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Assessment of neuronal cell type specific transcriptional effects of three pathological sequence variants of the FMR1 gene promoter

Maud Simansour

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par

SIMANSOUR Maud

Assessment of neuronal cell type specific transcriptional effects of three pathological sequence variants of the *FMRI* gene promoter

Soutenu le 09 Juin 2015

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Abstract

FXS often results from the silencing of the *FMRI* promoter triggered by a CGG triplet expansion. In 2010, Collins et al., found three variants in the *FMRI* promoter which are reducing its activity to 36,2%, 29,2% and 5,9% altering the transcription factors binding sites necessary to initiate *FMRI* expression and decreasing the levels of its product FMRP. FMRP is important for synaptic plasticity in particular in mGluR-dependent LTD which is exaggerated in FXS leading to impairments in learning and memory and dendritic spine morphogenesis. To validate these results, we created various plasmids controlled by the *FMRI* promoter containing one of these three variants. Plasmids were transfected in HeLa cells, rat cortical neurons and human neuronal precursors. In parallel, mouse hippocampal slice cultures were shot with gold particles coated by the same plasmids. Confocal microscopy highlighted that the variant located near the first transcriptional start site was found to reduce *FMRI* expression to 42.08% in human neuronal precursors and to 45.7% in mouse hippocampal slices. The cells and slices were treated with neuronal activity modulators. Bicuculline was found to rescue *FMRI* expression suggesting that the inhibition of GABA receptors triggers a sufficient increase of excitatory glutamatergic activity to modulate *FMRI* expression.

Key words: FXS, *FMRI* promoter, transcription factors, FMRP, synaptic plasticity, neuronal activity modulators, GABA receptors, glutamatergic activity.

Résumé

Le FXS proviendrait de l'expansion d'un triplet de CGG qui provoquerait l'inactivation du promoteur du gène *FMRI*. Trois variants de séquence, altérant les sites de liaison des facteurs de transcription et diminuant la production de FMRP, furent aussi trouvés dans le promoteur *FMRI*. FMRP est importante pour la plasticité synaptique principalement dans la LTD dépendante des mGluR qui est exagérée dans le FXS menant à des déficits d'apprentissage, de mémoire et de morphogénèse des épines dendritiques. Pour valider ces résultats, nous créâmes différents plasmides contenant ces trois variants et nous les transfectâmes dans des cellules HeLa, des neurones corticaux de rats et des précurseurs neuronaux humains. Des cultures de coupes hippocampales de souris furent aussi transfectées par ces mêmes plasmides avec un Genegun. Nous observâmes par microscopie confocale que le variant situé proche du plus important site start de transcription réduisait l'expression de *FMRI* de 42.08% dans les précurseurs neuronaux humains et de 45.7% dans les coupes hippocampales de souris. Les cellules et coupes furent ensuite traitées avec des modulateurs de l'activité neuronale. La Bicuculline permit de rétablir l'expression de *FMRI* suggérant que l'inhibition des récepteurs GABA déclencherait une augmentation suffisante de l'activité glutamatergique excitatrice pour moduler l'expression de *FMRI*.

Mots clé: FXS, promoteur *FMRI*, facteurs de transcription, FMRP, plasticité synaptique, modulateurs de l'activité neuronale, récepteurs GABA, activité glutamatergique.

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The best for the end, I would like to thank Vincent for his presence by my side, his help and his patience all along the writing of this thesis.

Abbreviations

AMPA : α amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BDNF: Brain-Derived Neurotrophic Factor
Bic: Bicuculline
BSA : Bovin Serum Albumin
BZD : Benzodiazepin
CC: Coiled coils
CMV: Cytomegalovirus
CNS : Central nervous System
CREB: cAMP/Ca²⁺ -Response Element Binding
CTD: C Terminal Domain
DDR: DNA Damage Repair
DHPG: DiHydroxyPhenyl Glycine
DMP: Development Molecular pathway
DNA: Desoxyribo Nucleic acid
FMR1: Fragile X Mental Retardation 1
FMRP: Fragile X Mental Retardation Protein
FXS: Fragile X Syndrom
GABA: γ Amino Butyric Acid
GFP: Green Fluorescent Protein
IRES: Internal Ribosome Entry Site
KH: K homology
KO: Knock out
LTD: Long Term Depression
LTP: Long Term Potentiation
L-VSCC: L-type-Voltage-Sensitive Ca²⁺ Channels
MAP: Microtubule Associated Protein
mGluR: Metabotropic Glutamate Receptor
MPS: Massively Parallel Sequencing
NEO: Neomycin
NES: Nuclear Export Signal
NGS: Next Generation Sequencing
NIBR: Novartis Institute for Biomedical Research
NLS: Nuclear Localization Signal
NMDA: N-Methyl-D-Aspartate
NO: Nitric Oxyde
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PKC: Protein Kinase C
PKG: Protein Kinase G
PLC: Phospholipase C
PPI: Prepulse Inhibition
RISC: RNA induced Silencing Complex
RNA: Ribo Nucleic Acid
TBP: TATA Binding Protein
TSS: Transcriptional Start Site
TTX: Tetrodotoxin
USF: Upstream Stimulating Factor
UTR:Untranslated Region

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1 Introduction

The most common cause of the loss of Fragile X Mental Retardation Protein (FMRP) in Fragile X Syndrome (FXS) is the result of a CGG expansion (>200 repeats also called full mutation) full mutation in the 5'UTR (5-prime untranslated region) of *Fragile X Mental Retardation-1* gene (*FMRI*) (Mandel et al., 1991). This expansion triggers epigenetic silencing of the promoter which becomes DNA methylated. However, deletions in *FMRI* can also cause Fragile X Syndrome leading to the absence of the gene product, FMRP (Coffee et al., 2008). In addition, variants affecting the expression and function of FMRP can represent the third important cause of FXS. However, since *FMRI* was identified in 1991, only one missense mutation (I304N) and two small nonsense deletion mutations have been reported (De boulle et al., 1993; Lugenbeel et al., 1995). Sequencing of *FMRI* is rarely performed in clinical setting and methodological constraints bound to the cost-effectiveness and throughput of traditional Sanger sequencing have previously prevented a thorough assessment of *FMRI* sequence variations in a large number of patients, leaving the true significance of pathogenic sequence variants in *FMRI* unknown.

Recently, massively parallel sequencing (MPS) also called new generation sequencing (NGS) vastly improved detection of sequence variation at a scale that was previously impractical (Shendure and Ji, 2008). Through the use of pooled-template massively parallel sequencing, 130 novel *FMRI* sequence variants were identified in a population of 963 developmentally delayed males without CGG repeat expansion (Collins et al., 2010). Among these, the authors identified a novel missense change p.R138Q which alters a conserved residue in the nuclear localization signal of FMRP, ten non coding variants in conserved non coding regions and 3'untranslated region (UTR) of *FMRI* including two predicted splice site mutations and three promoter mutations which significantly reduce in vitro levels of *FMRI* transcription. These three promoter mutations, c.-332G>C, c.-293T>C and c.-254A>G, occurred in only one of the 963 sequenced developmentally delayed males. They were not detected in 1308, 1266 and 1304 control males of European descent. In addition, these variants were found to be near much conserved sites of the *FMRI* promoter region when comparing the sequence to foreign mammalian sequences.

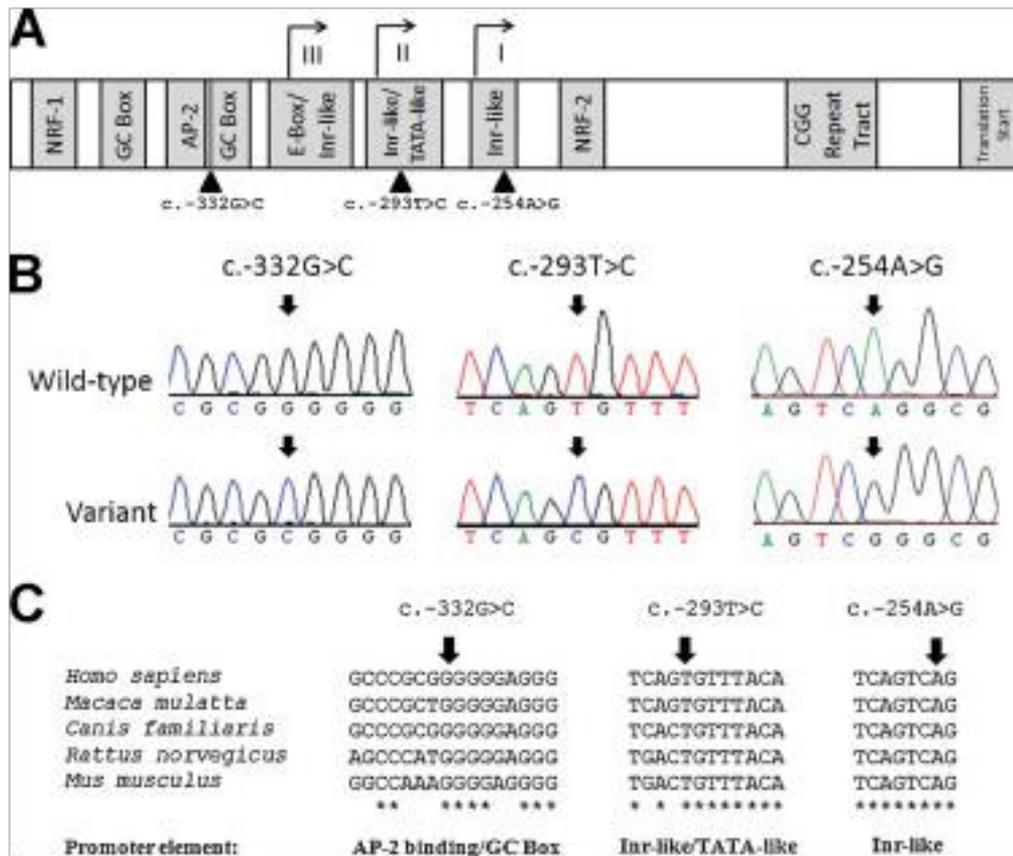


Figure 1 : The novel promoter variants c.-332G>C, c.-293T>C, and c.-254A>G. **A:** Diagram of the minimal promoter and promoter region of *FMRI*. Roman numerals I–III represents the three transcription start sites of *FMRI*. The GC boxes bind the transcription factor SP1. **B:** DNA chromatograms of the three promoter variants. **C:** Mammalian conservation of the overlapping AP-2 binding site and GC box, the overlapping Inr-like and TATA-like sequences at transcription start site II, and the Inr-like sequence at transcription start site I. (Collins et al., 2010).

The c.-332G>C variant is located within overlapping binding sites for the SP1 transcription factors and AP-2 α transcription factors (Smith et al., 2004 and Lim et al., 2005). The c.-293T>C variant is located near transcription start site II, within both an initiator-like (Inr-like) and a TATA like sequence (Hwu et al., 1993; Beilina et al., 2004). The third variant, c.-254 A>G is located within an Inr-like sequence near the primary transcription start site (Kumari and Usdin, 2001; Beilina et al., 2004). These three novel variants in the *FMRI* promoter are the first to be identified in much conserved defined elements of the *FMRI* promoter. These elements have been described as important and close to important binding sites for transcriptional factors important in regulatory mechanisms for the transcriptional initiation of *FMRI* (Beilina et al., 2004).

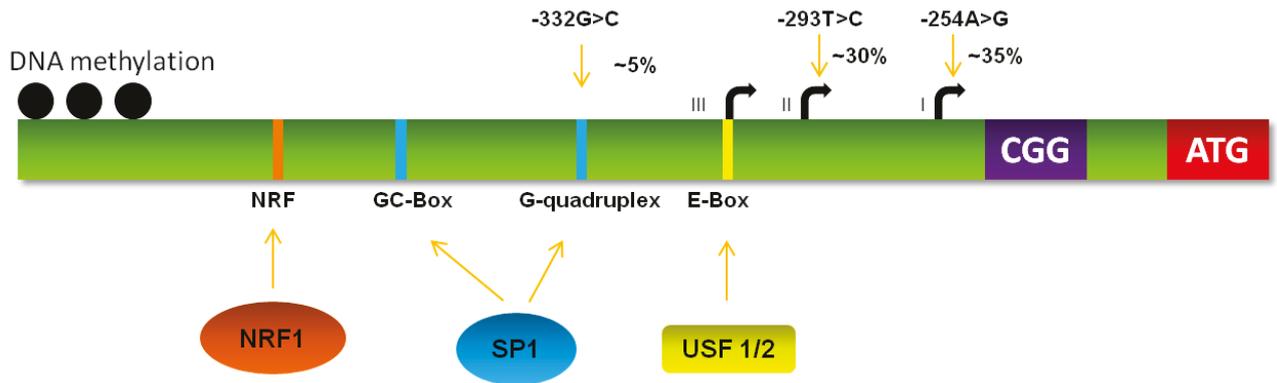


Figure 2: *FMRI* promoter and the location of mutations. The black arrows indicate the three TSS. The decreased expression for each mutation is indicated in percent compared to the wild type close to the variant. The most important transcription factors NRF1, SP1 and USF1/2 and their binding sites are indicated by the colored bar corresponding to the color of each transcription factor.

