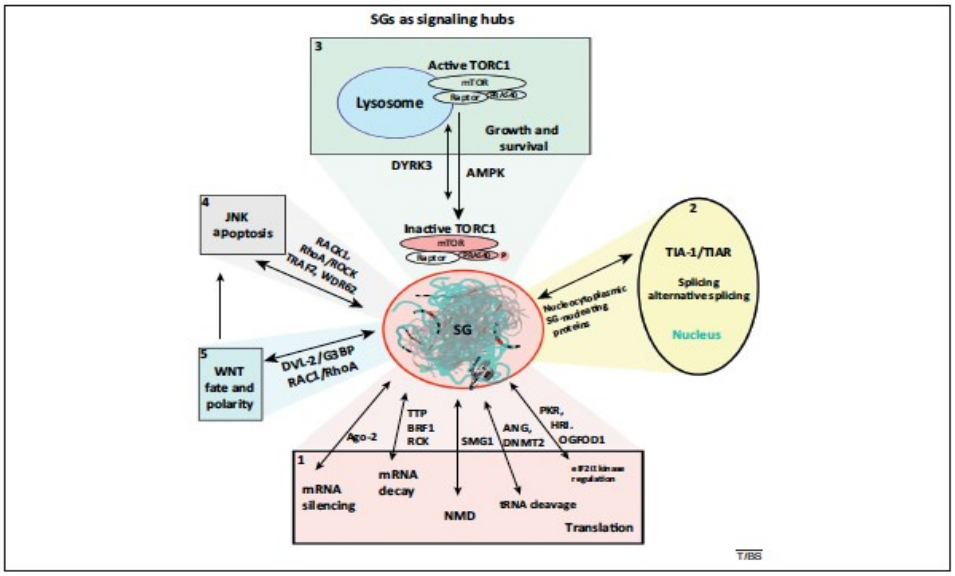

Figure 14. SGs signaling. From Kedersha et al., 2013

5) SGs disassembly

SGs are reversible aggregates useful during stress condition to preserve mRNAs. Thus, in cells recovering from stress is common to observe SGs disassembly. Before disassembly, SGs are relatively few and large (microns in diameter) and within few minutes they disappear completely (Kedersha et al., 2005).

5.2 SGs and polysomes: a dynamic equilibrium

As mentioned before, SGs formation regulation is in equilibrium with translation. The effect of pharmacological inhibitors of protein translation reveal this dynamic equilibrium between polysomes and SGs (Anderson and Kedersha, 2002). Indeed, cycloheximide and emetine, drugs that freeze ribosomose on translating mRNAs, dissolve SGs in arsenite-stressed cells and simultaneuosly promote polysomes assembly; in contrast puromycin, that destablize polysomes by promoting premature termination, causes releasing of untranslated mRNAs and promotes SGs assembly (Kedersha et al., 2000). Thus, the effects of these drugs on polysome stability is useful to explain the mechanism which links SGs and protein synthesis and furthermore, these data indicate that mRNAs can shuttle between SGs and polysomes during stress (Kedersha et al., 2000). During SGs formation a migration of mRNAs and proteins from their sites to SGs takes place. To examine the dynamic characteristics of these molecules, Kedersha and collaborators used two constructs: GFP-TIA-1 and GFP-PABP that exhibit the same behavior of endogenus proteins TIA-1 and PABP-1. Specifically, TIA-1 is a RNA binding protein localized in the nucleus; in response to different type of stress, TIA-1 moves to SGs and become a SGs core component. TIA-1 or GFP-TIA-1, are useful to monitor the assembly and disassembly of SGs in living cells for their shuttle proprierties (http://www.jcb.org/cgi/content/full/151/6/1257/DC1). In response to arsenite-induced stress GFP-TIA-1, like TIA-1, translocates in the cytoplasm within 3-6 minutes and after approximately 10 minutes, GFP–TIA-1 is visible like cytoplasmic aggregates. When arsenite is washed out, SGs fuse each other, became few and large and finally dissolve (Kedersha et al., 2000).

5.3 SGs and neurodegeneration

Recently, a possible role of stress granules in neurodegeneration has become object of increasing interest. SGs formation is generally considered to be a protective mechanism charcterized by synthesis of stress protective proteins and sequestering

of pro-apoptotic proteins. Even though SGs show cytoprotective features, they may become neurotoxic when the SGs pathway is overactive or when SGs disassembly fails (reviewed by Wolozin, 2012).

Abnormal neuronal inclusions and dysfunction in RNA metabolism has been observed in several neurodegenerative diseases (van Blitterswijk and Landers, 2010; Polymenidou et al., 2012; Wolozin, 2012; Bentmann et al., 2013). In many of these disorders, such as in Alzheimer's disease, pathological inclusions characterized by protein aggregates are existent. Similarly, in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) mutant RNA-binding proteins TDP-43 (TAR DNA-binding protein of 43 kDa) or FUS (fused in sarcoma) are abnormally deposited in cytoplasmic inclusions of neuron and glia cells in the majority of ALS and FTLD patients (Arai et al., 2006; Neumann et al., 2009). Similar scenario was observed for SMN protein, which when mutated leads to ALS or spinal muscular atrophy (SMA). Interestingly, all of these proteins have been found recruited in SGs. Thus, several RNA-binding proteins recruited in SGs have been associated with neurodegenerative diseases. This suggests that SGs might play an important role in the pathogenesis of proteinopathies. Moreover, pathological inclusions of TDP-43 in ALS, FUS in FTD or tau in Alzheimer's disease, show robust co-localization with several SG markers (TIA-1, PABP-1 or eIF4G). Other neurodegenerative related proteins, without mRNA-binding properties, are connected to SGs. One example is mutant superoxide dismutase 1 (SOD1), which has been observed with SGs-associated proteins and co-localizes with SGs markers in cell culture and murine spinal cord extracts (Lu et al., 2009). Another example is mutant huntingtin (Htt) with pathological poly Q expansion, which co-localizes with TIA-1 in different cell lines (Waelter et al., 2001). It is important to underline that SGs do not have insoluble aggregate properties of typically cytoplasmatic inclusion associated with neurodegenerative diseases, in fact their nature is fully reversible after stress. Thus, the question that remains to clarify is whether SGs contribute to development of pathological protein inclusions (Bentmann et al., 2013).

5.4 SGs and Fragile X Syndrome

Another aspect under investigation is the role of SGs in mental retardation. Oxidative stress is found in various neurodegenerative diseases and in autism like FXS and several studies suggest that there is a link between abnormal SGs formation and intellectual disabilities (Mazroui et al., 2002; Kim et al., 2006; Didiot et al., 2009; Gareau et al., 2013a).

FMRP has been found to be associated with the pool of mRNAs that go into SGs upon cellular stress and can be involved in the inhibition of protein synthesis occurring during stress (Mazroui et al., 2002). FMRP moves in SGs during heat stress (Mazroui et al., 2002) and during oxidative stress (Kim et al., 2006). FMRP, with its nuclear localization and nuclear export signals, is able to shuttle between the cytoplasm and the nucleus. It is known that in the cytoplasm FMRP is associated with mRNP complexes bound to polyribosomes (Corbin et al., 1997; Feng et al., 1997a; Feng et al., 1997b), and that FMRP has a role in translation acting as a translational repressor by trapping mRNAs into RNA granules which are transported out of the soma in a repressed state until they reach their destination (Bassel and Warren, 2008). Furthermore, FMRP might also promote translation repression of its mRNA targets under stress conditions by trapping them into SGs (Mazroui et al., 2002). In addition, the other members of FXR family, FXR1P and FXR2P, co-localize together with FMRP in SGs (Mazroui et al., 2002) as well as PQBP1 and TDP-43, other proteins involved in several X-linked intellectual disability disorders and neurodegenerative diseases (Kunde et al., 2011; Linder et al., 2008). It has been observed that FMRP moves in SGs after neuronal injury induced by sodium arsenite in hippocampal rats (Kim et al., 2006). Furthermore, it has been shown that the lack of FMRP impairs SGs formation (Didiot et al., 2009) and maybe the defect in SGs formation observed in the cells lacking FMRP could be due to the loss of an active role of FMRP in the process of translation inhibition (Gareau et al., 2013b). Thus, FMRP is involved in mRNA storage process during stress conditions, indeed it leads mRNAs in SGs and its absence impairs the formation of SGs, but the exact contribution of FMRP in SGs formation appears

unclear. Based on this knowledge FMRP may have a positive role in stress response, facilitating and enhancing SGs formation to prevent stress damages. Thus maybe this process is useful to understand what happens in neurodegenerative disorders and autism.

CHAPTER I

Fragile X mental retardation protein (FMRP) interacting proteins exhibit different expression patterns during development

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ABSTRACT

Fragile X syndrome is caused by the lack of expression of Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein involved in mRNA transport and translation. FMRP is a component of mRNA ribonucleoprotein complexes and it can interact with a range of proteins either directly or indirectly, as demonstrated by two-hybrid selection and co-immunoprecipitation, respectively. Most of FMRP-interacting proteins are RNA-binding proteins such as FXR1P, FXR2P and 82-FIP. Interestingly, FMRP can also interact directly with the cytoplasmic proteins CYFIP1 and CYFIP2, which do not bind RNA and link FMRP to the RhoGTPase pathway. The interaction with these different proteins may modulate the functions of FMRP by influencing its affinity to RNA and by affecting the FMRP ability of cytoskeleton remodeling through Rho/Rac GTPases. To better define the relationship of FMRP with its interacting proteins during brain development, we have analyzed the expression pattern of FMRP and its interacting proteins in the cortex, striatum, hippocampus and cerebellum of wild type mouse (WT) at different ages. FMRP and FXR2P were strongly expressed during the first week and gradually decrease thereafter, more rapidly in the cerebellum than in the cortex. FXR1P was also expressed early and showed a reduction at later stages of development with a similar developmental pattern in these two regions. CYFIP1 was expressed at all ages and peaked in the third post-natal week. In contrast, CYFIP2 and 82-FIP (only in forebrain regions) were moderately expressed at P3 and gradually increased after P7. In general, the expression pattern of each protein was similar in the regions examined, except for 82-FIP, which exhibited a strong expression at P3 and low at later developmental stages in the cerebellum. Our data indicate that FMRP and its interacting proteins have distinct developmental patterns of expression and suggest that FMRP may be preferentially associated to certain proteins in early and late developmental periods. In particular, the RNA binding and cytoskeleton remodeling functions of FMRP may be differently modulated during development.

Key words:
FMRP, FXR1P, FXR2P, CYFIP1, CYFIP2, 82-FIP

Highlights

- Levels of FXR proteins in mouse brain are high in the first/second post-natal week.
- Levels of FXR proteins gradually decrease after the first/second post-natal week.
- Levels of CYFIP1 and CYFIP2 gradually increased in the first two post-natal weeks.
- CYFIP1 and CYFIP2 are maximally expressed in the third post-natal week.
- Levels of 82-FIP increase in forebrain and decrease in cerebellum post-natally.

1. INTRODUCTION

Fragile X syndrome (FXS) is the most frequent form of inherited cognitive deficit and the second genetic cause of intellectual disability after Down syndrome, affecting about 1 in 4000 male and 1 in 8000 females. It is caused by the lack of Fragile X Mental Retardation protein (FMRP), a RNA binding protein involved in several steps of post-transcriptional control of target mRNAs such as transport (Dictenberg et al., 2008), translation repression (Brown et al., 2001; Darnell et al., 2001; Laggerbauer et al., 2001; Castets et al., 2005; Darnell et al., 2011; for recent reviews see Wang et al., 2012, Darnell and Klann, 2013) and translation activation (Bechara et al., 2009; Gross et al., 2011). A role of FMRP as an effector of stress granule assembly has been also described (Didiot et al., 2009).

FMRP is a component of mRNA ribonucleoprotein (mRNP) complexes and it can interact with a range of proteins either directly or indirectly, as demonstrated by two-hybrid selection and co-immunoprecipitation, respectively. Fragile X related protein (FXR) 1 and 2 are very similar in overall structure to FMRP, they are encoded by two *FMR1* paralog genes and form a family of FXR proteins. The three members of the FXS family can homo and heterodimerize and are endowed with different RNA binding motifs, namely KH domains and RGG box (reviewed by Bardoni et al., 2006). Given the high degree of protein similarity, it is possible that these genes have overlapping functions. Most of FMRP-interacting proteins are RNA-binding proteins such as FXR1P, FXR2P, NUFIP and 82-FIP (Bardoni et al., 2006). Interestingly, FMRP can also interact directly with the cytoplasmic proteins CYFIP1 and CYFIP2, which do not bind mRNA and link FMRP to the RhoGTPase pathway (Schenk et al., 2003). The interaction with these different proteins may influence the affinity of FMRP to mRNA, as it was shown for some FXR1P isoforms (Bechara et al., 2007).

Here, we have analyzed the expression pattern of FMRP and its interacting proteins FXR1P, FXR2P, CYFIP1, CYFIP2 and 82-FIP in the cortex, striatum, hippocampus and cerebellum of wild type (WT) mice at different ages with the aim of better defining the relationship of FMRP with its interacting proteins during

brain development.

2. MATERIALS AND METHODS

2.1 Experimental animals

We used brains of *Fmr1* knockout (KO) mice (FVB strain) and their WT littermates, which derive from a colony of *Fmr1* KO mice originally provided by Prof. Ben Oostra. Brains were taken from mice at postnatal (P) day P3, P7, P14, P23, P45 and adult mice (three month age-1 year-old). Day of birth was considered postnatal day 0 (P0). Different brain areas were dissected, immediately frozen in dry-ice and stored at -80°C until use. 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).

2.2 Primary Antibodies

The following primary antibodies were used for Western blotting: monoclonal anti-FMRP 1C3 (1:1000) (Devys et al., 1993), monoclonal anti-3FX (1:2000) (Dubè et al., 2000), polyclonal anti-FXR1 830 (Dubè et al., 2000) (1:12000), polyclonal anti-CYFIP1 (1:2000) (Schenck et al., 2001), polyclonal anti-CYFIP 2 (1:2000) (Schenck et al., 2001), and polyclonal anti 82-FIP (1:2000) (Bardoni et al., 2003), and were kindly provided by Dr Barbara Bardoni. Monoclonal anti-FXR2P clone A42 (1:2500) was purchased from Abcam. Polyclonal rabbit anti-tubulin or polyclonal rabbit anti-actin (1:1000, Cell Signaling) antibodies were used as a loading control.

For immunocytochemistry we used a polyclonal mouse anti-FMRP antibody (R65), which was raised against the C-terminus domain spanning aminoacids 516-632 of the longest isoform of human FMRP ISO1 (Adinolfi et al., 1999). The sequence of this domain is highly divergent from the corresponding C-terminal domains of the two paralogs of FMRP, namely FXR1P and FXR2P. The His-tagged FMRP C-

terminus was purified as described previously (Bechara et al., 2009) and used to produce polyclonal antibodies in mouse using standard protocol (Bardoni et al., 1999). The anti-FMRP IgG were then affinity purified from mouse R65 serum with the same fusion protein used for immunization as described previously (Bardoni et al., 1999). To assess the specifity of the R65 antibody, 20 □g of lysates from *Fmr1* KO fibroblasts (STEK) stably transfected with empty vector pTL1 or expressing pTL1-*FMR1* ISO isoforms1 [described in (Castets et al., 2005)] as well as 30 □g of brain extracts from *Fmr1* KO and WT mouse brain were loaded on a 10% SDS-PAGE. Proteins transferred onto a 0.45 □m nitro-cellulose membrane were revealed using the affinity-purified R65 antibody diluted 1:1000. No bands were detected in the *Fmr1* KO samples while FMRP was detected in the STEK expressing FMRP and in WT brain lysates, confirming that these antibodies specifically recognize FMRP in Western blotting (not shown).

2.3 Western blotting

Frozen brain areas from control and *Fmr1* KO mice were allowed to thaw on ice and homogenized in 10% (wt/vol) extraction buffer [40 mM TRIS–HCl pH 6.8, 20 µg/ml Leupeptin, 20 µg/ml Aprotinin, 0.1 mM phenyl-methyl-sulpho-fluoride (PMSF), 1 mM EDTA, 5 mM EGTA]. The homogenate was centrifuged for 10 min at 18000 g (4°C), the pellet was discarded and the supernatant was used. After quantification of proteins by using the bicinchoninic acid method (BCA kit, Pierce, Rockford, IL, USA) and their denaturating step, an equal amount of proteins (100 µg) were loaded onto 10% SDS–polyacrylamide gels. Gels were transferred to PVDF (Invitrogen) or nitrocellulose (Hybond C-extra 0,45 µm; Amersham Biosciences, Buckinghamshire, UK) membranes by using a transblot SD apparatus (Bio-Rad, Hercules, CA). Filters were blocked for 1h in 5% not fat dried milk in TTBS (Tris 100 mM, 0.9% NaCl, and 1% Tween 20) and then were incubated for 1h with primary antibodies.

After washing in TTBS buffer, filters were incubated for 1h with the anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Amersham, Buckinghamshire, UK). A chemiluminescent detection method (ECL

plus, Amersham, Buckinghamshire, UK) was carried out to develop filter signals.

2.4 Cell culture

Hippocampal cultures were prepared from WT and *Fmr1* KO P0-P1 newborn pups of litters obtained by mating a heterozygous female with a *Fmr1* KO male (FVB strain). All experiments have been performed without previous knowledge of the genotype of the culture being tested. The genotype of each pup was defined by PCR as previously described (Musumeci et al., 2007). Hippocampi from individual newborn pups were dissected, dissociated with Trypsin 0,25% and plated, at a density of ~ 30.000 cells per dish, onto 35 mm cell culture dishes (Nunc) coated with poly-L-ornithine (10 µg/ml, Sigma). Hippocampal cultures were plated in Neurobasal medium (GIBCO) supplemented with 2% B27 (GIBCO), 1% penicillin/streptomycin (GIBCO) and 0,5% Glutamax (GIBCO). Cytosine arabinofuranoside (Sigma) was added (5 µM) on days in vitro (DIV) 4 to prevent glial cell proliferation. Culture media were changed every 7 days.

2.5 Immunofluorescence staining

Once removed the medium, cells were fixed with 4% paraformaldeyde (PFA) and 4% sucrose for 15 min at room temperature (R.T.), washed and first permeabilized in PBS containing 0.2% Triton for 10 min and then incubated with blocking solution containing NGS 4% for 20 min at R.T. For double-labelling experiments, the cultures were incubated for 1h and 30 min at RT with the following primary antibodies: anti-FMRP (R65) (mouse, 1:500), anti-FXR1P (830) (rabbit, 1:5000) and anti-FXR2P (mouse, 1:2000, Sigma). We also used anti-MAP2 (rabbit, 1:1000, Millipore or mouse, 1:1000, Sternberg), anti-Tau1 (mouse, 1:150, Millipore) and anti-GFAP (rabbit, 1:500, Dako or mouse, 1:500, Millipore) antibodies. Afterwards, cultures were incubated for 45 min at RT with the appropriate secondary fluorescent antibody: Cy3 anti-mouse (1:500, Jackson Immunoresearch) or Cy3 anti-rabbit (1:500, Jackson Immunoresearch) and FITC DyLight488 anti-rabbit (1:300, Jackson Immunoresearch) or FITC anti-mouse (1:300, Jackson Immunoresearch). Specificity of the mouse monoclonal anti-FMRP antibody was

tested in cultures prepared from *Fmr1* KO litter, which did not exhibit any labeling (data not shown).

Imaging of hippocampal neurons. Images were obtained with the LSM-510 Meta confocal microscope (Zeiss) using a 63X lens.

2.6 Data analysis

Signal obtained from films of Western blotting experiments was quantified by computer-assisted densitometry, using the MCID system (Imaging Research, St. Catharines, Ontario, Canada). Images were visualized on a video monitor connected to the illuminator through a video camera. The integrated optical density (OD) was obtained by the software-operated conversion of absolute gray values in arbitrary OD units.

2.7 Statistical analysis

Statistical analysis was carried out using SIGMA STAT 3.1 software. All data were analyzed using multifactorial ANOVA followed appropriate post-hoc comparison test.

3. RESULTS

3.1 FMRP, FXR1P and FXR2P are differently expressed in mouse brain regions.

The regional distribution of FMRP, FXR1P and FXR2P was studied by examining their expression in different brain areas such as cerebellum, cortex, hippocampus, striatum, olfactory bulb, brain stem and spinal cord in 45 days old mice. **FMRP** was present in all examined areas, although at different expression levels (**Fig. 1**). In particular, higher levels of expression of FMRP were found in the cortex and olfactory bulb. Hippocampus, cerebellum and striatum showed moderate levels, while brain stem and spinal cord revealed less amount of protein.

FXR1P was studied with two different antibodies: the polyclonal anti-FXR1P

antibody (830) (Dubè et al., 2000), which identifies two isoforms of 78 and 80 kDa, and the polyclonal anti-3FX antibody which recognizes different FXR1P brain isoforms at 70, 74, 78 and 80 kDa and the FXR2P band at 92 kDa (Dubè et al., 2000). The regional distribution of FXR1P appeared comparable when studied with both antibodies, and similar but not identical to that of FMRP (**Fig. 1**). In general, FXR1P levels of expression were high in the cortex, hippocampus, striatum, olfactory bulb and brain stem. Instead, a lower signal was detected in the cerebellum and spinal cord. Interestingly, in some areas the 78 and 80 kDa isoforms of FXR1P are expressed differently. In particular, the 80 kDa isoform appeared absent or low expressed in the brain stem, spinal cord and cerebellum.

FXR2P distribution in the different examined areas mimicked that shown by FMRP. Indeed, FXR2P presented the highest levels of expression in the cortex and the olfactory bulb, while lowest levels were found in the spinal cord (**Fig. 1**). Identical results were obtained with the use of two antibodies (clone A42 and 3FX).

3.2 FMRP, FXR1P and FXR2P exhibited a similar pattern of expression decrement during development in different brain areas and cultured neurons.

The expression patterns of FMRP and its paralogs FXR1P and FXR2P during development was studied in the cortex, cerebellum, hippocampus and striatum (**Fig. 2** and **6**). The three FXR proteins were present at all ages and exhibited a characteristic profile of decrement, with high levels of expression during the first/second week of post-natal life and a progressive reduction thereafter. A semiquantitative analysis of signal in Western blots suggests that levels of FMRP and FXR2P decreased earlier in the cerebellum than in the cortex, while FXR1P profile of reduction is similar in both regions (**Fig. 2**). The expression of FMRP, FXR1P and FXR2P was also studied by immunocytochemistry in cultured hippocampal neurons at different DIV. Interestingly, the expression profile observed in cultured neurons was strikingly similar to that observed in brain tissue. All three proteins were indeed strongly expressed in neurons as early as 3 DIV, their expression was still high at 7 and 13 DIV but decreased at 20 DIV (**Fig. 3**).

FMRP, FXR1P and FXR2P were maximally expressed in neurons, whereas its expression was lower in astrocytes as confirmed by double-labelling experiments (**Fig. 4**). In cultured hippocampal neurons FMRP, FXR1P and FXR2P are particularly abundant in the soma, with lower but detectable levels in dendrites and axons as revealed by double labelling immunocytochemical experiments using anti-MAP2 and anti-tau antibodies, respectively (supplementary Figures S1 and S2).

3.3 CYFIP1, CYFIP2 and 82-FIP exhibit a different expression pattern than FXR proteins during development

The developmental expression profile of CYFIP1, CYFIP2 and 82-FIP was studied in the cortex and cerebellum. In both regions, these FMRP interacting proteins were detected at each post-natal age, but were characterized by a different pattern of expression than FXR proteins. Levels of CYFIP1 were high in the cortex as early as P3, reached maximum levels up to P23 and then slightly decreased in the adult; this developmental pattern of expression was also observed in the cerebellum. CYFIP2 also increased from P3 to P23 in both cortex and cerebellum, but in the cerebellum the levels of CYFIP2 were higher at P3-P7 than levels at same age in the cortex (**Fig. 5**). In contrast, 82-FIP exhibited an opposite developmental pattern in the cerebellum respect to the development profile showed in cortex. In fact, 82-FIP was strongly expressed in the early stages of post-natal development in the cerebellum and drastically decreased during the first week of post-natal life (**Fig. 5**). Instead, it was barely detectable during the first week in the cortex, increased progressively up to P23 and slightly decreased thereafter (**Fig. 5**). A pattern of expression similar to that observed in the cortex was observed in both the hippocampus and striatum (**Fig. 6**).

4. DISCUSSION

During the last decade several aspects of FMRP function have been elucidated,

however how FMRP works in the context of a complex with its interacting proteins is still unclear. Elucidating the expression pattern of FMRP interacting proteins during development may help to unravel this interesting biological problem and may contribute to a better understanding of the molecular bases of FXS. The main finding of this study is that the FMRP, FXR1P and FXR2P are highly expressed during the first two weeks of post-natal brain development and exhibit a similar pattern of expression decrement during development in different brain regions. In contrast, we found that other FMRP interacting proteins, such as CYFIP1, CYFIP2 and 82-FIP show a different developmental profile than FXR proteins. Furthermore, we have highlighted that, although present in all the brain regions examined, the relative amount of each FXR protein is different in distinct brain areas. Cortex and olfactory bulb appear to be the regions where all the three FXR proteins are maximally expressed, whilst spinal cord has lower levels of all three proteins. The distribution of FMRP in different brain areas of mouse brain revealed by our semi-quantitative analysis of Western blotting experiments is strikingly similar to that described in adult monkey (Zangenehpour et al., 2009). Interestingly, while FMRP and FXR2P exhibited a similar distribution pattern suggesting a similar relative abundance, FXR1P showed a more distinct expression profile, being more uniformly present in the regions of the forebrain compared to the other two proteins. In addition, comparison of the expression pattern of FXR1P with two different antibodies revealed that the 80 kDa isoform of FXR1P is present only in regions of the forebrain, but not in posterior regions of brain and in the spinal cord.