To assess the functional significance of these three promoter variants, the authors introduced each variant individually into the pFMR1-Luc (*FMRI* promoter region coupled to a luciferase reporter gene) plasmid which uses the human *FMRI* promoter to drive expression of firefly luciferase. Firefly luciferase activity, measured 48h post transfection into HeLa cells, was normalized to Renilla luciferase activity and expression relative to the wild type *FMRI* promoter activity.

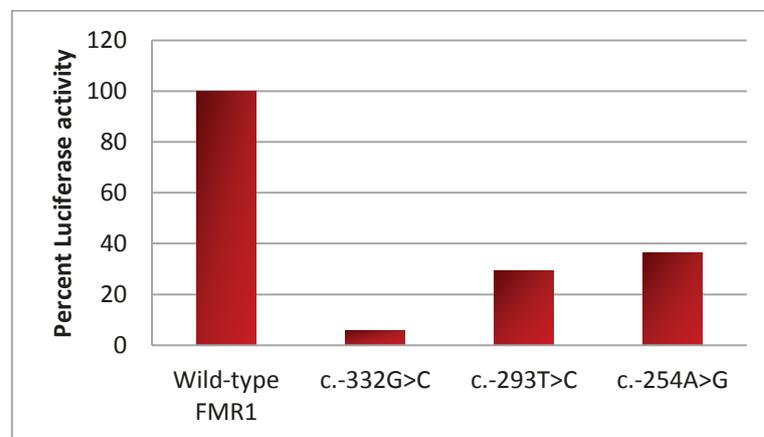


Figure 3: Functional effects of the novel promoter variants: c.-332G>C, c.-293T>C, and c.-254A>G. Luciferase activity is depicted as the ratio of firefly luciferase to Renilla luciferase and normalized to the activity of the wild-type *FMRI* promoter. The results shown represent six independent transfections. Data are represented as mean -1 SD. (Collins et al., 2010).

As seen in the figure 3, the c.-332G>C variant reduced *FMRI* promoter activity to 5.9% of wild type levels, the c.-293T>C variant reduced *FMRI* promoter activity to 29.2% of wild type levels and the c.-254A>G variant reduced *FMRI* promoter activity to 36.2% of wild type levels. All of these reductions from wild type promoter activity were statistically significant ($P < 1.10^{-7}$). The authors have proved in this luciferase assay that all these three mutations independently reduced the

activity of the *FMRI* promoter. This reporter assay data suggests that the three identified variants are likely to have a functional effect.

In order to verify these results, we decide to first clone these three mutations of the *FMRI* promoter region (pFMR1) into plasmids containing three various fluorescent reporter genes: the red mCherry and tdTomato and the green GFP. Second, we transfect these plasmids in human neuronal precursors, rat cortical neurons and mouse hippocampal slices to compare the expression level of each mutated pFMR1 plasmids to wild type pFMR1 expression. In this work, we expected to detect significant differences between the mutated *FMRI* (M1FMR1, M2FMR1 and M3FMR1) and the wild type *FMRI* (WTFMR1) expression levels in several cellular models.

The green/red fluorescence reporters can be detected in parallel without interference enabling us to do co-transfection experiments with the mutant *FMRI* and wild type promoter constructs. Thus we could normalize the expression from the mutant *FMRI* promoters to wild type levels to evaluate net difference. Then, we treat the rat cortical neurons and the mouse hippocampal brain slices with three neuronal activity modulators, TTX (Tetrodotoxin) that block the action potential discharges, Bic (Bicuculline) that serves to increase the glutamate release by inhibition of the GABA receptors and glycine that serves to induce NMDA-dependent LTP (chemical LTP). TTX is a potent neurotoxin that blocks voltage gated sodium channels (VGSCs) known to play a critical role in neuronal function under both physiological and pathological conditions (Nieto and al., 2011). Bic is an antagonist of the inhibitory effect of γ -aminobutyric acid (GABA) (Curtis and al., 1971). Application of glycine, which has a binding site on the NR1 subunit of the NMDA receptors, was first used to induce LTP in rat hippocampus in field recording (Shahi et al, 1993). Gly-LTP may provide a unique way to study long term synaptic transmissions while at the same time measuring changes in transgenic mice models (Shang et al., 2009). After transfections and treatments of the various cells, we visualize the FMRP expression level variations by immunofluorescence.

These experiments address the question how the neuronal activity plays a role in the regulation of the wild type and mutated *FMRI* gene. I will introduce this study by describing Fragile X Syndrome, neuronal activity and features of the *FMRI* gene and its product FMRP. In addition, I will present the various FXS *in vivo* animal models and the two theories around FXS pathomechanisms. In the second part, I will elucidate the specific goals and requirements for this work as well as the materials and methods that I used. At the end, I will describe the outcomes and possible future directions of our study.

2 The Fragile X Syndrome

Mental retardation occurs in 2-3% of the general population either in isolation or in combination with facial dysmorphisms and /or malformations. It is actually a fundamental research axis on which various researchers are working on in various laboratories like the Developmental and Molecular Pathways (DMP) laboratory that belongs to the Novartis Institutes for Biomedical Research (NIBR) directed by Mark Fishman.

The first intellectual disability syndrome was the Martin-Bell syndrome described in 1943 by James Purdon Martin and Julia Bell in multiple male members of a family. This finding in the same family permitted to conclude that this syndrome was X-linked (Martin and Bell, 1943).

Years later, Herbert Lubs discovered, in 1969, the existence of a break on the X chromosome of males presenting intellectual disability. This break was called “Fragile site” by Frederick Hecht in 1970. This site was designated FRAXA (Fragile site, X chromosome, A site) at Xq27.3 near the end of the long arm (Sutherland, 1977).

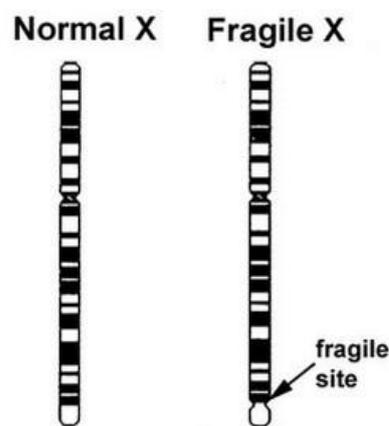


Figure 4: Fragile site of the X chromosome in Fragile X patients.
(<http://keytoflyaway.weebly.com/key-to-fly-away/fragile-x-syndrome>)

Martin Bell syndrome name was then changed to FXS. FXS is the most frequently encountered form of inherited mental disability in humans with a prevalence estimated to be 1/4000 in males and 1/7000 in females (Gustavson et al, 1986).

In addition to the mental retardation, the penetrant males exhibit language deficits, working and short-term memory problems, mathematics and visuospatial abilities and additional phenotypic involvements including abnormal facial features such as prominent jaw, large ears and macroorchidism in post pubescent males (Nussbaum and Lebtner, 1990). Therefore, many patients display subtle connective tissue abnormalities, hyperactive, attention deficit disorder and autistic behavior (Warren et al., 1994).

Moreover, abnormalities in dendritic spines and synaptic transmission, in the brain of adults suffering from FXS and *Fmr1* knockout mice, indicate perturbations in the development, maintenance and plasticity of neuronal network connectivity involved in learning and memory (Hinton et al., 1991).

These perturbations were found to be linked to the silencing of the *Fragile X Mental Retardation-1 gene (FMRI)* (Verkerk et al., 1991) and consequently the reduced expression or the complete absence of the gene product called Fragile X Mental Retardation Protein (FMRP).

The silencing of the *FMRI* gene, in the majority of cases, is caused by an expansion of a CGG repeat in the 5'UTR region of the *FMRI* gene (Oberle et al., 1991).

It was the first identified human disorder caused by a dynamic mutation. The CGG repeat is polymorphic and ranges from 5 to 40 CGGs with an average length of 30 CGG units in the normal population (Fu et al., 1991). However, in Fragile X patients, this expansion of CGG is found to be expanded beyond 200 repeats known as the full mutation. These repeats are hypermethylated and the cytosine methylation extends to the adjacent promoter region of the *FMRI* gene (Verkerk et al., 1991). The gene is also transcriptionally silenced and the Fragile X Mental Retardation Protein normally produced is absent (Verheij et al., 1993). When there are unmethylated expansions of 40-200 CGG units, it is called premutations. Premutations are unstable in meiosis and are found in both males and females and may expand to full mutation only upon maternal transmission in the next generation. The risk of transition to a full mutation is dependent on the size of the premutation, which accounts for the Sherman paradox (Fu et al., 1991). To date, the smallest known CGG repeat to expand to a full mutation is 56 (Fernandez-Carvajal et al., 2009).

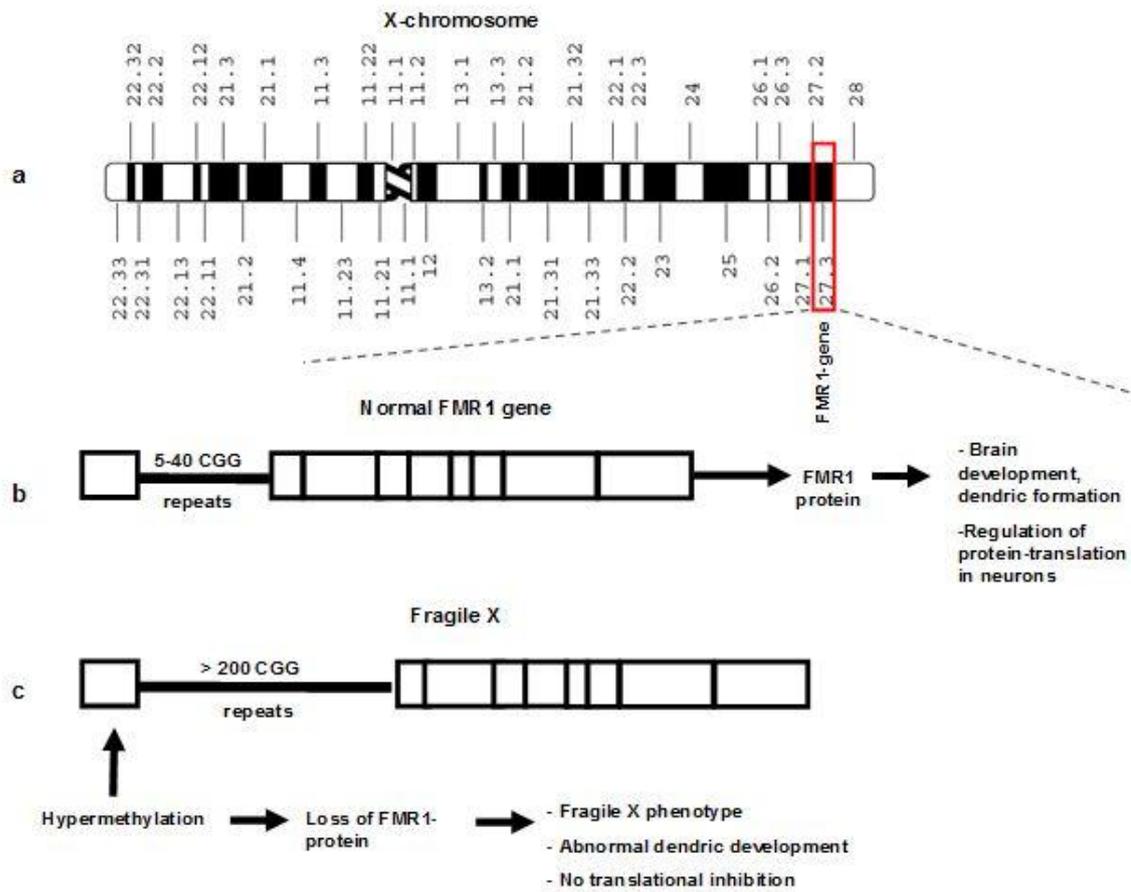


Figure 5: The unaffected, and mutated CGG repeat of the *FMR1* gene. A) location of the *FMR1* gene on the X chromosome. B) unaffected CGG repeat region. C) full mutation of the CGG repeat region. (<http://keytoflyaway.weebly.com/key-to-fly-away/fragile-x-syndrome>).

Loss of *FMR1* protein expression seems to be at the core of the intellectual disability and other features characteristic of FXS. In 1995, Feng et al., supposed that the expansion of CGG repeat in the 5'UTR of *FMR1* gene would form a secondary structure inhibiting the ribosome scanning and thus leading to a very low translational efficiency (see figure 5). In this aim, in 2011, Feng and Ting published in Nature a study that uncovers how Fragile X Syndrome causing gene mutations unleash a translation break that finally leads to overexpression of synaptic proteins that alter the proper transmission of signals at the synapse.

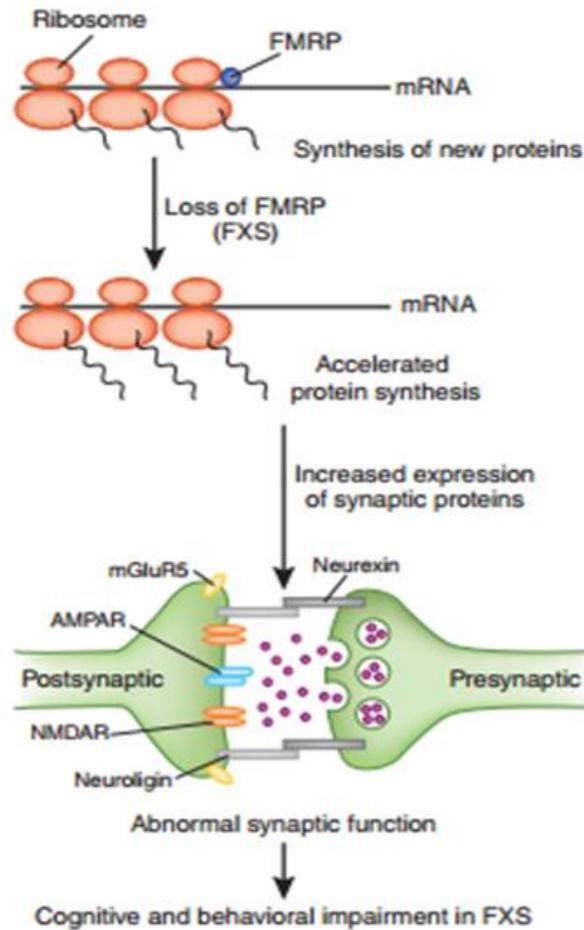


Figure 6: Synaptic dysfunctions in FXS. (Feng and Ting, 2011).

Thus, as shown in the figure 6, FMRP can function as a translational break to stall new protein synthesis. In the absence of FMRP, the translation of many synaptic proteins is accelerated leading to an aberrant synaptic function. This phenomenon contributes to cognitive and behavioral impairments in FXS. The lack of FMRP in neurons triggers an alteration of a wide array of mRNAs encoding proteins involved in synaptic structure and function such as NMDA (N-Methyl-D-Aspartate) receptors and GABA (γ -aminobutyric acid) receptors (Darnell et al., 2011). These receptor proteins are known to play a crucial role in regulating synaptic plasticity and neuronal activity. Some drugs modulating their function are available or under development. This complex dysregulation in the absence of FMRP creates impairment in spine morphology and functioning. Consequently, study of molecular and cellular mechanisms resulting in the dendritic spine anomalies and defects in learning and memory would permit to better understand the pathogenesis of FXS.

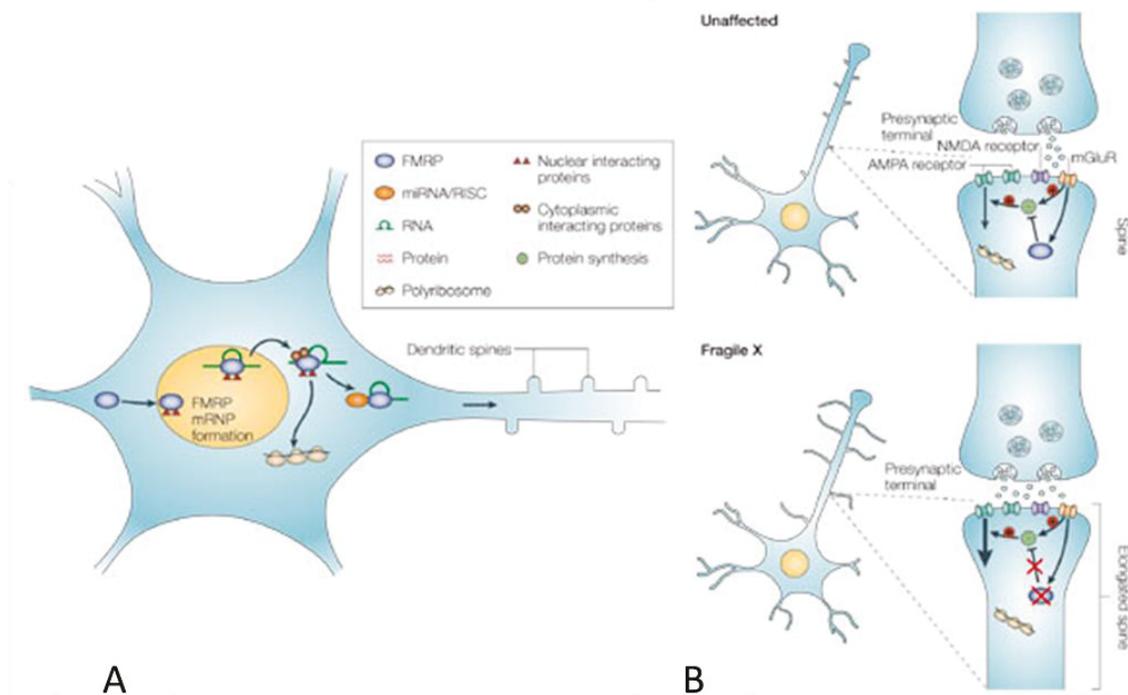


Figure 7: Mechanisms of pathogenesis in Fragile X syndrome A) FMRP modulates the translation of its targets, certainly through its association with the RNA-induced silencing complex (RISC). FMRP is transported to dendritic spines, together with its associated RNAs and proteins. B) Abnormal dendritic spine morphology in patients with FRAXA. An increased density of long, immature dendritic spines indicates that FMRP has a role in synaptic maturation and pruning, possibly through its regulation of gene products that are involved in synaptic development. (Gatchel, 2005).

It is interesting to indicate the mechanism by which FMRP controls the translation. FMRP is known to be an RNA binding protein that contributes to the posttranscriptional control of gene expression in the brain and gonads (Khandjian et al., 1999). Thus, on the figure 7A, to modulate the translation of its targets, we can see that FMRP makes an association with the RNA-induced silencing complex (RISC). At the end, FMRP is transported to dendritic spines, together with its associated RNAs and proteins to modulate the synaptic protein synthesis in the dendritic spines. Thus, on the figure 7B, in Fragile X neurons, we can see an increased density of long and immature dendritic spines compare to the unaffected dendritic spines shown on the neuron. These abnormalities present only in FXS patients, indicate that FMRP has a role in synaptic maturation and pruning, possibly through its regulation of gene products that are involved in synaptic development. FMRP might also have a regulatory role in activity-dependent translation at the synapse. In unaffected patients, stimulation of postsynaptic metabotropic glutamate receptors (mGluRs) results in increased protein synthesis and subsequent internalization of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. This protein synthesis is important in the expression of long-term depression (LTD, detailed later). The absence of FMRP in FXS results in over-amplification of this response.

3 Neuronal activity in hippocampus and neocortex

Neocortex and hippocampus are brain regions in which neuronal activity induces transcription of a large set of genes. Neuronal activity arising from sensory input induces the expression of new gene products that contribute to enduring adaptations in the central nervous system (CNS). These changes include the refinement of cortical circuitry during development (Katz and Shatz, 1996), the formation of long term memories (Koenig and Lu, 1967) and the development of complex behaviors such as learning (Clayton, 1997). Neuronal activity stimulates the influx of calcium ions into the postsynaptic neurons via the L-type-voltage-sensitive Ca^{2+} channels (L-VSCC) and the NMDA subtype of glutamate receptors stimulating a cascade of signaling events that lead to changes in gene expression. These changes in gene expression affect many aspects of nervous system development including dendritic morphogenesis, neuronal survival and synapse development which all drive the adaptive responses that underlie learning and memory in the mature nervous system. Mutations in components of the signaling pathways that participate in the process of experience-dependent brain development have been found to give rise to a variety of disorders of cognitive function including autism spectrum disorders.

Activation and localization to the nucleus of modulators of transcription and transcription factors activated by calcium bind to the regulatory regions of activity regulated genes to orchestrate tuned levels of gene expression. *BDNF* (encoding brain-derived neurotrophic factor). This *BDNF* gene is the most studied activity dependent gene. The *BDNF* gene product affects numerous processes in neuronal development including axonal and dendritic development, synapse formation and maturation, synaptic potentiation and neuronal survival. In addition, polymorphisms in the *BDNF* gene correlate with defects in learning and memory. For the activity dependent transcription of a large number of neuronal genes, including *BDNF* exon III, it was found that the cAMP/ Ca^{2+} - response element binding protein (CREB) is required and mutations in the *BDNF* exon III promoter in the CRE sequence lead to severely reduce the responsiveness of the promoter to Ca^{2+} influx (Shieh et al., 1998). However, activation of CREB is not sufficient to mediate alone the activity-dependent transcription of *BDNF* exon III. This finding indicates that there must to exist additional transcription factors which cooperate with CREB to regulate this gene in a Ca^{2+} dependent manner in neurons. The figure below resumes the various steps of Ca^{2+} dependent transcription in neurons.

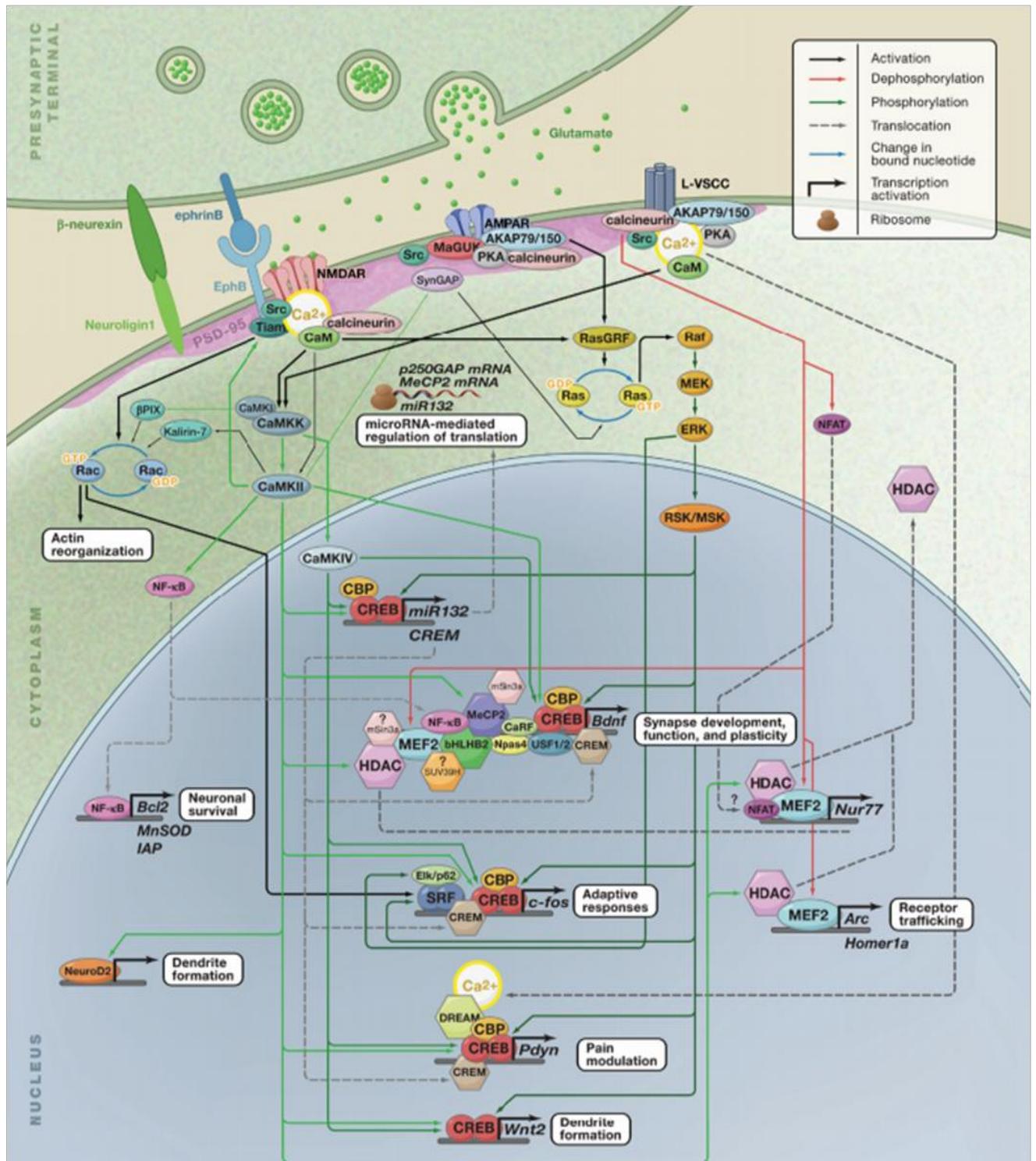


Figure 8: Ca²⁺ dependent transcription in neurons (Zieg and al., 2008). Activation of a number of signaling molecules which are required for the expression of new gene products is triggered by calcium influx through NMDA receptors, AMPA receptors and/or L-type VSCCs. The signaling cascades induced include the Ras/MAPK pathway, the calcium/calmodulin-dependent protein kinases, the phosphatase calcineurin and Rac GTPases. The activity of a large number of transcription factors which activate the transcription of number of genes, are modified in turn by these pathways in response to calcium influx. Numerous biological processes in neurons are affected by these calcium-responsive genes

Like *BDNF*, the expression of hundreds of others genes like *FMRI* is also regulated by synaptic activity. Investigating the regulatory mechanisms that control the transcription of these genes in neurons may provide important insight into activity-dependent neural development and synaptic plasticity.

4 The *FMRI* gene and its gene product FMRP

4.1 *FMRI* gene

In 1991, Mandel et al. identified *FMRI* as the gene mutated in FXS patients using a positional cloning strategy (Mandel et al., 1991). It belongs to a small gene family that includes the *Fragile X related gene 1 (FXR1)* and the *Fragile X related gene 2 (FXR2)*. These genes are autosomal genes mapping at 3q28 and 17p13.1 respectively. There is a high sequence similarity between *FMRI* and *FXR1* and *FXR2* especially in their functional domain and overlap in tissue distribution. Furthermore, *FMRI*, *FXR1* and *FXR2* are highly conserved in evolution and orthologous are present in vertebrates. Despite of this, FXR1P and FXR2P cannot compensate the lack of FMRP. This observation suggests that the FXR proteins have different functions (Coffee et al., 2010).