Most is known regarding the role of FMRP as major regulator of mRNA transport and translation, and key protein in mechanisms underlying synaptic plasticity both in adult and during development, whereas the role of FXR1P and FXR2P is far less studied. It is known that FXR proteins are able to homo- and hetero-oligomerize (Zhang et al., 1995), although not much is known about their stechiometry in the context of RNP complexes. Our results suggest a prominent role of all three FXR proteins in the first two weeks of post-natal development, a period characterized by active synaptogenesis and circuit formation in different brain areas. New findings

have showed that FXR1P, as FMRP, is also involved in local regulation of mRNA in dendrites and at synapses being colocalized with ribosomes and mRNA in hippocampal neurons during development (Cook et al., 2011). Furthermore, a prominent expression of FXR proteins has been detected in specialized microstructures defined granules in the axons at post-natal day 15 in both cortex and hippocampus (Christie et al., 2009). In these two regions the detection of these structures is drastically reduced at P30 and almost undetectable in the adult (P60 and P150) (Christie et al., 2009). Interestingly, the only region where FXR proteins containing granules are detected in the adult is the olfactory bulb, a region which is characterized by active synaptogenesis in the adult. These data are in line with our regional and developmental pattern of expression of FXR proteins and strongly suggested that FXR high expression during the first two weeks of age are related to synaptogenesis and circuit formation. In particular, it is possible that FXR proteins are abundantly expressed during the first two weeks of development in the axons and are important for the establishments of circuits during development, whereas localization in the dendritic/post-synaptic compartment may be maintained in the adult although at lower levels than during development.

We find a major abundance of FXR proteins in forebrain regions, namely cortex and olfactory bulb rather than more posterior regions such as cerebellum and spinal cord. Furthermore, the presence of the 80 kDa FXR1P isoform in forebrain regions might indicate a specific role of this protein in the function of distinct neuronal networks. It is also possible that a different combination of the three interacting proteins (and their isoforms) might give rise to different species of RNP complexes in an area-specific manner.

Despite the high levels of similarity in overall structure and common properties such as association with polyribosomes (Khandjian et al., 1996; Khandjian et al., 2004, Stefani et al., 2004; Darnell et al., 2009, Cook et al., 2011) and high expression in neurons (Devys et al., 1993; Tamanini et al., 1997; Cook et al., 2011), major evidence suggests that among FXR proteins FMRP has unique features that make it the prominent regulator of protein synthesis in neurons. Indeed, only human FMR1 gene, but not FXR1 and FXR2 genes, is able to rescue

molecular and cellular abnormalities in neurons of dfmr1 null mutants (Coffee et al., 2010). It is possible that in the brain the three proteins work in a cooperative way supporting and reinforcing the function of FMRP. While the longest FXR1P isoform, which is exclusively expressed in muscle, interacts specifically with a RNA sequence containing G-quadruplex structure, the shorter brain isoforms of FXR1P are not able to bind G-quadruplex RNA structures, but rather negatively regulate the affinity of FMRP for G-quadruplex RNA (Bechara et al., 2007; Davidovic, 2013). Thus, in certain tissues, each protein might be involved in a specific function on its own, while in others they may work together to similar activities, dependently on the pattern of expression and co-localization.

The expression pattern of FXR proteins during embryonic development has been previously described by others. These early studies highlighted a different expression pattern and levels for each protein at different developmental stages, suggesting a specific role for each member of the FXR family in different tissues (De Diego-Otero et al., 2000). In particular, these authors highlighted that FXR1P had a distinct developmental and distribution profile than that of FMRP and FXR2P, which instead exhibited a similar distribution in different tissues of mouse embryos. Accordingly, our data also suggest that FMRP and FXR2P share a more similar distribution pattern in different brain areas, while FXR1P exhibits a slightly different distribution pattern, which is characterized by a higher and more uniform expression in the regions of the forebrain. It has been proposed that the presence of FXR1P and FXR2P can partially compensate for the loss of FMRP in FXS. More data in support of a possible compensation of the FXS phenotype are available for FXR2P rather than FXR1P. Accordingly, *Fmr1* and *Fxr2* KO mice exhibit some similar behavioural abnormalities and *Fmr1/Fxr2* double KO have exaggerated behavioral phenotypes in locomotor activity, sensorimotor gating and cognitive processes, but not in anxiety-like behaviour, motor coordination and analgesic responses (Spencer et al., 2006). More recently, on the same line, it has been shown that *Fmr1* and *Fxr2* KO exhibit a similar shorter circadian rhythm, whereas double *Fmr1/Fxr2* KO have an exacerbated phenotype which is characterized by a loss of rhythmic activity (Zhang et al., 2008). In contrast, FMRP and FXR2P may

have distinct roles in the mechanisms of metabotropic glutamate receptor-induced Long Term Depression (LTD) (Zhang et al., 2009). Overall these data suggest that FXR2P can work in both a cooperative and separate manner with FMRP depending on the pathway involved in regulating a specific function/behaviour. The role of FXR1P in brain function is much less investigated because *Fxr1* KO mice are not viable, due to cardiac defects leading to premature death (Mientjes et al., 2004). FXR1P has been detected in neurons at dendritic spines where it is associated with ribosomes, suggesting a role in both mRNA transport and translation (Cook et al., 2011). Accordingly, a very recent paper using a conditional *Fxr1* KO reveals a role of FXR1P in Long Term Potentiation (LTP) and memory storage (Cook et al., 2014). Interestingly, FXR1P limits the synthesis and synaptic incorporation of the AMPA GluA2 subunit but this ability is not shared by FMRP or FXR2P, highlighting a specific role of FXR1P in synaptic plasticity (Cook et al., 2014).

An important step toward the understanding of the function of FMRP and the pathophysiology of FXS requires the study of other proteins able to interact directly with FMRP, such as CYFIP1 CYFIP2 and 82-FIP. CYFIP1 and CYFIP2, that are cytoskeleton associated proteins. Whereas CYFIP2 interacts with FMRP and FXR1P and FXR2P, CYFIP1 interacts only with FMRP. CYFIP1 and CYFIP2 are components of the WAVE complex that controls the actin cytoskeleton organization (Schenck et al., 2001, 2003), a process involved in the regulation of cell migration (Gautier et al., 2011), axonal polarity in neurons, cell adhesion, vesicle trafficking (Abekhoukh and Bardoni, 2014). CYFIP1 has been also shown to be involved in maturation and stabilization of dendritic spines (Oguro-Ando et al., 2014; Pathania et al., 2014). This is in line with our finding that CYFIP1 and CYFIP2 levels increase during the first three post-natal weeks an is maximal in the third week of post-natal development, a period characterized by stabilization of synaptic contacts by activity-dependent mechanisms. Importantly, *CYFIP1* is localized at the 15q11.2 a chromosomic region involved in intellectual disability, autism and other neurodevelopmental disorders (reviewed by Abekhoukh and Bardoni, 2014).

The role of the interaction between FMRP and CYFIP1/2 during development is

unknown. Interestingly, the domain of CYFIP1/2 interaction with FMRP is the same that mediates homo/heterodimerization of the FXR family (Schenk et al., 2001; Zhang et al., 1995), suggesting that CYFIP1/2 can modulate the interaction between FMRP and its paralogs. Our finding that FXR proteins and CYFIP1/2 express a different developmental pattern of expression implicates that the ability of CYFIP1/2 to modulate homo/heterodimerization of the FXR proteins changes during development with important implication for selection of FMRP target RNAs and their translation during development.

Another interesting finding of our study is the divergent pattern of expression of 82-FIP (also known as NUFIP-2) in the cerebellum and forebrain regions. 82-FIP is a nuclear and cytoplasmic partner of FMRP. 82-FIP interacts with FMRP and not with FXR1P and FXR2P, is associated to polyribosomes and exhibits an overlapping distribution with FMRP (Bardoni et al., 2003). Since not much is currently known concerning the function of these proteins in the brain, we cannot speculate on the functional significance of our finding. However it is known that, unique among the FMRP interactors, 82-FIP exhibits a cytoplasmic/nuclear distribution which is dependent of cell cycle phases. Thus, it is tempting to speculate that the high expression of 82-FIP at early phases in the cerebellum might be related to a specific function of this protein during cell cycle, which at this age occurs at the high rate for granule cells production.

4.1. CONCLUSIONS

All together, our study adds interesting information to the current knowledge of the expression of FXR proteins and FMRP interactors during development of the central nervous system. In particular the divergent pattern of expression of FXR proteins and CYFIP1/2 proteins suggests a different composition of the mRNP containing FMRP during post-natal life, and might indicate that the ability of FMRP to affect RNA metabolism and cytoskeleton reorganization are differently modulated during development.

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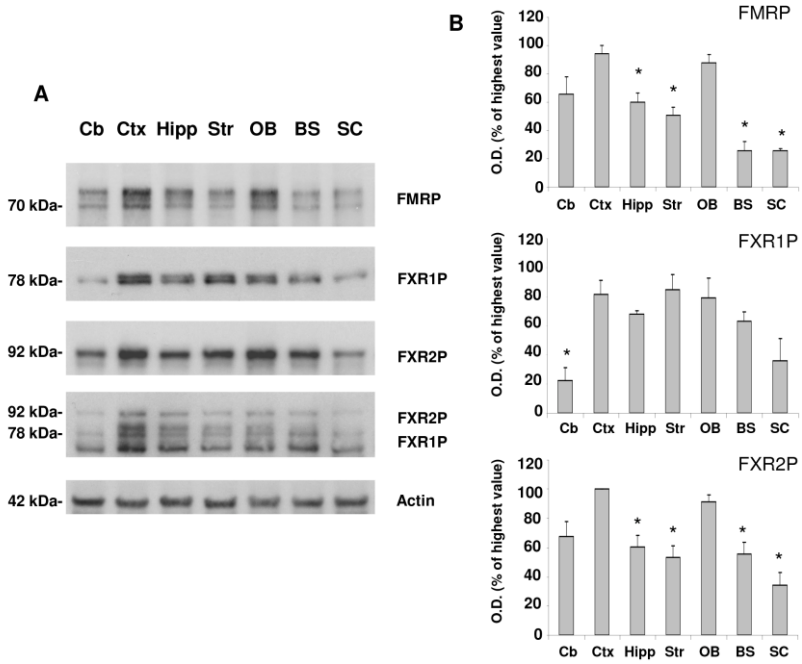

Figure. 1. Expression of FMRP, FXR1P and FXR2P in different mouse brain areas. (A) Representative Western blots of protein extracts from cerebellum (Cb), cortex (Ctx), hippocampus (Hipp), striatum (Str), olfactory bulb (OB), brain stem (BS) and spinal cord (SP) of mice at post natal day 45 carried out using the anti-FMRP (1C3), anti-FXR1P (830), anti-FXR2P (Clone A42), anti-FXR1P/FXR2P (3FX), anti-actin antibodies (from top to bottom). Equal amounts of proteins (60 μg) were loaded. (B) Graphs show semi-quantitative analysis of Western blots. Optical density (O.D.) of bands is presented as a percentage of the highest value. Data represent mean ± SEM of four experiments. *$p<0.05$ versus cortex by one-way ANOVA followed by Holm-Sidak method.

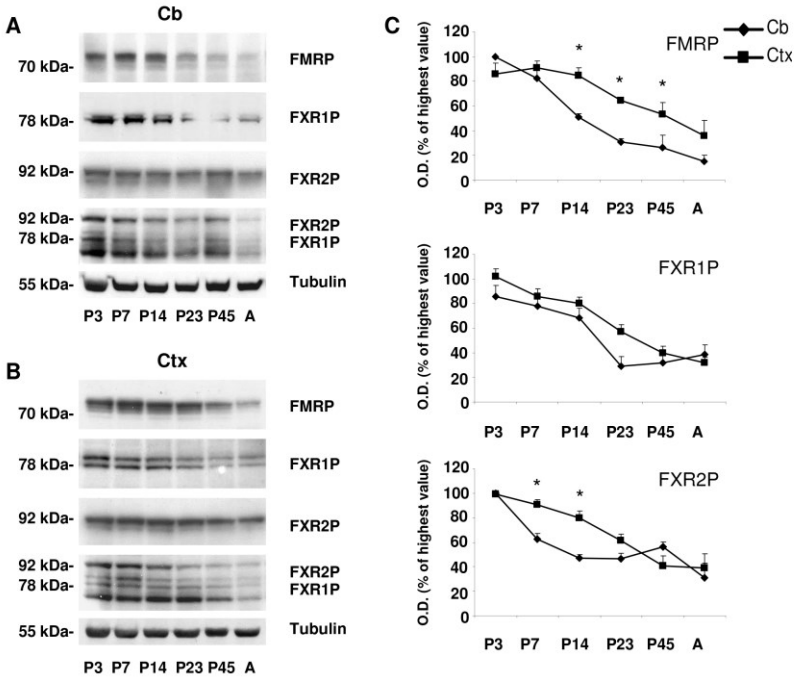

Figure 2. Expression pattern of FMRP, FXR1P and FXR2P in mouse cerebellum (Cb) and cortex (Ctx) during development. (A, B) Representative Western blots of protein extracts from cerebellum (A) and cortex (B) at post natal day (P) 3, 7, 14, 23, 45, adult (A). Equal amounts of proteins (100 µg) were loaded. (C) Graphs show semi-quantitative analysis of Western blots. Optical density (O.D.) of bands is presented as a percentage of the highest value. Data are mean ±SEM of four experiments. *$p<0.05$ between Ctx and Cb at indicated post-natal day by two-way ANOVA followed by Holm-Sidak method.