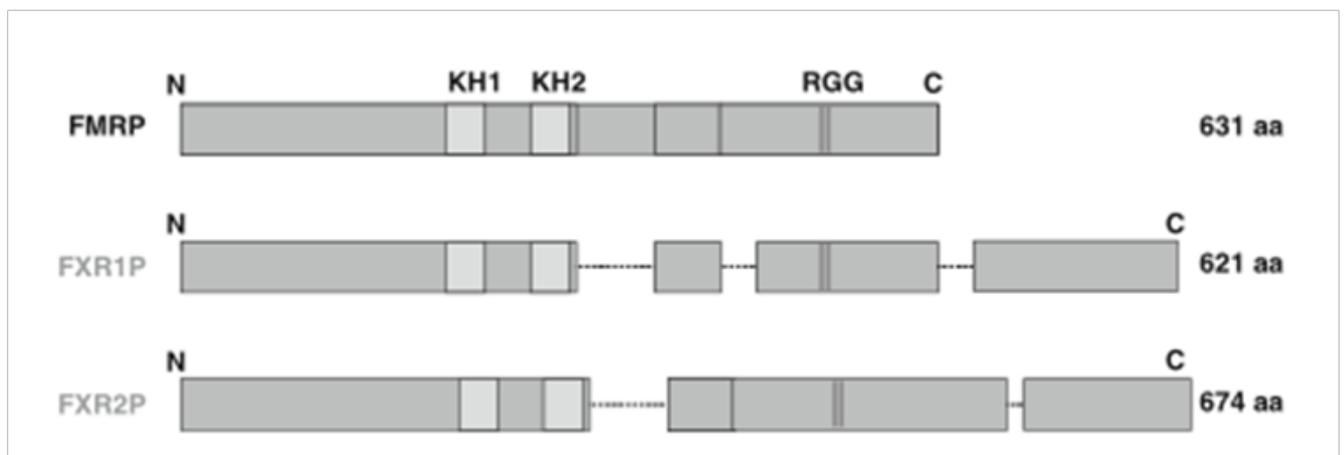


Figure 9: *FMRI* and its paralogs *FXR1* and *FXR2* encoding FMRP, FXR1P and FXR2P respectively. The KH domains and the C-terminal RGG box are the RNA-binding domains (Pop et al., 2013).

The *FMR1* gene is composed of 17 exons and subject to alternative splicing occurring preferentially at exons 12, 14, 15 and 17.

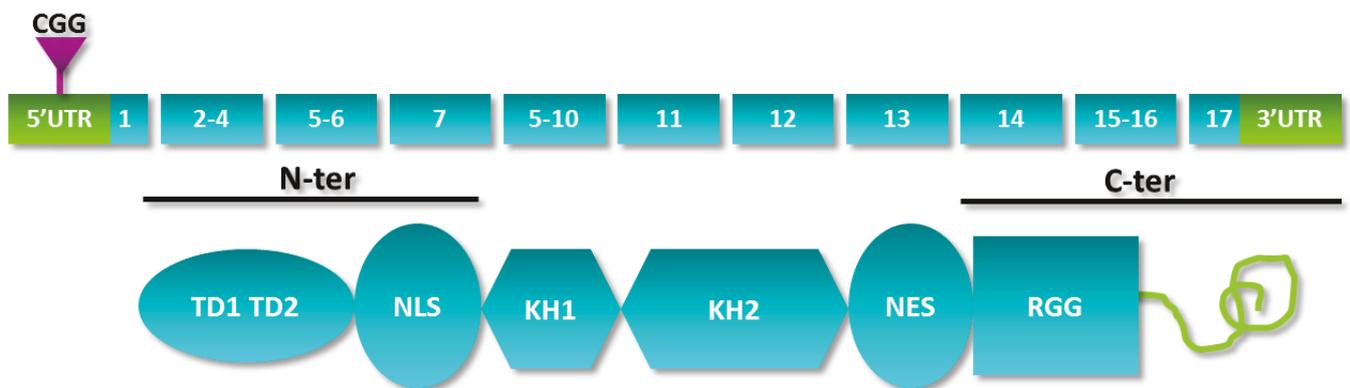


Figure 10: Scheme of the *Fragile X Mental Retardation 1 gene (FMR1)* and protein (FMRP). The CGG expansion is located in the 5' UTR of the gene. The protein shows several functional RNA binding domains as well as a nuclear localization signal (NLS) and a nuclear export signal (NES).

4.2 *FMR1* promoter

The *FMR1* promoter does not possess a typical TATA element but a putative TATA-like sequence and an initiator-like (Inr) sequence near the major TSS. A deletion of these two elements does not abolish gene activity (Kumari and Usdin, 2001). These observations support the idea that other transcription start sites as well as other important regulatory sequences are operating during transcriptional initiation from the *FMR1* gene. In this regard, Beilina et al. in 2004 demonstrated that transcription of the *FMR1* gene is initiated at three different start sites for both neuronal and non-neuronal cells. Indeed, they found that *FMR1* promoter region possesses three Inr sequences which correspond to TSS I-II-III. The Inr sequences are located close to the transcription start sites and have been involved in transcription initiation from TATA-less promoters. For this kind of promoters, binding of Inr-binding proteins to the Inr-elements on the promoter facilitates polII (polymerase II) complex formation either via interaction with TBP (TATA Box-binding Protein or TFIID) and/or other general and specific transcription factors, or by interacting with the CTD (C-Terminal Domain) of RNA pol II (Pugh and Tjian, 1991).

In addition, Beilina *et al.*, demonstrated that the transcriptional initiation from the *FMR1* gene is modulated by the downstream CGG repeat. This coupling of CGG repeat size and start site appears to direct the activation of the more upstream start sites. Two mechanisms are implied. One possible mechanism by which the downstream CGG element could influence start site utilization would be

through the interaction of a CGG binding protein with both component of the transcriptional regulatory machinery like USF1 and USF2 transcription factors and the CGG element itself. The other possibility would be that the expanded CGG repeat element exerts a direct effect in cis by modulating the local chromatin architecture (nucleosome positioning). This mechanism would lead to the altered accessibility of transcription factors to regions within the proximal *FMRI* promoter.

In order to identify the most important conserved promoter elements, in 2001 Kumari and Usdin compared the sequences of the Human *FMRI* promoter sequence with that from two other primates *Pan Troglodytes* and *Maccaca arctoides* and two more evolutionary distant species: *Mus domesticus* and *Canis familiaris* (Kumari and Usdin, 2001). For the amplification of the *FMRI* 5'UTR from the five species, they used a primer derived from the conserved 5'boundary of the *FMRI* promoter and a primer derived from exon 1. The sequences were then aligned with the human and mouse promoter sequences to identify various regions conserved in all five species.

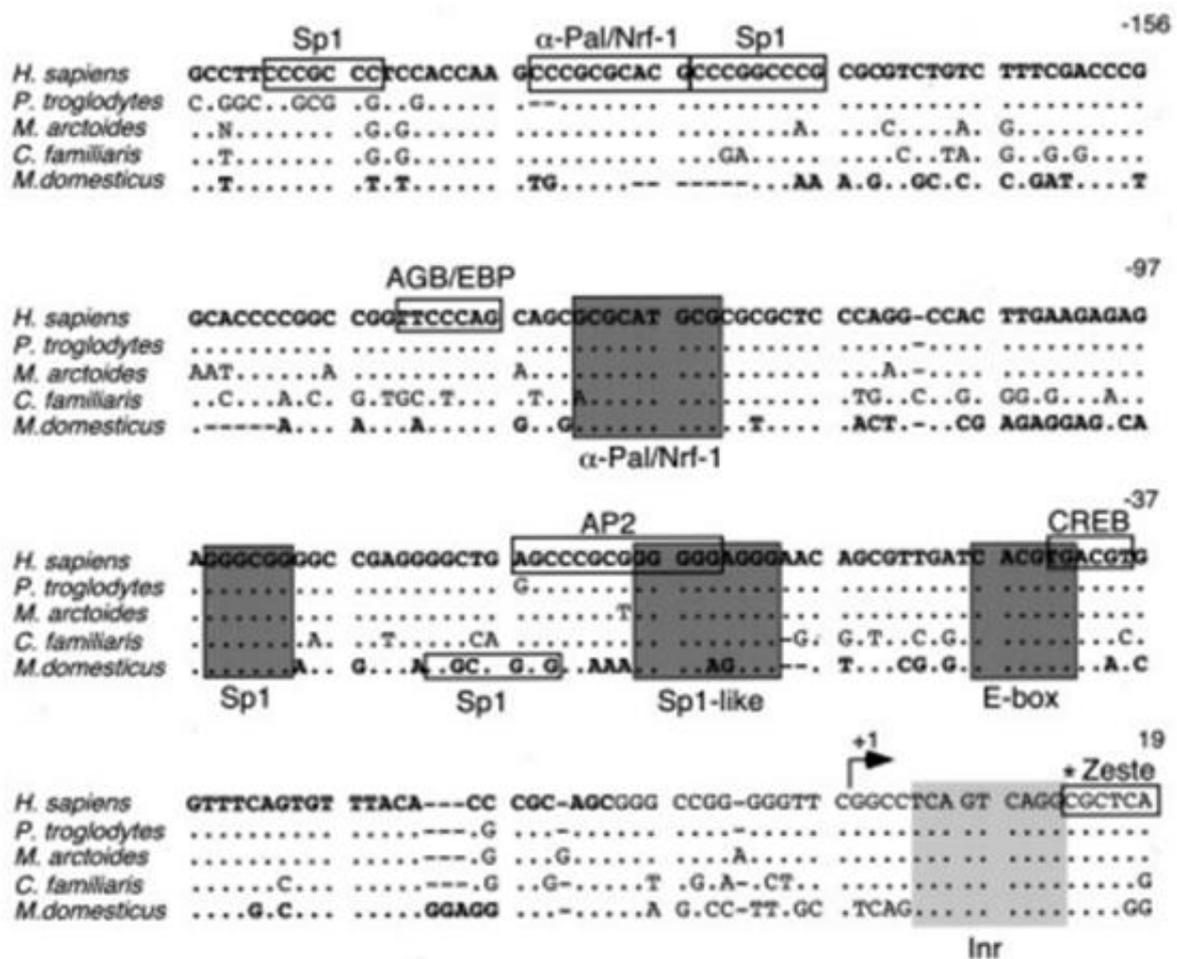


Figure 11: *FMRI* promoter sequences alignments (Kumari and Usdin, 2001). In dark grey boxes, the most conserved transcription factor binding sites for: α PAL/Nrf1, SP1, SP1-like and USF1/2 (E-box). In light grey boxes, the Inr-like sequence corresponding to the TATA-like sequence located near the TSS I (+1).

The minimal conserved *FMRI* promoter may only be 131bp long. No transcription factors (TF) binding sites are conserved between mouse and Human 5' of the α PAL/Nrf1 site and 131 bases 5' of the start of transcription.

Five evolutionary conserved sites are identified in the *FMRI* promoter and four of them are important for transcriptional activity in neuronal derived cells. These include the four protein binding sites α PAL/Nrf1, the two Sp1 or two GC boxes and the E-box (USFs binding site) (shown in the dark gray boxes in figure 11). The conservation of these sites, among the five evolutionary divergent species who shared a common ancestor 80-120 million years ago, indicates that these sites are important for regulation of *FMRI* gene.

For CREB and AP-2 α binding sites, the lack of evolutionary conservation and the failure of these factors to bind in brain and testis extracts, suggests that these two sites may be less important for *FMRI* expression.

Among the several putative cis-regulatory elements within the promoter region of the *FMRI* gene, USF1/USF2 (USFs) and α Pal/Nrf-1 appear to be major regulatory factors involved in activation of the *FMRI* gene in brain and testis extracts. USFs have been reported to be Inr-element binding proteins suggesting that these factors perhaps via the Inr-like sequences associated with sites I-III, seem to play a role in the regulation of *FMRI* transcription (Beilina and al., 2004). This finding is correlated by elevated levels of these factors accounting in part for the elevated *FMRI* expression in these organs. USFs are two similar helix-loop-helix/leucine zippers and are involved in the regulation of several tissues specific developmentally- or metabolically- regulated genes (Chen at al., 2003). α PAL/Nrf-1 are positive regulators of *FMRI* promoter and putative bZip factor, involved in the regulation to cellular proliferation.

These transcription factors are required for normal central nervous system development and may be relevant given the role of *FMRI* in learning and memories (Smith et al., 2004). Thus, these three factors seem to positively affect transcription of the *FMRI* promoter. Moreover, it is known that methylation abolishes α PAL/Nrf-1 binding to the promoter and affect the binding of USF1/USF2 to a lesser degree. Methylation may inhibit *FMRI* transcription not only by recruiting histones deacetylases but also by blocking transcription factor binding. Therefore, deletion of the binding site (by mutational analysis) for these factors reduces transcriptional activity in neuronal derived cell lines (Kumari and Usdin, 2001).