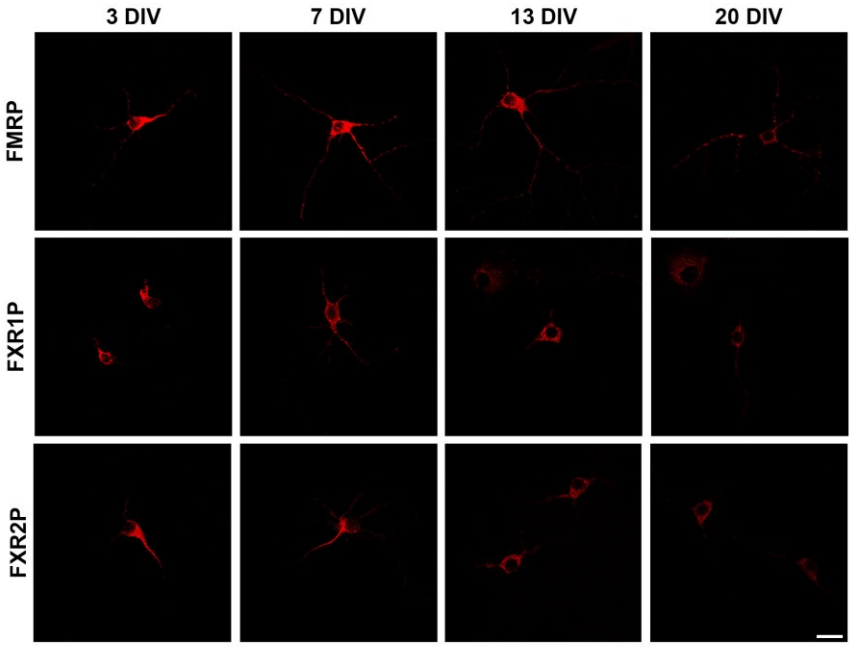

Figure 3. The expression of FXR proteins was high in cultured hippocampal neurons up to 13 DIV and decreased substantially at 20 DIV. The panel shows representative images of cultured hippocampal neurons from WT mice at different days in vitro (DIV, 3, 7, 13, 20) stained with anti-FMRP, anti-FXR1P and anti-FXR2P antibodies. Scale bar 20 μm.

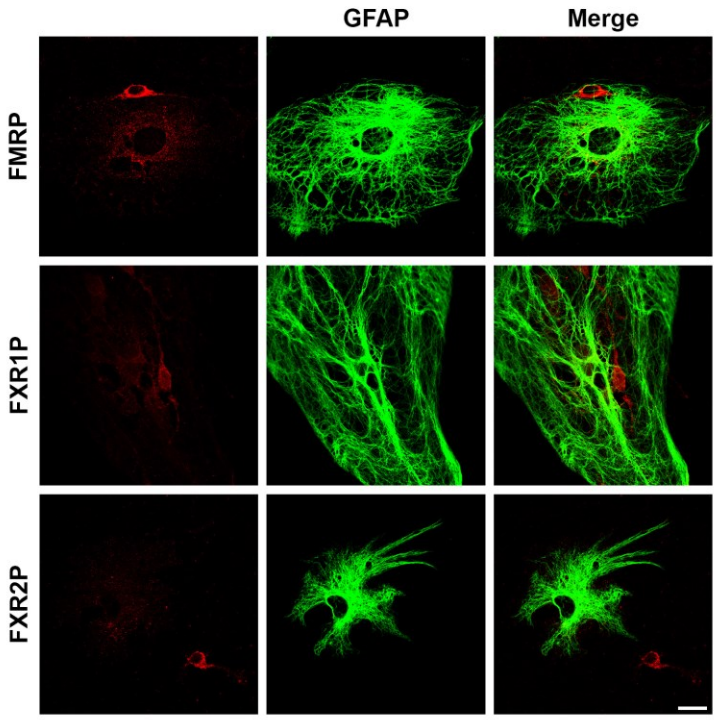

Figure 4. The expression of FXR proteins is higher in neurons than astrocytes. The panel shows representative images of cultured hippocampal neurons from WT mice at 7 DIV double-stained with the primary antibodies anti-FMRP, anti-FXR1P, anti-FXR2P and anti-GFAP. Scale bar 20 μm.

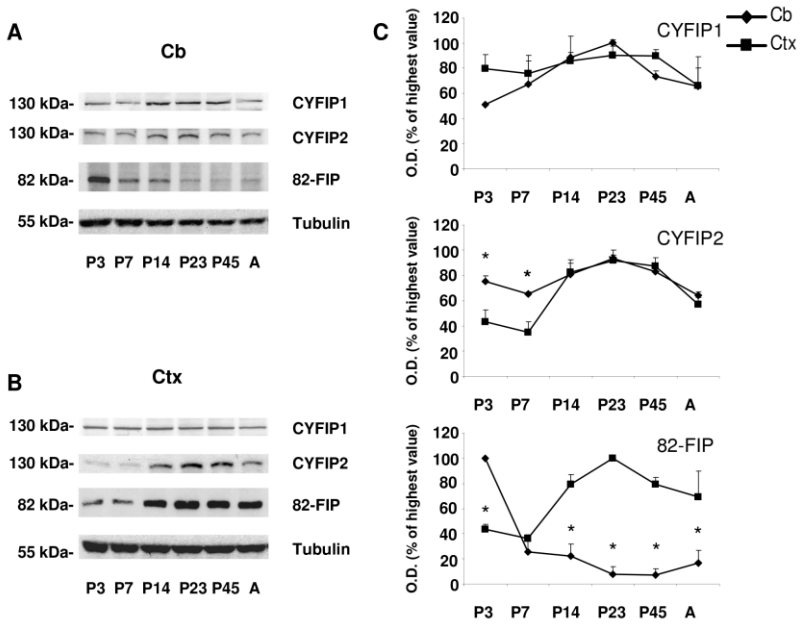

Figure 5. Expression pattern of CYFIP1, CYFIP2, 82-FIP in mouse cerebellum (Cb) and cortex (Ctx) at different ages. (A, B): Representative Western blots of protein extracts from cerebellum (A) and cortex (B) at post natal day (P) 3, 7, 14, 23, 45, adult (A). Equal amounts of proteins (100 μg) were loaded. (C) Graphs show semi-quantitative analysis of Western blots. Optical density (O.D.) of bands is presented as a percentage of the highest value. Data are mean ±SEM of four experiments. *$p<0.05$ between Ctx and Cb at indicated post-natal day by two-way ANOVA followed by Holm-Sidak method.

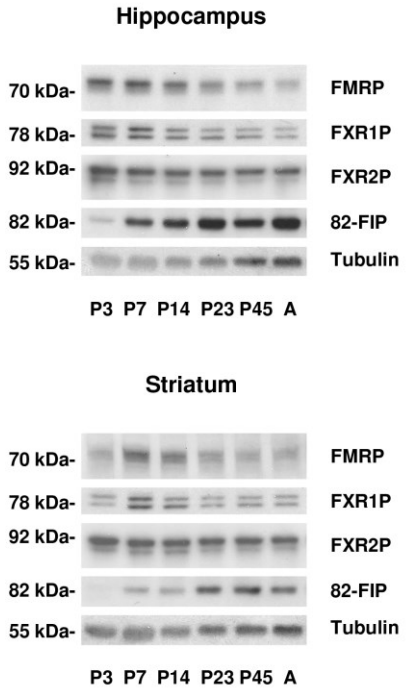

Figure 6. Western blots showing the expression pattern of FMRP and FMRP-interacting proteins in mouse hippocampus and striatum during development. Hippocampus and striatum were taken at different ages of FVB mice strain. 100 µg of proteins extracts were loaded.

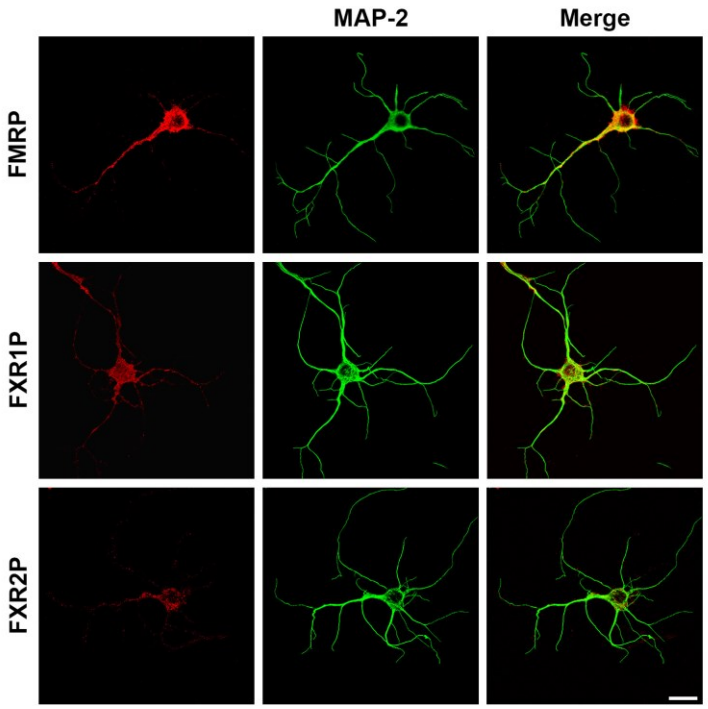

Figure. S1. The panel shows representative images of cultured hippocampal neurons from WT mice at 7 DIV double-stained with the primary antibodies anti-FMRP, anti-FXR1P, anti-FXR2P and anti-MAP-2. Scale bar 20 μm.

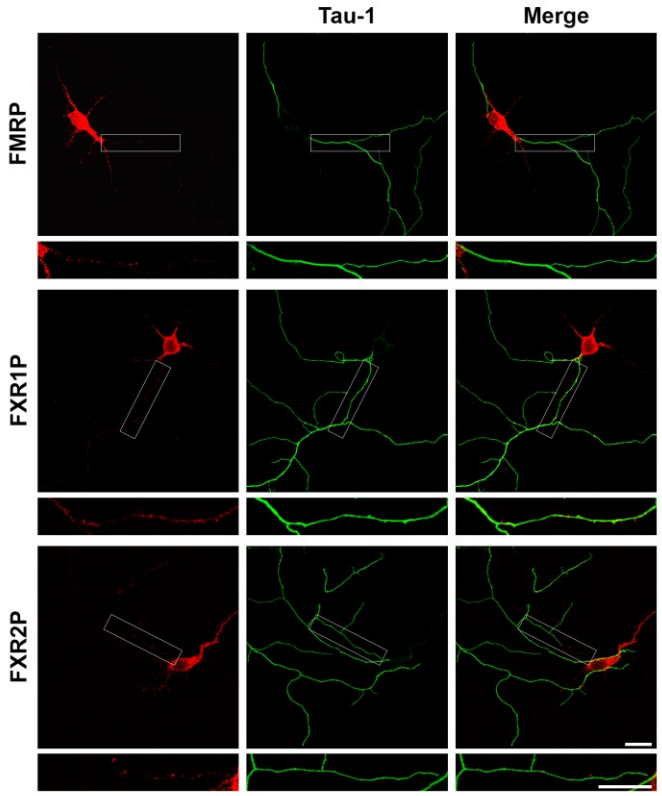

Figure S2. The panel shows representative images of cultured hippocampal neurons from WT mice at 7 DIV double-stained with the primary antibodies anti-FMRP, anti-FXR1P, anti-FXR2P and anti-Tau-1. Scale bar 20 μm. Boxed regions are shown magnified in insets. Scale bar 10 μm.

CHAPTER II

Group-I metabotropic glutamate receptor activation reduces stress granules formation via a mechanism mediated by FMRP

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Key words: Fragile X, FMRP, FXR1P, FXR2P, SGs, mGlu5 receptor, astrocytes

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Abstract

Fragile X Syndrome (FXS), a common form of inherited intellectual disability and autism, is caused by the lack of Fragile X Mental Retardation Protein (FMRP), an RNA binding protein involved in RNA transport and protein synthesis. Upon cellular stress, canonical protein synthesis is blocked and mRNAs are recruited into stress granules (SGs), ribonucleoproteic structures containing translation components and RNA binding proteins. Activation of group-I metabotropic glutamate (mGlu) receptors stimulates FMRP-mediated mRNA transport and protein synthesis, but their role in SGs formation is unknown. To investigate the effect of mGlu receptors activation on SGs, wild type (WT) and *Fmr1* knockout (KO) astrocytes were treated with the group-I-mGlu receptor agonist, DHPG, and then exposed to sodium arsenite ($NaAsO_2$). Immunocytochemistry for TIA-1 protein, a marker of SGs, revealed a lower number of SGs in *Fmr1* KO than WT astrocytes after stress. In WT cultures activation of mGlu receptors reduced SGs formation, TIA-1/FMRP co-localization, and phosphorylation of eIF2α and FMRP, whilst it did not lead to any of these in *Fmr1* KO astrocytes. Phosphorylation of eIF2α and FMRP are crucial key events in SGs formation and modulation of protein synthesis, respectively. Thus, mGlu receptors may act by shifting the balance from inhibition to activation of protein synthesis during stress.