Concerning SP1, it is a finger zinc transcription factor gene that binds to GC rich motifs of many promoters. The encoded protein is involved in many cellular processes including cell differentiation, cell growth, apoptosis, immune response, response to DNA damage and chromatin remodeling. Post translational modification such as phosphorylation, acetylation, glycosylation and proteolytic processing significantly affect the activity of this protein. Gel mobility and foot printing assays showed that SP1 and AP-2 α transcription factors could be involved in the functioning of the *FMRI* promoter. These transcription factors were found within the 5'UTR of *FMRI* in normal cells with a transcriptionally active *FMRI* but were absent in cells with Fragile X mutations (Carillo C. et al., 1999).

In the *FMRI* promoter, SP1 and Nrf-1 are potent and synergistic activators of transcription from a *FMRI* driven reporter in Drosophila SL2 cells. SP1 is known to be methylation insensitive (Holler M. et al., 1988). Indeed, dense methylation of the *FMRI* promoter results in loss of Nrf-1 stimulation but only partially reduces SP1 activity. In addition, mutation of these sites in reporter plasmids results in a loss of promoter activity (Carrillo C. et al., 1999).

In 2004, Smith et al. found that Nrf-1, SP1 and E-box binding USFs 1 and 2 bind the endogenous *FMRI* promoter in normal cells but not in Fragile X cells. The *FMRI* promoter also harbors several potential silencer elements recognized by such factors as NF1 and MAX (Carillo et al., 1999). This finding was reported by Smith et al in 2004. In the same study, they showed in HeLa cells that USFs can likely be also repressor of *FMRI* transcription. This finding was in opposition with the study of Kumari and Usdin in 2001. It seems clearly that these results need to be interpreted in the context of the type of cell line or tissue used in these studies. Indeed many transcription factors such as AP-2 α and α PAL/Nrf-1, identified to be important in *FMRI* transcription, show unique expression patterns at both the cellular and tissue level. In addition, AP-2 α is highly expressed during embryonic and prenatal development and is thought to be essential for CNS development in embryos (Zhang et al., 1996).

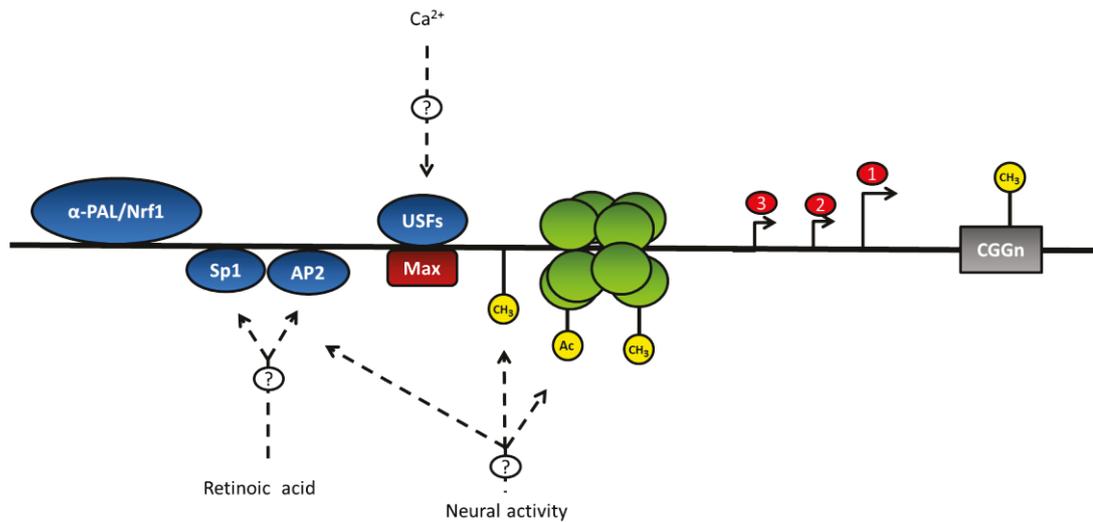


Figure 12: Model for the transcription regulation of *FMR1* (adapted from Lim et al., 2005). Transcription factors α -Pal/Nrf1, Sp1, AP-2 α , and USFs bind the *FMR1* promoter and activate its expression. Some of these factors might be influenced by signaling cascades that involve retinoic acid, Ca²⁺, and/or neural activity. MAX has been identified as repressor of *FMR1* transcription. Epigenetic factors also play an important role in the regulating *FMR1* expression. Acetylation and methylation of the N-terminal tail of histones as well as the methylation of CGG repeats and CpG sites on the promoter has been shown to modulate *FMR1* activity. Three different transcription start sites are shown (numbered 1–3). Upstream start sites are preferentially used.

Some of these factors, described above, might be influenced by signaling cascades that involve retinoic acid, Ca²⁺ and/or neuronal activity (Figure12). The work of Aoto et al in 2008, demonstrated that tetrodotoxin (TTX) and D-2-amino-5-phosphonovalerate (APV) treatments drive intracellular synthesis of retinoic acid which reduces the association of the RNA binding protein RAR α with selected mRNAs such as GluA1, thus removing their translational block. USFs can modulate the expression of several activity dependent genes via calcium mediated signaling pathway (Chen et al., 2003). And neural activity plays an important role in gene expression and, in turn, the activity responsive genes play important roles in synaptic function (West et al., 2002).

In the vast majority of cases, the Fragile X Syndrome is caused by the absence of the *FMR1* gene expression. From a molecular point of view, the pathology results from the absence of FMRP encoded by this gene (Pieretti, Zhang and al 1991, Verkerk and al. 1993).

4.3 Fragile X mental retardation protein : FMRP

FMRP is an RNA binding protein that is maximally 631 amino acids long. Twelve FMRP isoforms, with a molecular weight ranging between 70 and 80 KDa (Verkerk et al., 1993), are expressed in many tissues and organs with a tissue-specific relative abundance (Kaufmann et al., 2002). During a specific period in human embryonic development, this protein has been shown to bind to nitric oxide synthase 1 transcript which is important for the normal development and function of the nervous system especially in some processes like language recognition, speech production, attention, decision making, complex social behaviors and emotional processing (Kwan et al., 2012).

4.3.1 Spatial and temporal expression pattern of FMRP

Particularly high expression of FMRP is observed in ovary, thymus, eye, spleen and esophageal epithelium with an abundant expression in brain and testis. In brain, FMRP expression is restricted to differentiated neurons, mainly in the hippocampus and granular layer of the cerebellum. FMRP expression is absent from non-neuronal cells (Devys et al., 1993, Hinds et al., 1993). The mostly neuronal FMRP is concentrated in the perikaryon and proximal dendrites, expression is also detected in synapses but not in axons (Feng et al., 1997).

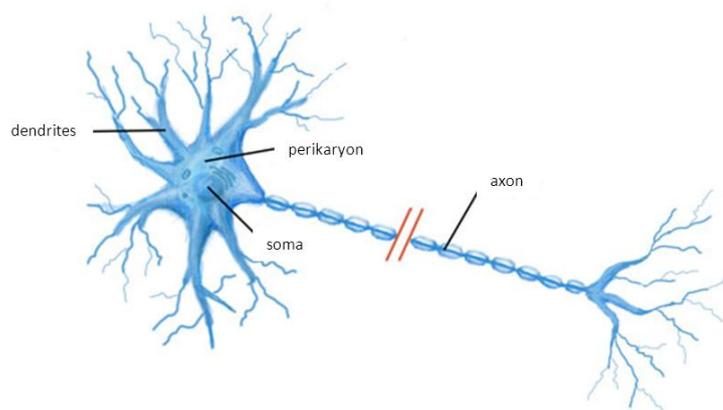


Figure 13: Scheme of a neuron.

(<http://www.solunetti.fi/fi/histologia/perikaryon>/<http://www.solunetti.fi/fi/histologia/perikaryon/>).

During development, FMRP is particularly found in body tissues including brain, muscle tissue and internal organs of mice. In adulthood, FMRP is enriched in the brain and testis of mice and becomes

much less abundant in muscle tissue (Khandjian et al., 1995). In addition, FMRP expression is mostly confined to neuronal cells in adult brain (Tamanini et al., 1997).

Moreover, immunohistochemical studies proved that FMRP is highly expressed in the cytoplasm of many types of fetal and adult neurons including cerebellar Purkinje cells, brainstem and cortical neurons. In monkeys, three dimensional mapping techniques revealed that FMRP expression is especially high in the cerebellum, striatum and temporal lobe. Temporal lobe structures, especially hippocampus, play an important role in mediating memory and learning process. This finding suggests that deficits in behavior and cognition in FXS patients may be linked to the loss of FMRP from specific sub regions of the brain (Zangenehpour et al., 2009).

In watching more closely at the subcellular distribution of FMRP, in using cultured mouse hippocampal neurons, it exhibits a strong expression in the cytoplasm and proximal dendrites. Thus, FMRP exhibits largely overlapping cytoplasm and dendrites expression patterns in developing and adult mammalian neurons suggesting that FMRP controls some aspect of mRNA metabolism in the somatodendritic compartment (Tamanini et al., 1997).

These results suggest that FMRP may play an important role in the presynaptic compartment and that the various behavioral deficits seem to be explained in FXS by the loss of FMRP from a large number of important neuronal circuits.

4.3.2 FMRP structure

FMRP is structurally divided in three regions: N-terminal region, central region and C-terminal region (Nelson et al., 2013). Most of the protein-protein and protein-mRNA interactions occur at the N-terminal and central regions of FMRP. A high degree of conservation is seen for these two regions among the different family members (FMRP-FXR1P and FXR2P), in comparison to the C-terminal region (Menon et al., 2004). In the N-terminal region, we can find two Tudor domains that can bind single strand nucleic acids and a nuclear localization signal (NLS). The central region is composed of two K homology domains (KH) that share a high degree of homology with the HnRNP K domain, a Nuclear Export Signal (NES) and two coiled coils (CC) involved in protein-protein interaction (Valverde et al., 2008). The last region called the C-terminal region is the least conserved region among different species. This region contains a RGG box characterized by a

conserved Arg-Gly-Gly triplet (Darnell et al., 2001). The KH domains and RGG box are motifs characteristics of RNA binding proteins (Siomi et al., 1994).

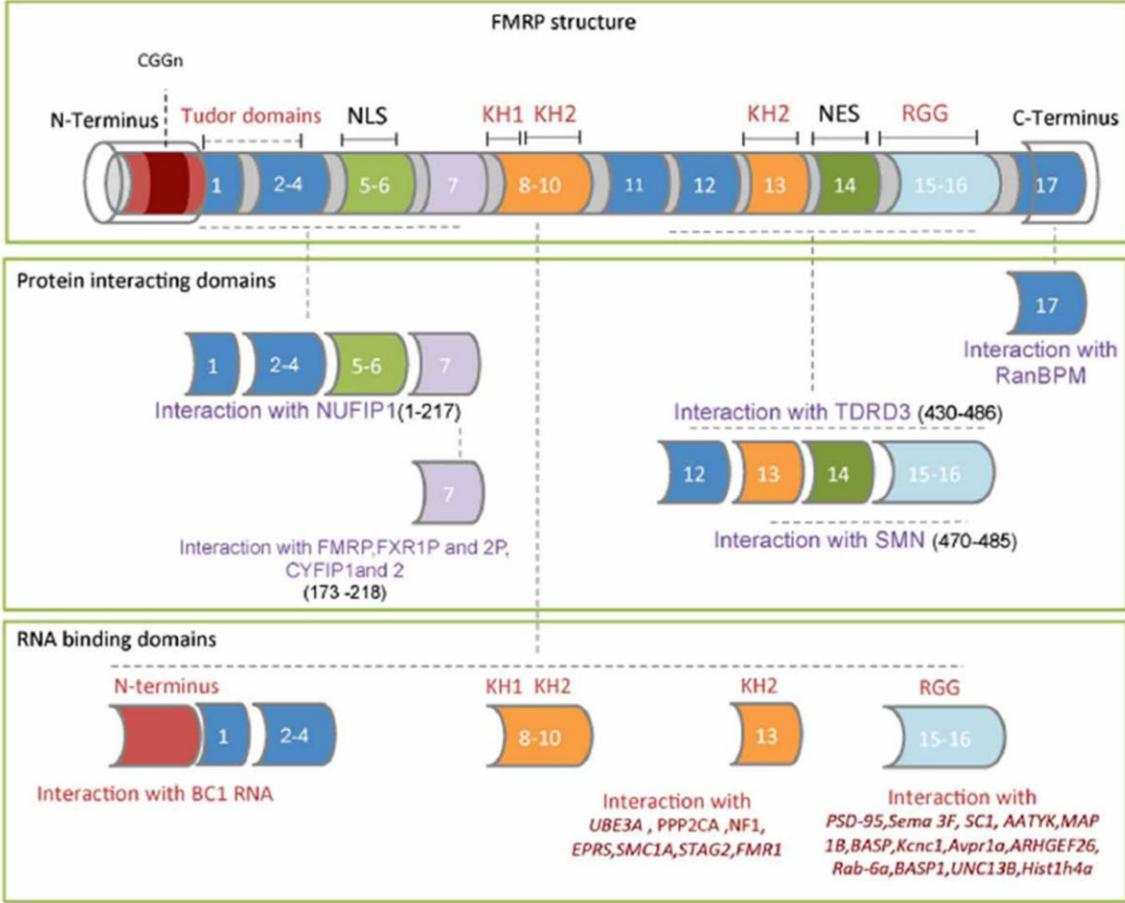


Figure 14: FMRP exon structure comprising its functional domains. FMRP structure frame: The red box at the N-terminus of exon 1 indicates the location of the CGG triplet repeat within the 5'UTR region of the mRNA. The four RNA binding domains are: the N-terminus, the two K homology domains (KH1 and KH2) and the RGG box. Protein interacting domains frame: FMRP domains interacting with NUFIP1, CYFIP1, CYFIP2, FXR1P, FXR2P, TDRD3, and SMN proteins. The FMRP amino acid sequence involved in these interactions is shown between the brackets. The nuclear localization signal (NLS) and the nuclear export signal (NES) are also indicated. RNA binding domains frame: The FMRP RNA binding domains and the RNA/mRNA targets directly bound are indicated (Fernandez et al., 2013).