Introduction

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID) and a leading cause of autism. In addition to a moderate to severe cognitive impairment, increased susceptibility to epilepsy, hyperactivity and altered pain sensitivity are frequently associated to FXS (Dutch-Belgian Fragile X Consortium, 1994). In most cases, FXS is caused by the amplification of CGG trinucleotide repeat in the promoter region of the *FMR1* gene, a mutation that ultimately leads to transcriptional silencing of the *FMR1* gene and ensuing lack of the *FMR1* encoded protein FMRP (**F**ragile X **M**ental **R**etardation **P**rotein) (Devys et al., 1993; O'Donnel and Warren, 2002). FMRP is an RNA binding protein involved in the regulation of target mRNA transport and translation. FMRP acts mainly as a negative regulator of translation, although the underlying mechanisms have been only partially unravelled. FMRP is also implicated in mRNAs transport along dendrites and axons being a component of RNA granules, the mRNPs (messenger ribonucleoprotein particles) that escort mRNAs in repressed conditions from soma to synapses where mRNAs are delivered to be translated upon specific signals. The absence of FMRP causes abnormal distribution of its mRNAs cargos (Miyashiro et al., 2003). FMRP interacts with two close paralogs, FXR1P and FXR2P (Fragile X Related Protein 1/2) although the significance of this interaction is not clear (reviewed by Bardoni et al., 2006). FMRP, FXR1P and FXR2P show similar structure, which suggests that they may play a similar role but this aspect is also poorly understood.

A new aspect of FMRP function in the cytoplasm can be related to its presence in peculiar structures called Stress Granules (SGs), cytoplasmic aggregates that are formed only under stress conditions, such as exposure to heat, oxidative agents, UV irradiation, where mRNAs are recruited and protected during stress (Anderson and Kedersha, 2002). FMRP has been found to be associated with the pool of mRNAs that go into SGs upon cellular stress and can be involved in the inhibition of protein synthesis occurring during stress (Mazroui et al., 2002). Lack of FMRP impairs SGs formation but the function of FMRP in SGs is not clear (Didiot et al.,

2009). During SGs formation an initial event is the phosphorylation of a translation initiation factor eIF2α, which, once phosphorylated, is no longer available for the canonical protein synthesis and makes up the core of these structures (Kedersha et al., 1999). After this initial event a number of RNA binding proteins, including TIA-1, TIA1R and FMRP, are recruited to SGs (Wolozin et al., 2012). After an environmental stress, mRNAs stored in this abortive translation initiation complex can be routed to either translation initiation or degradation (Buchan and Parker, 2009). The link between SGs formation and cell survival after stress is unclear; a few studies suggest that SGs may play a protective role against cell death (Arimoto et al., 2008; Eisinger-Mathason et al., 2008), but a causal relationship between SGs formation and cell survival under stress condition is lacking. On the other hand, mutations in SG-associated RNA binding proteins and abnormal formation of inclusions containing SGs associated proteins are frequent in neurodegenerative diseases, suggesting a role for SGs in neurodegeneration (reviewed by Wolozin, 2012).

The role of group-I metabotropic glutamate (mGlu) receptors, namely mGlu5, in several FMRP-mediated functions such as mRNPs transport and protein synthesis is established (Dictenberg et al., 2008; Bear et al., 2004; Huber et al., 2002). The interplay between FMRP and mGlu receptors has been observed in neuronal cultures where the activation of group-I mGlu5 receptors increases the rapid translation of pre-existing mRNAs, including the FMRP mRNA (Weiler et al., 1997, 2004). Furthermore, the activation of group-I mGlu receptors is necessary for FMRP trafficking from the cell body into dendrites, but reduces FMRP localization at synapses (Antar, 2004). The role of this receptor in modulating cell death in several experimental paradigms has been previously identified (Bruno et al., 2001; Nicoletti et al., 1999), but its involvement in the formation of SGs has never been investigated.

In this study we investigated the effect of mGlu5 receptor activation on SGs formation in cultured astrocytes from wild type (WT) and *Fmr1* knock out (KO) mice. We found that activation of mGlu5 receptors reduced the formation of SGs in WT astrocytes only and thus, we examined a possible link between these effects

and mechanisms related to activation of protein synthesis.

Results

FMRP and its paralogs are expressed in astrocytes

FMRP and its paralogs FXR1P and FXR2P are expressed in primary cultured astrocytes as revealed by Western blotting analysis (Fig. 1). Levels of FMRP, FXR1P and FXR2P in cultured astrocytes were lower than levels present in cortical lysates at P7 (data not shown). No substantial difference was detected in levels of both FXR1P and FXR2P between WT and *Fmr1* KO astrocytes either grown in the presence of serum or shifted in serum-free media for 16 hours. Immunocytochemistry revealed that FMRP was mainly expressed in the cytoplasm and localized in fine puncta distributed in the perinuclear region of the cells (Fig.6). A similar distribution was observed for FXR1P and FXR2P (Fig. S1, S2).

Fmr1 KO astrocytes show less SGs than WT astrocytes.

In order to induce SGs formation, WT and *Fmr1* KO astrocytes were exposed to different stressors such as sodium arsenite ($NaAsO_2$, 200 μM for 60 min), hydrogen peroxide (H_2O_2, 500 μM for 60 min) and heat (43°C for 60 min). SGs were studied by means of immunocytochemistry aimed at revealing the **T** cell **i**nternal **a**ntigen **1** (TIA-1) protein, a known marker of SGs; TIA-1 protein has a nuclear localization under control condition, whereas under stress condition it translocates in the cytoplasm and takes part in SGs formation (Kedersha et al., 1999). As expected, exposure to both oxidative stress and heat significantly increased the formation of SGs in both WT and *Fmr1* KO cultured astrocytes as revealed by the increased the number of cells bearing TIA-1^+ SGs (Fig. 2). However, we detected a significantly lower number of cells with SGs both under basal condition and after exposure to stress in *Fmr1* KO than WT astrocytes (Fig. 2). Furthermore, *Fmr1* KO show less number of SGs per cell than WT astrocytes

(Fig. 3C, 3E). SGs formation was observed in astrocytes grown both in the presence of serum (Fig. 2B) or grown in the presence of serum and then shifted to serum–free medium for 16 hours before exposure to stress (Fig. 2C). The percentage of cells exhibiting SGs was slightly higher in the absence of serum; however the percentage of *Fmr1* KO astrocytes bearing SGs was significantly lower than WT astrocytes in both conditions. Subsequent experiments were carried out in serum-deprived cell cultures.

Activation of mGlu5 receptors before exposure to stress reduces SGs in WT but not in *Fmr1* KO astrocytes .

mGlu5 receptors are known to be expressed in astrocytes and their activation regulates several functions in this cell type, such as release of gliotransmitters and glutamate transport under both physiological and pathological conditions (D'Antoni et al., 2008). We confirmed that mGlu5 receptor is expressed in cultured primary astrocytes; levels were very low as compared with that of cortex at post-natal day 7, as expected (Ciccarelli et al., 1997; Janssens and Lesage, 2001) and comparable in WT and *Fmr1* KO mice (Fig. 3A).

Activation of mGlu5 receptors with the group-I mGlu receptor agonist (*S*)-3,5-Dihydroxyphenylglycine (DHPG, 100 μM for 5 min) before exposure to $NaAsO_2$ (200 μM for 60 min; Fig. 3B, 3C, or 500 μM for 30 minutes and 90 minutes, not shown) induced a significant reduction in the number of SGs per cells and percentage of cells showing SGs in WT (Fig. 3B, 3C, 4A), but had no effect in *Fmr1* KO astrocytes (Fig. 3D, 3E, 4B). The DHPG-induced reduction of cells bearing SGs observed in WT cultures treated for 5 minutes with DHPG was antagonised by the highly selective non competitive antagonist, 2-methyl-6-(phenylethynyl)pyridine (MPEP, 3 μM) (Fig. 4A). Conversely, the exposure to MPEP alone before stress induction did not affect SGs formation, while in *Fmr1* KO cultures increased the percentage of astrocytes with SGs to levels similar to those exhibited by WT cultures (Fig. 4B). This result suggests that an increased constitutive activity of mGlu5 receptor might be responsible for a lower number of astrocytes with SGs in *Fmr1* KO cultures.

To get an insight into the mechanisms underlying the effect of mGlu5 receptor activation on the modulation of SGs formation, stress was induced in cells treated with the protein synthesis inhibitors puromycin and cycloheximide (CHX), which act by destabilizing polysomes and freezing ribosomes on translating mRNAs, respectively. In line with published results (Kedersha et al., 2000) puromycin induced a significant increase of cells bearing SGs, whereas CHX inhibited SGs formation in both WT and *Fmr1* KO astrocytes (Fig. 4A, 4B). Interestingly exposure to DHPG for 5 minutes reduced the number of cells with SGs also in puromycin-treated WT cells (Fig. 4A), but had no effect in *Fmr1* KO astrocytes (Fig. 4B). Since polysomes and SGs are in dynamic equilibrium, this result suggests that DHPG may reduce SGs formation by shifting the equilibrium towards polyribosome formation.

Activation of mGlu5 receptors reduces phosphorylation of translation initiation factor eIF2α in WT but not in *Fmr1* KO astrocytes.

Since the stress-induced phosphorylation of eIF2α factor is a key player in SGs formation, we tested if mGlu5 receptor activation modulates eIF2α phosphorylation during stress.

Western Blot analysis showed that eIF2α was highly phosphorylated in stress condition, as expected, in both WT and *Fmr1* KO astrocytes. We observed a reduced eIF2α phosphorylation after mGlu5 receptor activation in WT, but not in *Fmr1* KO astrocytes. (Fig. 5).

Activation of mGlu5 receptors reduces phosphorylation of FMRP and its recruitment in SGs.

Phosphorylation/de-phosphorylation state of FMRP is modulated by activation of mGlu receptors and regulates the association of FMRP to actively translating polyribosomes (see discussion). Activation of group-I mGlu receptors reduced levels of phosphorylated FMRP, both under control and stressed conditions (Fig. 6A).

Double-labelling immunocytochemistry and confocal microscopy revealed a

remarkable co-localization of FMRP and TIA-1 protein in WT astrocytes exposed to $NaAsO_2$ (Fig. 6B) indicating that FMRP is recruited in SGs as observed in other cell types (Mazroui et al., 2002, Didiot et al., 2009). Astrocytes exposed to DHPG (100 μM, 5 minutes) before $NaAsO_2$ showed a lower degree of TIA-1/FMRP co-localization than cell exposed to $NaAsO_2$ only. Furthermore, FMRP and TIA-1 signal appeared more diffuse throughout the cytoplasm in DHPG-treated stressed cells compared to untreated stressed cells (Fig. 6B). Similar results were obtained for FXR1P and FXR2P (Fig. S1 and S2). We noticed a high degree of co-localization of both FXR1P and FXR2P with the protein TIA-1 in WT cultures treated with $NaAsO_2$, while co-localization was less evident when stress was induced after activation of mGlu5 receptors (Fig. S1 and S2). Interestingly, in *Fmr1* KO cultures the co-localization of FXR1P and FXR2P with TIA-1 was reduced compared to WT astrocytes, whereas no change in FXR1P and FXR2P co-localization with TIA-1 in response to DHPG before $NaAsO_2$ was detected (Fig. S1, S2). This last result suggests that upon mGlu5 receptor activation, SGs formation is impaired and FXR proteins are not entirely recruited in SGs (Fig. 6B, S1, S2). Interestingly FMRP seems to be crucial for SGs formation and for the recruitment of FXR1P and FXR2P in SGs.

Discussion

In this study we investigated SGs formation in WT and *Fmr1* KO astrocytes and its modulation of SGs formation after activation of mGlu5 receptors. Astroglial cell cultures represented a perfect *in vitro* model to assess SGs formation because SGs were clearly visible in astrocytes after stress treatment and expressed both FMRP and related proteins FXR1P and FXR2P and mGlu5 receptors. The importance of glial cells in Central Nervous System (CNS) function is crucial and is not only due to their role in supporting the neurons, but astrocytes are directly involved in different neurodegenerative diseases and brain trauma (Bradley et al., 2012). The role of FMRP has been investigated in neurons, but emerging evidence suggests

that it can have a role also in astrocytes, supporting neuronal morphology.