As indicated by the presence of the KH domains and RGG box, FMRP is an RNA-binding protein able to recognize some coding and non-coding RNAs including the brain cytoplasmic RNA BC1/BC2 (Ashley et al., 1993) and micro RNAs (Gessert et al., 1993). Most of these mRNA targets have been found to be located in the dendrites of neurons. These mRNAs are required to increase contact with other neurons. FMRP binds a significant percentage of brain mRNAs and has a preference for two classes of mRNAs that contain either a G quartet structure or a U rich sequence (Darnell et al., 2001).

In 2003, Miyashiro et al, identified some 80 mRNAs of which 60% were directly associated with FMRP. In the brain of KO mice, some of these mRNAs and their corresponding proteins display subtle changes in location and abundance. Furthermore, brain tissue from humans with FXS and mouse models also show abnormal dendritic spines. The subsequent abnormalities in the formation and function of synapses and development of neural circuits result in impaired neuroplasticity, an integral part of memory and learning.

FMRP can interact directly or indirectly with several cytoplasmic and nuclear proteins. In 2003, Bardoni et al, used yeast to hybrid or co-immunoprecipitation techniques to describe direct interaction of FMRP with NUF1P1 (nuclear FMRP interacting protein), 82-FIP (82kDa FMRP interacting protein) and MSP58 (microspherule protein 58).

Among the best characterized protein interactions, the part of FMRP protein encoded by exon 7 is responsible for the interaction with the CYF1P1 and CYF1P2 (Cytoplasmic interacting protein 1 and 2), which link FMRP to the RhoGTPase pathway (Schenck et al., 2001). This interaction permits the formation of the eIF4E-CYF1P1-FMRP complex that blocks mRNAs translation process (Napoli et al., 2008).

The other best characterized interaction is with the two paralogs Fragile X related proteins 1 and 2 (Zhang et al., 1995). By inducing structural changes in conformation, these proteins can modulate the affinity of FMRP for different classes of mRNAs.

In addition, residues 470-485 are also essential for the interaction with SMN, and the residues 430-486 in the second KH domain are crucial for the binding with TDRD3 (Linder et al., 2008).

At the end, the C terminal region is involved in the interaction with RanBPM, this interaction permits to modulate the FMRP-mRNA binding activity (Menon et al., 2004).

4.3.3 FMRP functions

The exact functions of FMRP are still poorly understood. FMRP has been suggested to play roles in the formation of mRNP complex in the nucleus, in the regulation of mRNA transport, in the control of the mRNA translation and protein synthesis by the miRNA pathway, in the mRNA stability, in the dendritic spine development and recently in DNA damage response (DDR). For more understanding, the various FMRP functions are resumed in the figure 15 below.

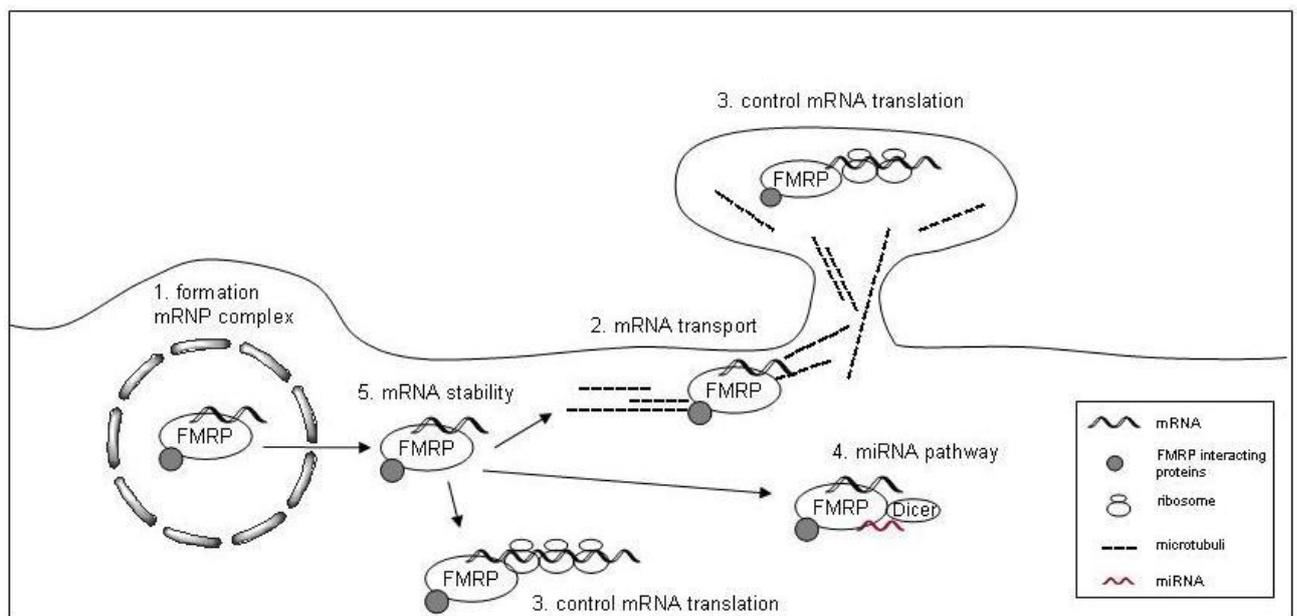


Figure 15: FMRP functions in neuron. 1) After FMRP binds target mRNA and proteins in the nucleus, forming an mRNP particle, it is exported to the cytoplasm, where it can exert multiple functions. 2) The complex can stay in the cell body or move to dendritic spines, transporting the mRNA. 3) Subsequently, it can associate with translating ribosomes, regulating mRNA translation. 4) FMRP may also function as a translational regulator via its role in the miRNA pathway. 5) A last known function of FMRP is the involvement in mRNA stabilization. (Heulens, 2011).

4.3.3.1 Regulation of the translation and the synaptic protein synthesis

FMRP is thought to play a role in synaptic plasticity through the regulation of mRNA transport and translational inhibition of local protein synthesis at the synapses (Jin P. and al., 2004).

The major molecular mechanism by which FMRP regulates translation seems to be by the RNA interference (RNAi) pathway. In this way, Qurashi and al, in 2007, observed specific interactions between dFmrp (Drosophila Fragile X Mental Retardation Protein) and two functional RNA interference silencing complex (RISC) proteins, dAGO (Argonaute 2) and Dicer, which mediate

RNAi. Micro RNAs seem regulate the translation of its target genes. Once FMRP binds to its specific mRNA ligands, it recruits RISC along with miRNAs and it permits to facilitate the recognition between miRNAs and their mRNAs ligands. One single miRNA can regulate multiple mRNA targets and a given mRNA can be regulated by multiple miRNAs. This transient and temporal translational regulation allows a rapid and reversible translation, a requirement for protein dependent synaptic plasticity. This protein synthesis occurs in the soma but also along axons, dendrites and postsynaptic sites.

A scheme of these mechanisms was resumed by Jin and Warren in 2003 and permitted to propose a model for FMRP neuronal functioning.

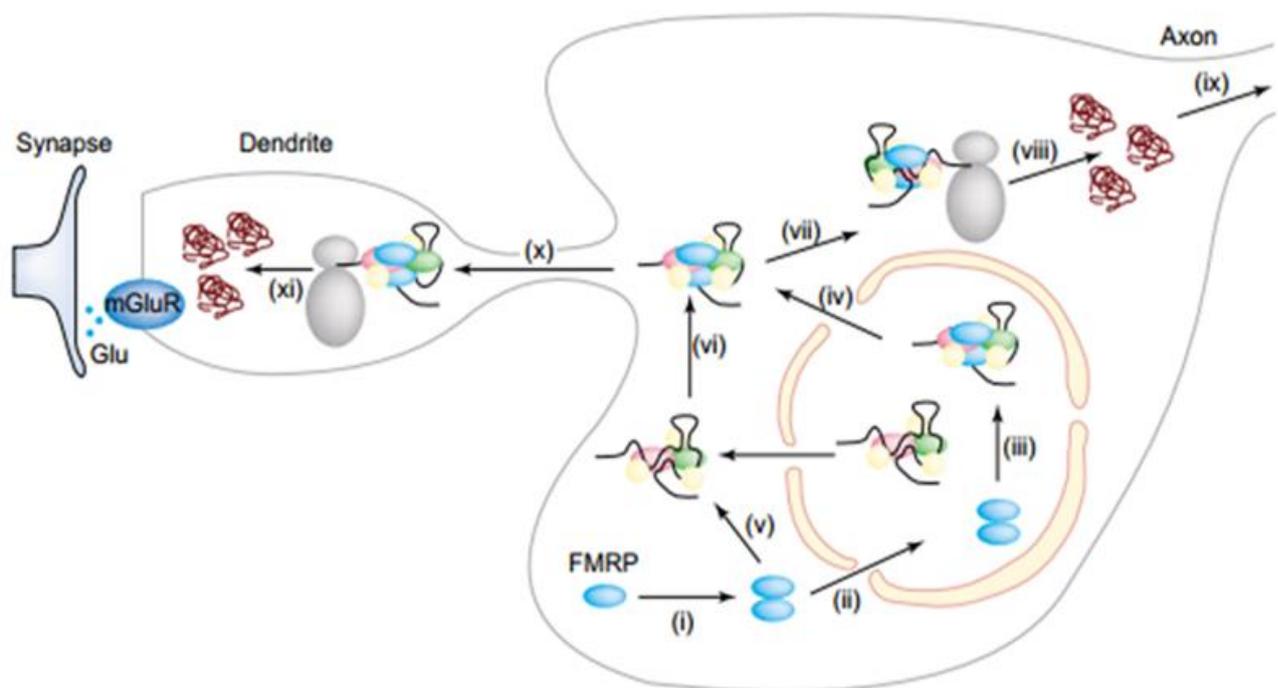


Figure 16: Model of Fragile X Mental Retardation Protein (FMRP) in the neuron (Jin and Warren, 2003).

As indicated in the figure 16, FMRP is dimerized in the cytoplasm (I) and is transported into the nucleus via its NLS (II). In the nucleus, dimerized FMRP assembles into a messenger ribonucleoprotein (mRNP) complex and interacts with specific RNA transcripts and others proteins (III). Then, the FMRP-mRNP complex is transported out of the nucleus via its NES (IV). In the cytoplasm, FMRP might bind to RNA and associate with the mRNP (V, VI). Once in the cytoplasm, the FMRP-mRNP complex interacts with the RISC before linking with ribosomes in the cell body (VII) to produce the proteins (VIII). Some of these proteins might be important for axon

guidance (IX). Alternatively, the FMRP-mRNP complex can be transported into dendrites (X) to regulate local protein synthesis of specific mRNAs in response to synaptic stimulation signals such as mGluR (metabotropic glutamate receptor) activation (at XI in the figure 16) or by the activation of glutamate receptors NMDAR and AMPAR respectively the ionotropic receptors N-methyl-D-aspartate and the alpha-amino-3-hydroxy-5-methyl-4-isoxazo-lepropionic acid (Shi et al., 1999). This synthesis is required for long lasting forms of synaptic plasticity that underlie consolidation of long term memories (Flavell and Greenberg, 2008).

The seat of these long term memories in the brain is the hippocampus. Since the late 1950s, with the pioneering work of Brenda Milner, the hippocampus is known to be important for aspects of long term memory storage in human and others mammals.

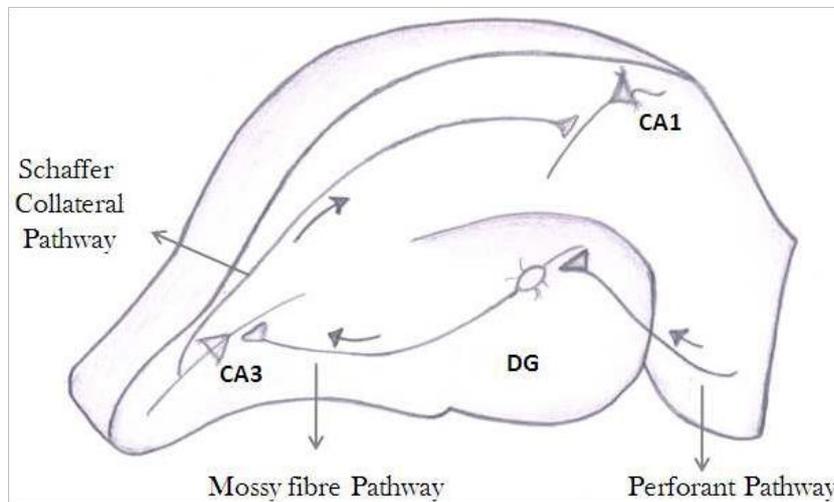


Figure 17: Scheme of hippocampal slices with the three detailed synaptic pathways.

The figure above represents the hippocampus which is divided in three distinctive regions composed of three distinctive kinds of cells. The dentate gyrus (DG) which is composed of granules cells, the CA3 and CA1 regions which are composed of pyramidal cells having different properties.

These regions are connected by well-defined pathways through which signals traverse the hippocampus. Three important pathways are visualized in the hippocampus: the perforant pathway which synapses onto the granule cells in the dentate gyrus, the mossy fibre pathway which synapse on the pyramidal cells in the CA3 region and the Schaffer collateral pathway which permits to the pyramidal cells in CA3 to send excitatory (Schaeffer) collaterals to the pyramidal neurons in the CA1 region of the hippocampus.

In 1973, Bliss and Lomo found that a brief high frequency action potential train in the perforant pathway, in the granule cells of hippocampus, produces an increase in the excitatory synaptic potential. This phenomenon could last for hours and even for days and weeks. They called this facilitation: long term potentiation (LTP).

Like LTP, long-term depression (LTD), also provides a means for regulating the strength of synaptic connections in the mammalian brain. In contrast to LTP, LTD requires a prolonged inhibition of synaptic transmission and several synaptic events including mRNA targeting, local protein synthesis and degradation.

LTD was discovered at the beginning of the 80's in the cerebellar Purkinje cell. This kind of cells is the backbone of the cerebellar cortex and the cerebellar function. It likely plays an essential role in the memorizing. Contrary to the LTP which is triggered by a high frequency synaptic stimulation, LTD is produced by a low frequency nerve impulse (1-5 Hertz). The synapse is then subjected to an inverse transformation to the LTP: instead of seeing their increased efficiency, the synaptic connection is weakened.

The reason for this difference comes from the fact that these two different activation patterns will have on the concentration of calcium ions inside the cell: a significant increase in calcium for LTP or a small increase for the LTD. These different levels of intracellular calcium concentration will cause the activation of distinct second messengers. In the case of LTP, much calcium would activate protein kinases, whereas the low calcium released by the LTD rather activated phosphatases.

It is these enzymes that modify the synapse to make it less effective to the passage of nerve impulses.

It is interesting to note that these are the same neurotransmitter (glutamate) and the same NMDA receptors that are involved in the massive or restricted entry calcium into the neuron.

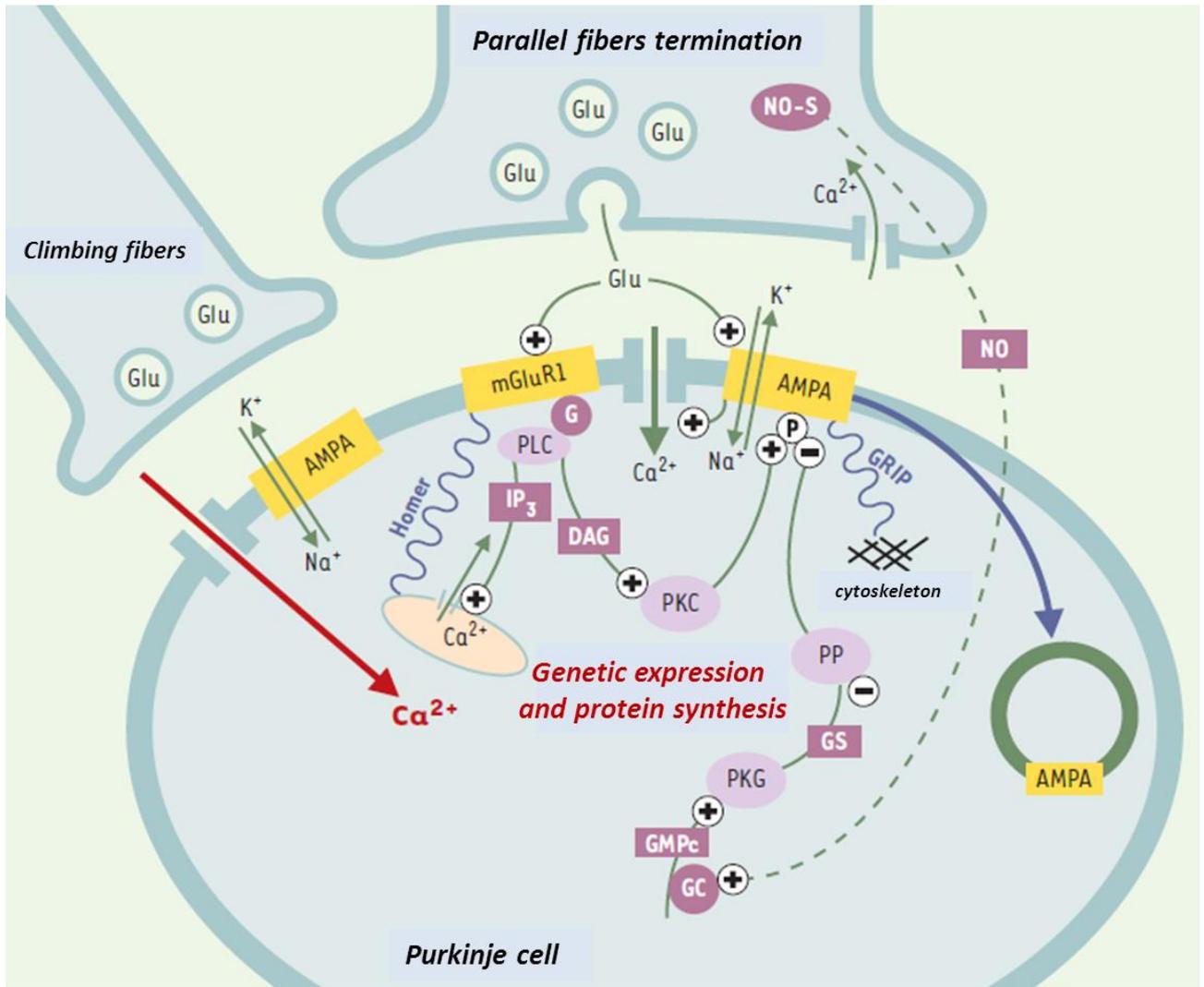


Figure 18: Schematic representation of the molecular and cellular mechanisms of the LTD (Vigot, 2003).

The figure 18 allows describing the various LTD mechanisms. The glutamate released by climbing fibers and parallel fibers activates AMPA receptors triggering the neuron depolarization and the opening of potential dependent calcium channels. The intracellular free Ca²⁺ increase, bound to opening of these channels, is an essential factor for LTD induction. The glutamate coming from the parallel fibers also activates the metabotropic glutamate receptor subtype 1 (mGluR1) triggering the phospholipase C (PLC) activation and the diacylglycerol inositol tri-phosphate formation. This one induces after receptors activation, the releasing of Ca²⁺ from intracellular stocks. Two kinase proteins called protein kinase C (PKC) and protein kinase G (PKG) have a central role in the control of the AMPA receptors phosphorylation state. Indeed, the PKC directly phosphorylates receptors only after its activation by the inhibition of the dephosphorylating of these receptors. The intracellular way using this kinase is activated by a diffusible neurotransmitter: nitric oxide (NO) which is synthesized and released by the parallel fibers termination. Its distribution in Purkinje cell

causes the guanylate cyclase activation and the cyclic GMP formation. This one is the activator of PKG which inactivates the phosphatases through a specific substrate: G substrate.

Two main types of LTD are found in hippocampus: one is dependent on NMDA receptors and the other is dependent on metabotropic glutamate receptors (mGluRs). These both forms of LTD lead to a decrease in postsynaptic AMPA receptors. The main distinction in these both forms is that early mGluR-triggered LTD requires the fast translation of mRNAs localized in postsynaptic dendrites, whereas hippocampal NMDA-triggered LTD does not require protein synthesis for its early expression (Huber et al, 2000).

Studies have established that FMRP is important for synaptic plasticity, in particular in mGluR-dependent LTD. This association came from a finding which revealed that synaptoneurosomal activation of Gp1-mGluRs stimulates the FMRP synthesis (Weiler and Greenhough, 1993). In *Fmr1* KO mice, DHPG-induced LTD plasticity is strongly increased in hippocampus. This effect on LTD is due to dysregulated local protein synthesis (Huber et al., 2002) and permitted to settle the bases to describe the “mGluR Theory” (Bear et al., 2004). This theory proposes that under normal conditions, in response to Gp1-mGluR activation, FMRP synthesis maintains the balance of mGluR-LTD by acting as a brake on the synthesis of new proteins upregulated by Gp1-mGluR signaling. In the absence of FMRP, Gp1-mGluR dependent mRNA translation continues unopposed, leading to an overabundance of “LTD proteins”.

Indeed, by repressing in vivo and in vitro translation, FMRP plays a role in both basal and activity dependent local protein synthesis (Zalfa et al., 2003). This phenomenon was visualized in lymphoblastoid cells from individuals affected by FXS, in which it has been observed that several hundred FMRP mRNA targets showed an abnormal polysomal distribution triggering to an increased translation (Brown et al., 2001). In addition, in *Fmr1* KO mice, protein synthesis of FMRP-targeted mRNAs is increased, extending to synapses the function of FMRP as a translation repressor (Zalfa et al., 2003). On the scheme below, we can resume the various effects of FMRP in translation dependent synaptic plasticity.

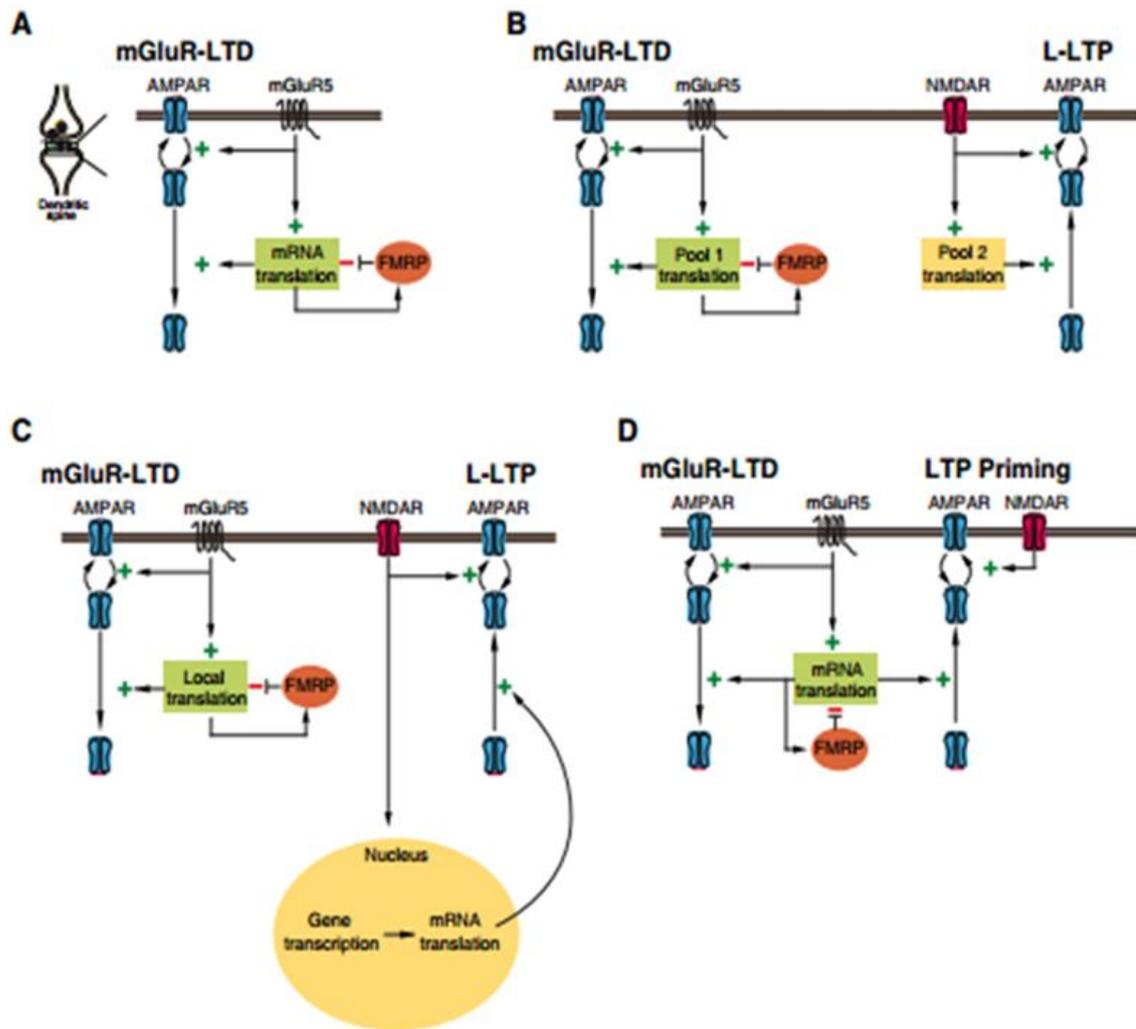


Figure 19: The role of FMRP in translation-dependent synaptic plasticity (Sidorov et al., 2013). (A), FMRP and mGluR5 impose opposite regulation on the local mRNA translation required for mGluR-LTD expression. We can deduce that in absence of FMRP, there is excessive protein synthesis and exaggerated LTD. In the (B), different pools of mRNA might be available and are differentially required for LTD versus LTP, and FMRP may specifically regulate the pool required for LTD. In the (C), FMRP is explicitly involved in the regulation of dendritically localized translation and not in the regulation of the somatic translation. By the way, FMRP impacts only on the forms of synaptic plasticity required for local translation such as mGluR-LTD. However, in the (D), FMRP also regulates protein synthesis involved in mGluR-dependent facilitation of LTP. This finding suggests that the translated proteins under the control of FMRP may be involved in bidirectional maintenance of plasticity rather than being specific to LTD.