Previous studies suggest that FMRP is among the RNA-binding proteins involved in SGs formation (reviewed in Wolozin, 2012) and, importantly for human pathology, that the lack of FMRP impairs SGs formation (Didiot et al., 2009). Here we showed that *Fmr1* KO astrocytes fail to form an adequate number of SGs confirming that FMRP is indeed involved in SGs formation in a cell type that is relevant for brain function and FXS.

mGlu5 receptors regulate translation and play a key role in pathophysiology of FXS. We investigated SGs appearance upon activation of group-I mGlu receptors. It has been shown that mGlu5 receptor and FMRP regulate translation of mRNAs at the synapse in a functionally opponent manner; where group-I mGlu receptors activate protein synthesis, FMRP suppresses it. In the absence of FMRP, like in FXS, mGlu5-dependent protein synthesis is unchecked, with consequent excessive translation (Dolen and Bear, 2008). On the basis of this knowledge, we hypothesized that activation of mGlu5 receptors might modulate SGs formation though a mechanism involving protein synthesis. During stress, protein synthesis is blocked and mRNAs recruitment in SGs occurs to preserve mRNAs from stress. When mGlu5 receptors are activated, they may create an imbalance in the relationship between SGs and protein synthesis, by favoring the latter. Our data support this hypothesis, because mGlu5 receptor activation before stress reduces SGs formation in WT astrocytes, but didn't further reduce SGs formation in *Fmr1* KO astrocytes. DHPG-induced reduction of SGs in WT was antagonised using the mGlu5 antagonist, MPEP, clearly indicating the involvement of mGlu5 receptors; interestingly, MPEP treatment in *Fmr1* KO cultures restored the ability of *Fmr1* KO cultures to form SGs to levels similar to those observed in WT cells. It is established that the absence of FMRP leads to a constitutive increased rate of protein synthesis (Qin et al., 2005; Dolen et al., 2007), which is antagonized by pharmacological blockade and genetic deletion of mGlu5 receptors (Dolen et al., 2007). In the light of these studies, our results suggest that an excessive constitutive activity of mGlu5 receptors leading to an increased rate of mRNA translation might facilitate mRNAs recruitment in polyribosomes and thus impair

SGs. To deepen the relationship between mGlu5 receptor, SGs and translation, we performed treatments with two different pharmacological inhibitors of protein translation: cycloheximide and puromycin. CHX stabilizes polysomes by freezing ribosomes on translating mRNAs, inhibits the assembly of SGs and actively dissolves pre-existing SGs. Conversely, puromycin destabilizes polysomes by releasing ribosomes from mRNA transcripts and promotes the assembly of SGs. The antagonistic effects of CHX and puromycin on SGs assembly have revealed that SGs-associated mRNA is in a dynamic equilibrium with polyribosomes (Kedersha et al., 2000). We have seen that even in the presence of these protein synthesis inhibitors, mGlu5 receptors activation is able to reduce SGs formation altering the balance "SGs-protein synthesis". Puromicyn added in stress condition caused a considerable increase in the number of cells with SGs in both genotypes, which was seen drastically reduced by pre-treatment with DHPG in WT astrocytes (Fig. 4A) but not in *Fmr1* KO (Fig. 4B). The deficit in puromycin-induced SGs reinforces the role of mGlu5 receptors in enhancing protein synthesis in stress conditions, although the latter should be blocked. For this reason we wondered whether eIF2α phosphorylation level was involved in this mechanism. In stressed cells, activation of one or more eIF2α kinases (PKR, PERK/PEK, GCN2, HRI) results in the phosphorylation of eIF2α, an essential subunit of the eIF2-GTP-$tRNA^{Met}$ ternary complex required to initiate protein synthesis (Kedersha et al., 2000). Phosphorylation of eIF2α inhibits translational initiation and promotes SGs assembly (Kedersha et al., 1999; Anderson and Kedersha, 2002). Once phosphorylated eIF2α is no longer available to the canonical assembly of the translation initiation complex, and takes part in abnormal complex 48S containing mRNAs that were destined for the translation. Furthermore, this complex is bound by various RNA binding proteins, such as TIA-1 and FMRP proteins (reviewed by Wolozin, 2012). There is general consensus that in stress condition protein synthesis is blocked and that phosphorylation levels of eIF2α are increased (Anderson and Kedersha, 2002). We have seen that DHPG treatment reduces the number of cells with SGs and a reduction of eIF2α phosphorylation was observed after DHPG pre-treatment. Remarkably, this result suggests an important

involvement of mGlu5 receptors in the modulation of "SGs-protein synthesis" balance. Indeed this reduced eIF2α phosphorylation indicates that under mGlu5 receptors activation eIF2α does not entirely partecipate in SGs formation but, at least in part, it could be engaged in polyribosomes even though is abnormal in stress condition. Also we investigated the phosphorylated form of FMRP under these conditions. It is known that when FMRP is associated with its mRNAs is in the phosphorylated form. The activation of mGlu5 receptors leads to activation of protein phosphatase 2A (PP2A) that can rapidly dephosphorylate FMRP. Dephosphorylated FMRP leads to an increased level of translation (Bassel and Warren, 2008; Narayanan et al., 2007). On the other hand, we showed here that mGlu5 receptor activation reduces recruitment of FMRP in TIA-1^+ SGs. Upon mGlu5 receptor activation, we observed a few SGs and FMRP appears to be more distributed in the cytoplasm, probably due to a its partial recruitment in SGs.

In conclusion our data suggest that mGlu5 receptors activation reduces SGs formation in the absence of FMRP by shifting the balance from inhibition to activation of protein synthesis during stress and contributing to altered protein synthesis observed in this disorder.

To our knowledge this is the first indication that a receptor for a neurotransmitter can modulate SGs formation. Our finding adds important information to a complex biological problem involved in the mechanisms of cellular response to stress and may have important implication for FXS pathophysiology. Recent data suggest that oxidative stress may play a role in FXS (El Bekay et al., 2007; Bechara et al., 2009). A reduced number of SGs may implicate that RNAs are less protected during stress in FXS, ultimately leading to RNA damage and degradation. Future studies may clarify whether this mechanism may further contribute to altered protein expression in FXS, especially under stress condition.

Materials and Methods

Astroglial cell cultures

Primary astroglial cultures were prepared from cortex of P0-P1 newborn pups of litters obtained from WT or *Fmr1* KO mice (FVB strain). All experiments were conducted in compliance with the European Council Directive (86/609/EEC) and Italian Animal Welfare Act for the use and care of laboratory animals. All experiments were performed without previous knowledge of the genotype of the culture being tested. The mice genotype was defined by PCR (Musumeci et al., 2007). Cortices were dissected at stereomicroscope (STEMI DV4 ZEISS) and tissue obtained were minced in small pieces into nutrient medium. The basal nutrient medium consisted of Dulbecco modified Eagle's medium (DMEM, Sigma), containing 10% heat inactivated fetal bovine serum (FBS, Sigma), 1% penicillin and streptomycin. Cells were seeded into plastic flasks of 25 cm^2 at a plating density of 0.5 x 10^5 cells/ cm^2 (one hemisphere/flask). Cultures were incubated at 37°C in a humidified 5% CO_2 / 95% air atmosphere. After 10-12 days cultures were treated with 5 mM leucin methyl ester (Sigma) and shaken (for 10-12 hours, 180 rpm) to purify cell cultures from oligodendrocytes and microglia. Subsequently purification 35 mm Ø dishes were prepared with 2000 cells/ dishes to perform immunocytochemistry. To evaluate specific proteins by Western Blot analysis we seeded cultures onto 100 mm Ø dish at density of $8x10^5$ cells/dish.

Treatments

To induce oxidative stress astrocytes were treated with different concentrations of sodium arsenite ($NaAsO_2$, Carlo Erba, 200 or 500 µM) or with hydrogen peroxide (H_2O_2, Fluka, 500 µM) for different times at 37°C in a humidified 5% CO_2 / 95% air atmosphere. To induce heat shock cells were maintained at 43°C for 1 hour in a humidified mix 5% CO_2 air atmosphere. A pre-treatment with agonist (S)-3,5-Dihydroxyphenylglycine (DHPG, 100 µM, Tocris) and/or antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP, 3 µM, Tocris) of group-I mGlu5 receptors was carried out before the exposure to stress-inducing agents. We also used Cicloexhimide (30µg/ml, TOCRIS) or Puromycin (20µg/ml, TOCRIS) like inhibitors of protein synthesis.

Immunocytochemistry

The appearance of SGs was studied by using antibody which recognizes the C-terminus of the TIA-1 protein. After treatments, cultures were fixed with 4% paraformaldeyde (PFA) for 15 min at R.T. followed by additional 10 min with cold methanol. Then, cultures were permeabilized in PBS containing 0.2% Triton for 10 min, incubated for 20 min at R.T. with blocking solution containing 4% normal donkey serum (NDS) or normal goat serum (NGS) and subsequently with polyclonal antibody anti-TIA-1 (goat, 1:250, Santa Cruz Biotechnology) for 2 hour at R.T. The expression of FXR proteins in astrocytes was studied by double-labelling immunocytochemistry using the following primary antibodies: anti-FMRP (rabbit 1:50, Cell Signaling), anti-FXR1P 830 (rabbit, kind gift of Dr B. Bardoni, University of Nice-France) and anti-FXR2P (mouse 1:1500, Abcam). After washing, cultures were incubated with correspondent fluorescent secondary antibodies. Images were obtained with the Axio Imager. D2 (Zeiss) or LSM-510 Meta Confocal microscopes (Zeiss) and analyzed using the AxioVision Imaging System and the ImageJ software to quantified SGs formation and SGs protein co-localization.

Western Blotting

Astroglial cell cultures (at 80% of confluence) after treatments were harvested by scraping them on ice. Cells were homogenate in lysis buffer (Tris-HCl 40mM pH 6,8, 1X Protease Inhibitor Cocktail-Roche, 1X Phosphatase Inhibitor Cocktail-Roche, 100 μM PMSF, 1mM EDTA, 5mM EGTA and 2% SDS), centrifuged for 10 min at 1000g (4°C) to separate the nuclear pellet and cellular debris. Protein concentration was determined by using the bicinchoninic acid method (BCA kit; Pierce Rockford, IL). Then proteins were denaturated and loaded onto 8% SDS-polyacrylamide gels. Gels were electroblotted to supported nitrocellulose membranes (Amersham Biosciences). Filtres were blocked for 30 min and incubated O/N with the following primary antibodies: anti-mGlu5 receptor (rabbit 1:6000 Upstate), anti-FMRP (rabbit 1:1000 Cell Signaling), anti-P-FMRP (rabbit 1,25:1000 Abcam), anti-FXR1P (830 rabbit 1:12000 kind gift of Dr B. Bardoni

University of Nice-France), anti-FXR2P (clone A42 mouse monoclonal 1:1000 Abcam), anti-eIF2α and anti-P-eIF2α (rabbit 1:1000 Cell Signaling), anti-GAPDH (rabbit 1:1000 Cell Signaling), anti-β-Tubulin (rabbit 1:1000 Cell Signaling) and anti-β-actin (mouse 1:1000, Cell Signaling). Alkaline phosphatase-conjugated secondary rabbit and/or mouse antibodies were used. Chemiluminescence was detected using the Western Breeze Chemiluminescent Immunodetection System (INVITROGEN) following instruction of manufacturer and visualized by VersaDoc™ 4000 Imaging System (BIORAD).

Statistical analysis

Statistical analysis was carried out by SIGMA STAT 3.1. All data were analyzed using multifactorial ANOVA (One-Way and Two-Way analysis of variance) followed by appropriate post-hoc comparisons (Holm-Sidak method).

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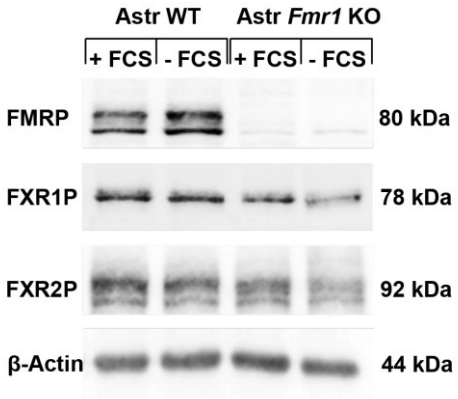

Figure 1 FMRP and its paralogs are expressed in astrocytes. Western blot analysis shows that WT and *Fmr1* KO cultured astrocytes express FMRP, FXR1P and FXR2P proteins. WT and *Fmr1* KO astrocytes are analyzed in presence and in absence of serum but no substantial difference was observed. The nitrocellulose membranes were incubated with the primary antibodies anti-FMRP, anti-FXR1P and anti-FXR2P.

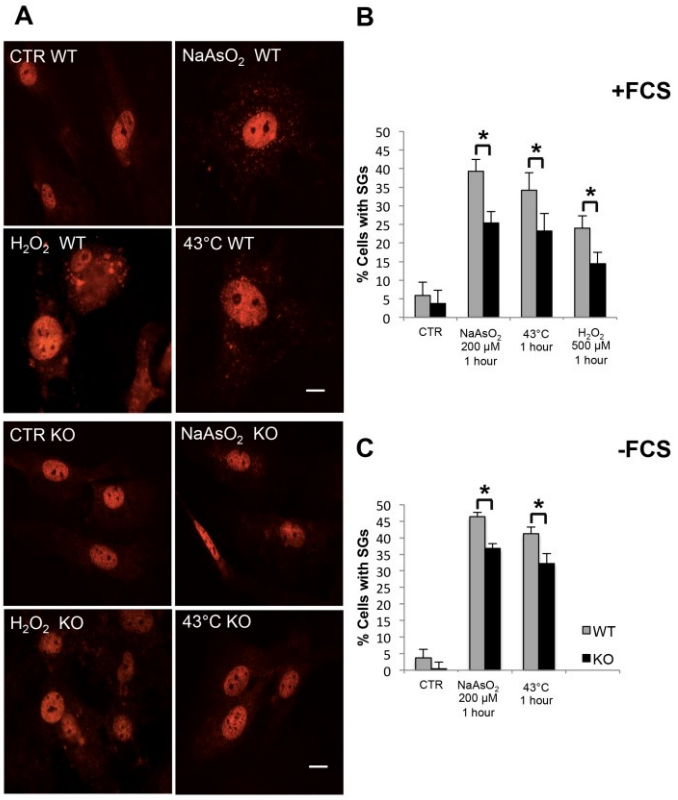

Figure 2 *Fmr1* KO Astrocytes show less TIA-1$^+$ SGs than WT astrocytes. (**A**) Cultured astrocytes from WT and *Fmr1* KO mice were stained with the primary antibodies anti-TIA-1 to detect stress granules formation. The selected cells are representative of the majority of cells expressing high or low level of SGs after treatments. Scale bar 10 µm. (**B**) Graph shows quantitative analysis of cells bearing SGs after exposure to different stress-inducing agents namely $NaAsO_2$ 200 µM for 1 hour, 43°C for 1 hour or H_2O_2 500µM for 1 hour, in serum medium. (**C**) Graph shows quantitative analysis of TIA-1$^+$ SGs cells after exposure to $NaAsO_2$ and 43°C treatments in serum free medium before e during the treatments. Values represent mean ± S.E.M. of data from 5 experiments each performed in triplicate (700-1000 cells/dish). *p= < 0,05 versus respective WT by Multifactorial Two-Way ANOVA followed by Holm-Sidak method.

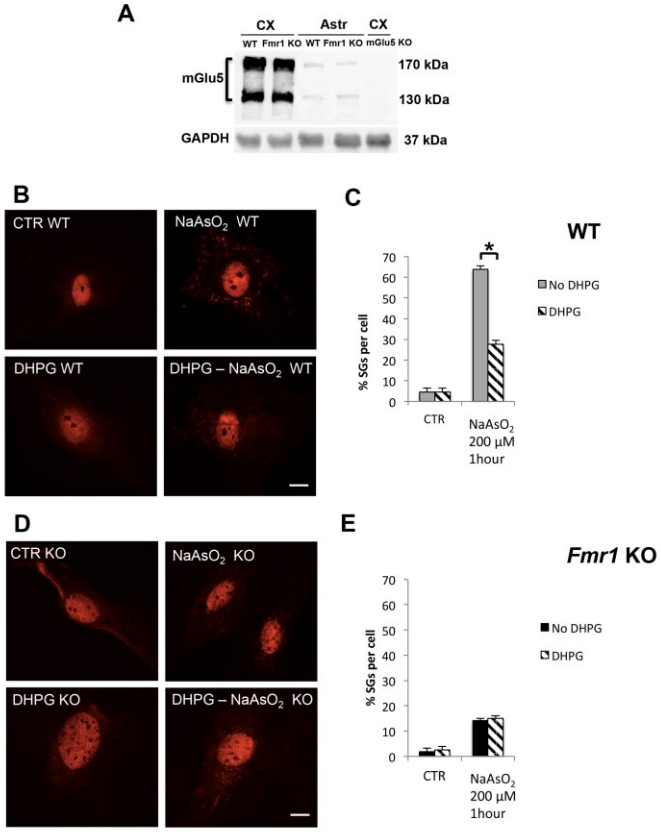

Figure 3 Activation of mGlu5 receptors reduces SGs in WT but not in *Fmr1* KO astrocytes. **(A)** Western blot analysis show expression levels of mGlu5 receptors in astroglial cell cultures from WT and *Fmr1* KO mice. (**B, D**) After exposure to $NaAsO_2$ with or without DHPG pre-treatment, cells were processed for immunofluorescence using specific TIA-1 antibody. The selected cells are representative of the majority of cells expressing high or low level of SGs. Scale bar 10 µm. (**C, E**) Graphs show quantitative analysis of the number of SGs per cell in WT (**C**) and *Fmr1* KO (**E**) cultures after exposure to different agents. Astrocytes were untreated, treated with $NaAsO_2$ 200 µM for 1 hour, treated with DHPG 100 µM for 5 min and with DHPG followed by $NaAsO_2$. Values represent mean ± S.E.M. of data from 4 experiments each performed in duplicate (700-1000 cells/dish). *p= < 0,05 versus respective control by Two-Way Anova followed by Holm-Sidak method.

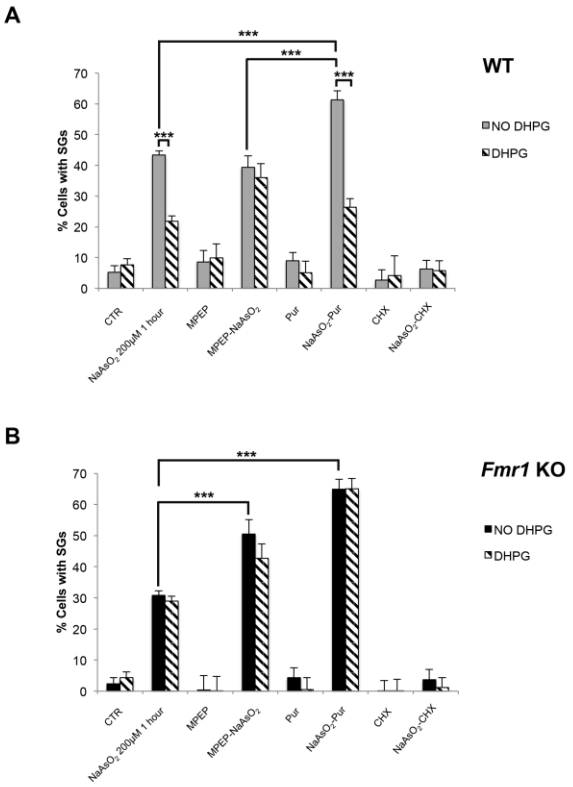

Figure 4 mGlu5 receptors modulate SGs differently in WT and in *Fmr1* KO astrocytes. Graphs show quantitative analysis of the number cell bearing SGs in WT (**A**) and *Fmr1* KO (**B**) cultures after exposure to different agents. Immunocytochemistry using TIA-1 antibody was performed. Astrocytes were untreated, treated with $NaAsO_2$ 200 µM for 1 hour, treated with DHPG 100 µM for 5 min and with DHPG followed by $NaAsO_2$; treated with MPEP 3 µM for 40 min alone, treated with MPEP with addition of DHPG in the last 5 min, with MPEP followed by $NaAsO_2$ and with MPEP-DHPG-$NaAsO_2$; treated with Puromycin (20µg) or Cycloheximide (30µg) for 1 hour, treated with $NaAsO_2$ for 90 min with addition of Puromicyn or CHX after 30 min, with or without a pretreatment with DHPG for 5 min before $NaAsO_2$ treatment. Values represent mean ± S.E.M. of data from 3 experiments each performed in triplicate (700-1000 cells/dish). *p= < 0,001 versus respective control by Multifactorial Two-Way ANOVA followed by Holm-Sidak method.

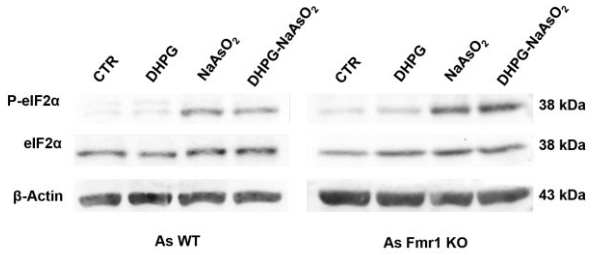

Figure 5 Activation of mGlu5 receptors reduces phosphorylation of eIF2α in WT astrocytes. Western blot analysis shows expression levels of eIF2α protein in phosphorylated and total forms in WT and *Fmr1* KO astrocytes. We compared untreated cells, cells treated with DHPG 100 μM for 5 min, treated with $NaAsO_2$ 500μM for 90 min and treated with DHPG for 5 min followed, after wash, by $NaAsO_2$ for 90 min. The selected blot is representative of the 3 experiments performed.

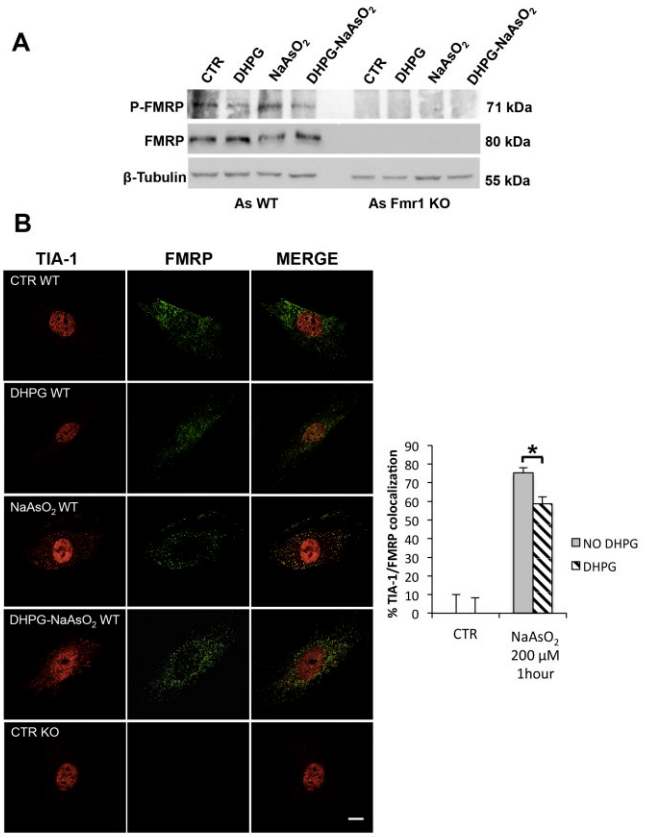

Figure 6 Activation of mGlu5 receptors reduces phosphorylation of FMRP and its recruitment in SGs. (**A**) Western blot analysis show phosphorylation of FMRP protein after different treatments. Astrocytes were cultured in media alone (CTR), with 100 µM DHPG for 5 min, with 500 µM $NaAsO_2$ for 90 min or with DHPG 100µM for 5 min before treatment with $NaAsO_2$. (**B**) WT and *Fmr1* KO astrocytes were stained with primary antobodies anti-TIA-1 and anti-FMRP. Astrocytes were untreated, treated with DHPG (100 µM for 5 min), with $NaAsO_2$ (500 µM for 30 min) or treated with DHPG followed by $NaAsO_2$. Red = TIA-1, Green = FMRP. The last column is merged images of TIA-1 and FMRP panels. Scale bar = 10µm. The graph represent the % of co-localization of TIA-1 protein with FMRP in SGs. *p= < 0,05 versus respective control by Multifactorial One-Way ANOVA followed by Holm-Sidak method.

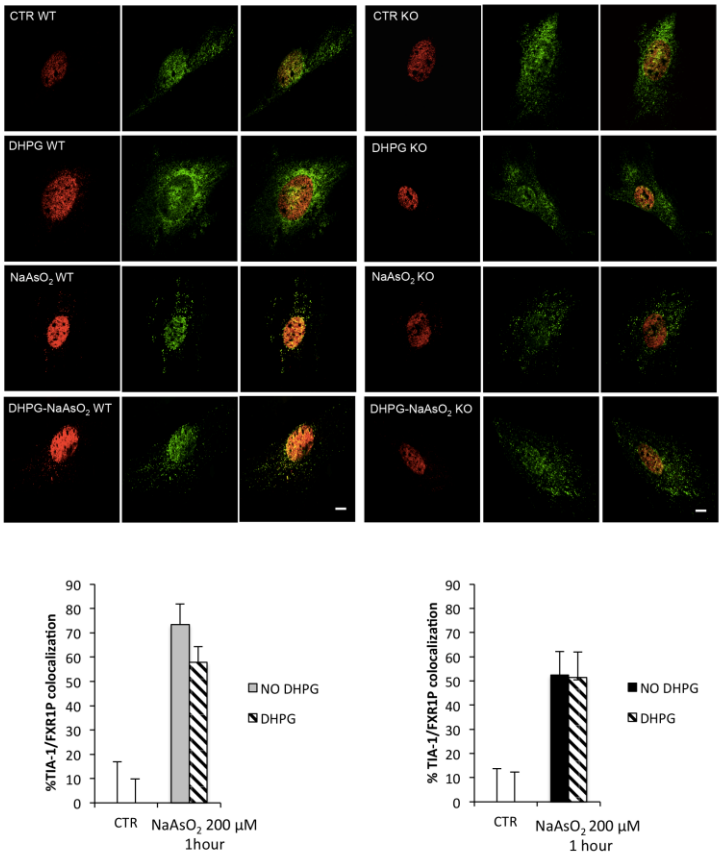

Figure S1 FXR1P recruitment in SGs. WT and *Fmr1* KO astrocytes were stained with primary antobodies anti-TIA-1 and anti-FXR1P after exposure to DHPG 100 µM for 5 min or $NaAsO_2$ 200 µM for 1 hour or DHPG followed by $NaAsO_2$.
Red = TIA-1, Green = FXR1P. The last columns are merged images of TIA-1 and FXR1P. Scale bar = 10µm.

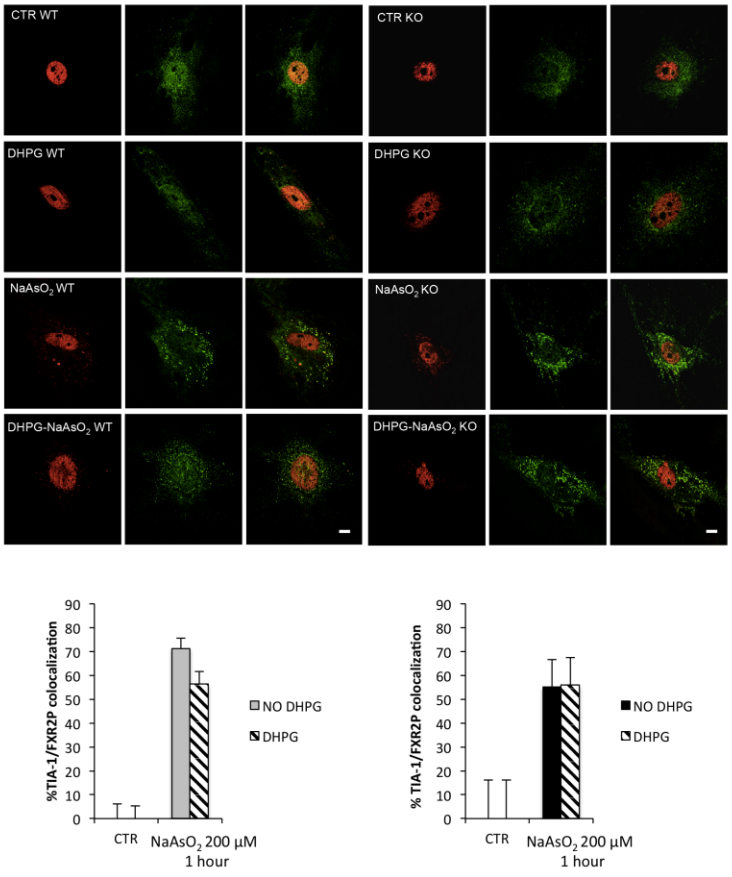

Figure S2 FXR2P recruitment in SGs. WT and *Fmr1* KO astrocytes were stained with primary antobodies anti-TIA-1 and anti-FXR2P (**S1**) after exposure to DHPG 100 µM for 5 min or $NaAsO_2$ 200 µM for 1 hour or DHPG followed by $NaAsO_2$. Red = TIA-1, Green = FXR2P. The last columns are merged images of TIA-1 FXR2P panels. Scale bar =10µm.

GENERAL DISCUSSION AND CONCLUSIONS

Intellectual disability (ID) is one of the largest unsolved problems of health care. Hereditary forms of ID and autism are recognized as neuro-developmental disorders, in which several genetic mutations (>80 genes involved) have been identified (Ropers, 2008; de Ligt et al., 2012). Several forms of hereditary ID are linked to X chromosome and are defined X-linked forms of ID (XLID). The human X chromosome contains only about 4% of the protein-coding genes in the human genome, but defects in these genes are responsible for 8-12% of the ID seen in males (Ropers and Hamel, 2005). Nowadays, X chromosome represents an attractive target for research into the molecular causes of ID.

Fragile X syndrome (FXS) is the most frequent form of inherited cognitive deficit and the second genetic cause of ID after Down syndrome, and is caused by the absence of FMRP. During the last decade many studies have been focused on elucidating the function of FMRP as repressor of mRNA translation, but much less is known about its function in the context of its complex with interacting proteins. FMRP is a component of mRNA ribonucleoprotein complexes (mRNPs) and it can interact with several proteins, interactions that may modulate the functions of FMRP. FMRP plays a crucial role in the regulation of RNA metabolism and it is a key protein in mechanisms underlying synaptic plasticity both in adult and during development. ID is a complex condition that starts before adulthood, with a lasting effect on development. For this reason we tried to better define the role of FMRP and the relationship of FMRP with its interacting proteins during brain development in different brain areas. FMRP interacts with its paralogs, FXR1P and FXR2P, with other RNA-binding proteins such as NUFIP and 82-FIP, and with proteins that do not bind RNA, like CYFIP1 and CYFIP2. We demonstrated that FMRP was strongly expressed during the first week and gradually decreases thereafter in the brain. FXR1P and FXR2P were also highly expressed in the first two weeks of post-natal brain and showed a decrement during development in different brain regions. Instead CYFIP1 and CYFIP2 showed different

developmental profile than FXR proteins.

In the **Paper I** we pointed out a prominent role of all three FXR proteins in the first two weeks of post-natal development, a period characterized by active synaptogenesis and circuit formation in different brain areas (**see Paper I**). FMRP interacts with FXR1P and FXR2P *in vitro* as well as *in vivo* (Siomi et al., 1996) and it is known that protein-protein interactions are involved in several mechanisms, such as DNA replications and transcriptions, RNA splicing, protein translations and modifications, biological responses, signal transduction, and metabolic processes (Miernyk and Thelen, 2008). Among FXR proteins, only FMRP is able to regulate protein synthesis, but it is possible that in the brain the three proteins work in a cooperative way supporting and reinforcing the function of FMRP. Our work suggest that FMRP interacts differently during development with its partners, and particularly that functions modulated by FXR1P or FXR2P, such as mRNA transport along axons and translation, are mainly regulated early during development whereas the function of FMRP as regulator of actin cytoskeleton may be more effective at later developmental stages.

On the basis of this knowledge, we considered a new role of FMRP in mRNA metabolism as an effector of stress granule assembly. It has been reported that FMRP is associated with the pool of mRNAs that go into stress granules (SGs) upon cellular stress (Mazroui et al., 2002). Stress-induced damage has been liked to several human disease (Thompson and Parker, 2009). Emerging evidence also indicates a potential role of elevated oxidative stress in neuro-developmental diseases such as autism, Rett syndrome and Down syndrome (De Felice et al., 2012; Lintas et al., 2012; Essa et al., 2013) and several studies suggest that there is a link between abnormal SGs formation and ID (Mazroui et al., 2002; Didiot et al., 2009). In our work we confirmed that in FXS there is a defect in SGs formation, due to the absence of FMRP **(see Paper II**). FMRP moves in SGs during stress (Mazroui et al., 2002; Kim et al., 2006), bringing with it several mRNAs to preserve them in SGs and contributing to repress translation under stress conditions (Mazroui et al., 2002). FXR1P and FXR2P co-localize with FMRP in SGs too (Mazroui et al., 2002). Furthermore, we investigated FMRP's role in stress

response under activation of mGlu5 receptor, being its involvement in the pathophysiology of FXS well established (Bear et al., 2004; Huber et al., 2002). In particular, we contribute, with **Paper II**, to define that the mGlu5 receptor is able to modulate SGs formation. Activation of mGlu5 receptor affects SGs formation through a FMRP-mediated mechanism in WT (see Discussion **Paper II**), instead, the blockade of mGlu5 receptor restore SGs formation in *Fmr1* KO astrocytes. Activation of mGlu5 receptor before stress reduced FMRP recruitment in SGs and reduced phosphorylation of eIF2α and FMRP. In a similar manner to that observed for FMRP, mGlu5 receptor modulates the recruitment of FXR1P and FXR2P in SGs. In contrast, mGlu5 receptor activation did not affect SGs formation in *Fmr1* KO astrocytes. Thus, FMRP plays a key role in SGs formation and mGlu5 receptor activation affects SGs formation and the recruitment of FXR proteins. We suggest that FMRP may have a positive role in stress response, facilitating and enhancing SGs formation to prevent stress damages. Thus maybe this process is useful to understand what happens in FXS, in which can occur abnormal modulation of different proteins during development with consequent abnormal response during adversal conditions. These findings suggest a potential novel role for mGlu5 receptor in SGs formation.

We want to underline, regarding the role of mGlu5 and FMRP in SGs formation, that these results are relevant because oxidative stress is a frequent component not only in ID and autism, but also in several neurodegenerative disorders. Interestingly in several neurodegenerative disorders mutations in SGs-associated RNA binding proteins cause abnormal SGs formation under stress conditions (Meyerowitz et al., 2011; Wolozin, 2012). In this case, SGs seem to lose their protective role to degenerate in insoluble protein aggregates commonly seen in Amiotrophic Lareral Sclerosis, Frontotemporal Dementia and in other forms of neurodegeneration. This suggests that SGs might play an important role in the pathogenesis of proteinopathies and in neurodegeneration (Bentmann et al., 2013; Wolozin, 2012; Meyerowitz et al., 2011). Furthermore, mutations in SGs-associated proteins, like TDP-43, represent the second leading cause in molecular neuropathogenesis of Alzheimer, Parkinson and Huntington diseases (Forman et

al., 2007). Interestingly, alterations in normal mGlu5 receptor signaling is associated with these neurodegenerative diseases (Ribeiro et al., 2010; Um et al., 2013), thus the pharmacological modulation of the relationship between SGs, SGs associate proteins and mGlu5 receptor, could be considered in a wide scenario. In addition, SGs formation is studied in tumors, but if SGs involve oncogenic signaling pathways is currently unknown. Fournier et al. reported that inhibition of mTOR blocks the association of antiapoptotic p21 pathway to SGs in cancer cells; thus, they proposed this type of modulation to sensitizes cancer cells to death (Fournier et al., 2012).

Taking into account these considerations, further perspectives will be aimed at studying other biochemical pathways, modulated by mGlu5 receptor, that can influence SGs formation, like mTOR and ERK.

Finally it would be useful to better understand the protective role of SGs against cell death. Several studies suggest a link between SGs formation and cell survival (Arimoto et al., 2008; Eisinger-Mathason et al., 2008), but a causal relationship between SGs formation and cell survival needs clarification. Several SGs-RNA binding proteins can influence the apoptosis; among them, eIF4E, TRAF, FAST. Also FXR1P seems to be implicated in the regulation of the cell apoptosis mechanism by interacting with BTF (Bcl-2-associated transcription factor 1), a death-promoting transcriptional repressor (Ma et al., 2014), but this aspect in FXS is still unknown. It could be important to better explore the roles of FXR1P and other proteins involved in cell apoptosis, in correlation with SGs formation, to add further clarification on these biochemical mechanisms that are common to many worldwide diseases.

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LIST OF PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

Publications

- **Fragile X mental retardation protein (FMRP) interacting proteins exhibit different expression patterns during development**
  C. M. Bonaccorso, M. Spatuzza, **B. Di Marco**, A. Gloria, G. Barrancotto, A. Cupo, S. A. Musumeci, S. D'Antoni, B. Bardoni, M.V. Catania
  Submitted to *International Journal of Developmental Neuroscience*

- **Group-I metabotropic glutamate receptor activation reduces stress granules formation via a mechanism mediated by FMRP**
  B. Di Marco, P. Dell'Albani, M. Spatuzza, C. M. Bonaccorso, S. D'Antoni, B. Bardoni, F. Drago, M. V. Catania
  Submitted to Journal of *Molecular Cell Biology*

- **Blockade of serotonin2B receptor reduces cocaine-induced hyperlocomotion independently of subcortical DA outflow**
  C. Devroye, A. Cathala, **B. Di Marco**, F. Drago, P. V. Piazza, U. Spampinato
  In preparation

Conference proceedings

- **Activation of group-I metabotropic glutamate receptors impairs stress granules formation and enhances cellular damage under stress condition**
  B. Di Marco, P. Dell'Albani, M. Spatuzza, C. M. Bonaccorso, S. D'Antoni, B. Bardoni, F. Drago, M.V. Catania
  September 28– October 3, 2014, 8th International Meeting on Metabotropic Glutamate Receptors, Taormina (Italy)

- **Metabotropic glutamate subtype 5 (mGlu5) receptor-mediated modulation of the interaction between Fragile X Mental Retardation Protein (FMRP) and the Fragile X related (FXR) proteins FXR1P and FXR2P**

  C.M. Bonaccorso, S. D'Antoni, M. Spatuzza, **B. Di Marco**, G. Barrancotto, S.A. Musumeci, L. Davidovic, B. Bardoni, M.V. Catania

  September 28– October 3, 2014, 8th International Meeting on Metabotropic Glutamate Receptors, Taormina (Italy)

- **Activation of group-I metabotropic glutamate receptor reduces stress granules formation through a mechanism mediated by FMRP**

  M.V. Catania, **B. Di Marco**, M. Spatuzza, C. M. Bonaccorso, S. D'Antoni, F. Drago, P. Dell'Albani

  July 5-9, 2014, 9th FENS (Federation of European Neuroscience Societies), Milan (Italy)

- **Group-I metabotropic glutamate receptor activation modulates Stress Granules formation undermining the role of FMRP under stress condition**

  B. Di Marco, P. Dell'Albani, M. Spatuzza, C. M. Bonaccorso, S. D'Antoni, F. Drago, M.V. Catania.

  June 28 - July 4 2014, 12th Summer School of Neuroscience: "Dopamine", Catania (Italy)

- **Stress granules formation and cell survival are differently modulated by group-I metabotropic glutamate receptor activation in wild type and fmr1 knockout astrocytes**

  B. Di Marco, P. Dell'Albani, M. Spatuzza, C. M. Bonaccorso, S. D'Antoni, F. Drago, M. V.Catania.

  November 9-13, 2013, Neuroscience 2013, San Diego (USA)

- **Stress granules formation and cell survival are differently modulated**

by group-I metabotropic glutamate receptor activation in wild type and fmr1 knockout astrocytes

B. Di Marco, P. Dell'Albani, M. Spatuzza, C. M. Bonaccorso, S. D'Antoni, F. Drago, M. V.Catania.

7-8 November 2013**,** 8th Brain Research Conference-RNA Metabolism in neurological Disease, Satellite Meeting to Society for Neuroscience, San Diego (USA)

- **Stress granules formation and cell survival in WT and Fmr1 KO astrocytes under stress-inducing conditions**

  B. Di Marco, P. Dell'Albani, M. Spatuzza, C. M. Bonaccorso, S. D'Antoni, M. V. Catania

  03-07 October 2012, Jacques Monod Conference, Roscoff (France)

- **Increased surface expression and lack of internalization of group-I metabotropic glutamate receptors subtype 5 (mGlu5) in Fragile X Syndrome.**

  Spatuzza M., D'Antoni S., Aloisi E., **Di Marco B**., Bonaccorso C.M., Musumeci S., Catania M.V.

  13-14 April 2012, 7th International Meeting on CNVs and genes in intellectual disability and autism, Troina (Italy)