4.3.3.2 Regulation of mRNA transport

FMRP contains nuclear localization signal (NLS) and a nuclear export signal (NES), indicating that FMRP has the ability to act as a chaperone for trafficking mRNAs from the nucleus into the cytoplasm (Eberhart et al., 1996) (see figure 16). Loss of FMRP impairs the activity-dependent transport of these mRNAs into dendrites by reducing their interaction with kinesin-1 (Dictenberg et al., 2008). In *Drosophila* neurons, FMRP regulates the movement of FMRP target mRNAs, suggesting the idea that FMRP, in acting as an adaptor protein between its target mRNAs and microtubule-based motors, influences mRNA transport (Estes et al., 2008).

4.3.3.3 Regulation of mRNA stability

In 2007, Zalfa et al. reported a new cytoplasmic regulatory function for FMRP: control of mRNA stability. They found that in mice, FMRP binds the mRNA encoding PSD-95 (postsynaptic density protein 95). This protein is a key molecule in the regulation of the neuronal synaptic signaling and learning. Increasing message stability appears when this interaction occurs at the 3'untranslated region of the PSD-95 mRNA. In addition, this stabilization is increased by mGluR activation. These results suggest that dysregulation of mRNA stability might contribute to the cognitive impairments in individuals with FXS.

4.3.3.4 Regulation of the dendritic spine development

The dendritic spines are crucial for the processing of synaptic inputs. One way in which neuronal activity regulates synaptic processing is by altering the length or complexity of dendritic spines.

In the brain and particularly in neurons, FMRP is localized at the base of dendritic spines in association with polyribosomes. This association is RNA and microtubule dependent, suggesting a role for FMRP in mRNA trafficking and dendritic spine development. Dendritic spines are the postsynaptic compartments of the most excitatory synapses in mammalian brains. In Fragile X Syndrome, dendritic spines are denser apically, thin, tortuous and elongated and an immature-appearing distribution profile of spine morphologies in the neocortex was found compare to the normal brain controls (Irwin et al., 2002).

Harris et al. in 1992 showed that the distribution of spines along apical shafts of pyramidal cells in hippocampal subfield CA1 shifts from an abundance of thin spines in young animals to predominantly stubby or mushroom-shaped spines by adulthood. In *Drosophila*, loss of *dfmrp* function (which acts as a regulator of cytoskeleton stability) in neurons results in inappropriate sprouting, branching and growth, it gives rise to gross changes in both axon and dendrite projections in motor, sensory and central neurons (Zhang et al., 2004).

In addition, loss of FMRP results in altered microtubule dynamics that affect neural development and indicate a potential role for FMRP in synaptic plasticity (Zhang et al., 2001). MAP1b is among the most abundant neuronal microtubule associated proteins (MAPs) in the brain. An increasing number of reports indicate the critical role for MAP1b in modulating microtubule dynamics in the growing neurite essential for neural network development. Indeed, the lack of FMRP-dependent translation suppression results in overproduction of MAP1b and aberrantly increased microtubules stability in *FMR1* KO neurons suggesting that FMRP is involved in regulating microtubules dynamics during brain development (Lu et al., 2004). In addition, microtubule dynamics also controls the trafficking of neurotransmitter receptors to the synaptic junction (Serge et al., 2003). Indeed, abnormalities in glutamate receptor mediated long term plasticity could result to the altered microtubule dynamics in *FMR1* KO neurons which could affect neurotransmitter dependent synaptic communication (Huber et al., 2002).

4.3.3.5 DNA Damage Response

In the work of Alpatov in 2014, using mouse spermatocytes as a model, the authors showed that FMRP decorates meiotic chromosomes and regulates γ H2A.X induction, BRCA1 and ATR recruitment, and resolution of single-strand repair intermediates during meiosis (Alpatov et al., 2014). These findings identified FMRP as a chromatin-binding protein and showed that FMRP plays a role in the DNA damage response at the chromatin interface independently from the canonical role of FMRP in translational regulation.

5 *In vivo* models for FXS

Different FXS *in vivo* models have been generated to study the effects of the lack of FMRP on behavior, mRNA translation and pharmacological interventions. Thus, FXS studies have been performed on fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*).

The *Drosophila melanogaster* orthologous of FMRP, dFmrp, exhibits considerable amino acid sequence similarities with the vertebrate FMRP, FXR1P and FXR2P. It exhibits many functional properties comparable to FMRP such as RNA-binding capacity, repression of translation and modeling neurons in the nervous system (Wan et al., 2000). Morphological abnormalities of neurons were observed in the central nervous system. Loss of dFmrp resulted in mushroom body defects and altered axonal branching (Zhang et al., 2001). This animal model was ideal for screening a chemical library of 2000 compounds and identified nine molecules that rescued lethality phenotype including three molecules implicate in the GABAergic inhibitory pathway. GABA treatment rescued mushroom bodies defects, abnormal male courtship behavior. These data are consistent with GABAergic impairment in Fragile X syndrome (Chang et al., 2008).

To study embryonic development in a vertebrate system, zebrafish is a very convenient animal. All organs are developed in 24 hours and the transparency of the animal allows direct observation of the embryonic development. The zebrafish genome encodes for all three orthologs of FMRP, FXR1P and FXR2P (Engels et al., 2004). Using the morpholino antisense oligonucleotide knockdown technique, a FXS phenotype has been observed including abnormal axonal branching, neuronal guidance and defasciculation defects (Tucker et al., 2004). However these results are under debate and this approach appears to result from potential experimental artifacts and seems not suitable to use as FXS disease model (den Broeder et al., 2009).

FMRI is conserved between human and mouse with a nucleotide and amino acid identity of 95% and 97% respectively (Ashley et al., 1993). The *Fmr1* KO mice have been studied extensively and it was demonstrated to be the most useful animal model to study FXS. The *Fmr1* KO mice show increased testicular weight (Bakker et al., 1994), epileptic seizures occur spontaneously in 20% of the individuals with FXS (Mumeci et al., 2000) and numerous behavioral tests were performed in the *Fmr1* KO mice and permitted to highlight the behavioral deficits in the *Fmr1* KO mice. In addition *Fmr1* KO mice show deficits in spatial learning, a defect of prepulse inhibition (PPI) of acoustic startle response and increased locomotor activity (Bakker and Oostra, 2003). Anxiety and

repetitive behavior have been found in *Fmr1* KO mice too. An important finding is that *Fmr1* KO mice exhibit abnormal spine morphology in different brain areas similar to the abnormalities found in FXS patients. Some studies found a higher dendritic spine density in cortex and hippocampus of *Fmr1* KO adult mice (Galvez and Greenough, 2005). In addition, most of the studies report an immature dendritic spine phenotype both in the cortex and in the hippocampus of *Fmr1* KO mice compared to the wild type controls (Irwin et al., 2002). The same immature dendritic spine phenotype was found in primary hippocampal neuron culture and in dissociated neuron cultures from FXS patients (Levenga et al., 2011b).

Moreover, in the *Fmr1* knockout mouse, a delay in somatosensory map formation was found, alterations in the morphology profile of dendrites and spines of neurons and a decrease in the synaptic levels of proteins involved in glutamate receptor signaling at times corresponding to the highest levels of FMRP expression. These results suggest that bad timing of developmental processes caused by the loss of FMRP would lead to alterations in neural circuitry that underlie behavioral and cognitive dysfunctions associated with FXS (Till et al., 2012).

6 Therapeutic interventions

6.1 The mGluR theory

According to the “mGluR theory” (Bear et al., 2004) described above in the text, in FXS, absence of FMRP leads to exaggerated protein synthesis following Gp1-mGluR activation and consequently to increased AMPAR internalization. By this way, in blocking the mGluR5 using a specific antagonist, it might reduce the excessive protein synthesis and altered synaptic plasticity in FXS.

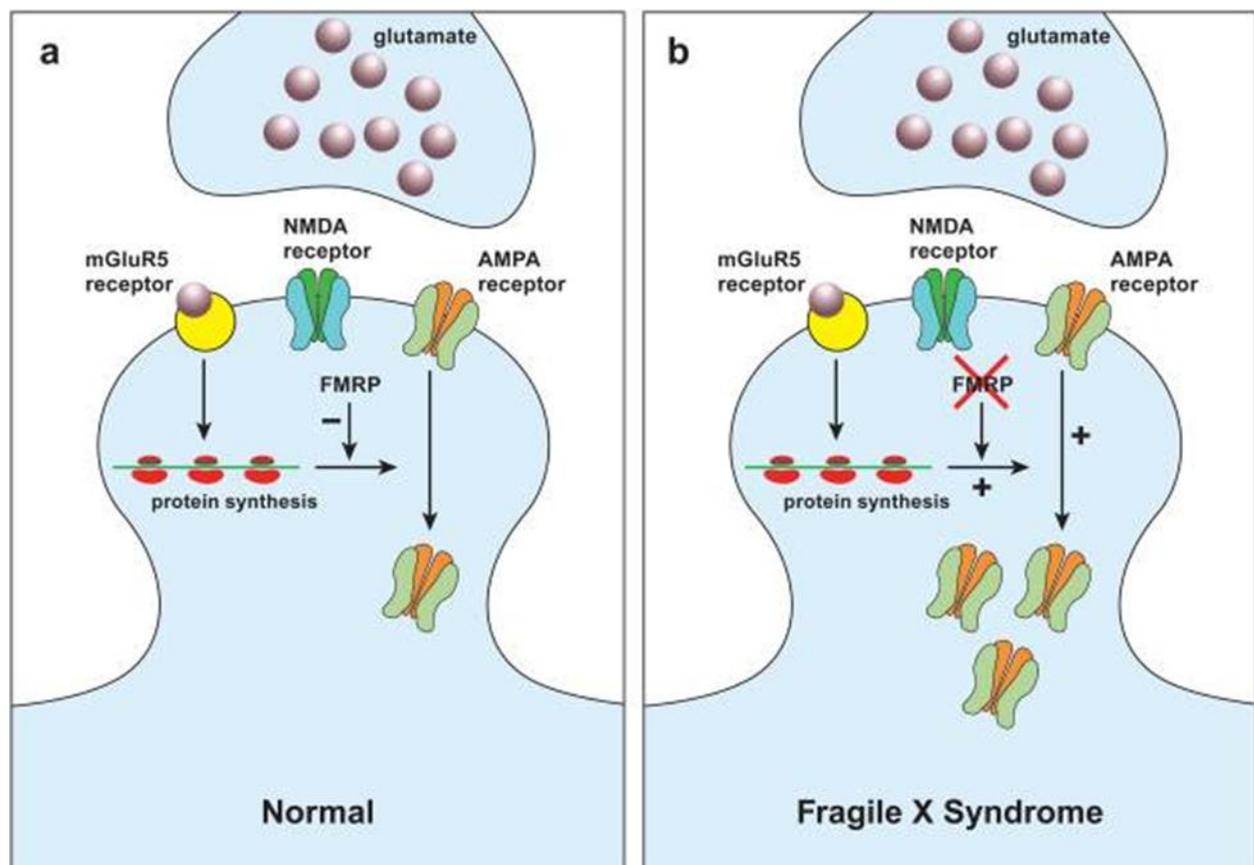


Figure 20: mGluR theory. (a) Stimulation of mGluR5 by glutamate induces local mRNA translation at the synapse. Local protein synthesis stimulates the internalization of AMPA receptors, which is essential for long-term synaptic plasticity. FMRP negatively regulates transcription and reduces the internalization of AMPA receptors. (b) By extrapolation from findings in *Fmr1* KO mice, neurons from patients with FXS have increased internalization of AMPA receptors in the absence of FMRP, which weakens the synapse.(Levenga, 2010).

Previous studies proved this finding in using treatment with 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and other mGluR5 antagonists (Fenobam, Mavoglurant (AFQ056), STX107, R04917523, CTEP). Moreover, it has been shown that phenotypic characteristics including audiogenic seizures, altered prepulse inhibition (PPI) and open field hyperactivity were reversed in using these treatments (Chuang et al., 2005, Lindemann et al., 2011). In human studies, the use of selective

mGluR5 antagonists (as Fenobam) in FXS showed clinical benefits including reductions in hyperactivity and anxiety (Berry-Kravis et al., 2009). In addition, in another study, the AFQ056 was tested in a seven subject subsets marked by full *FMRI* gene methylation, the authors observed a significant response to AFQ056 compared to placebo. They hypothesized that AFQ056 may permit to treat abnormal behaviors associated with FXS in a subgroup of patients with full *FMRI* gene methylation. AFQ056 (Novartis, Basel, Switzerland) was studied in large scale phase III clinical trials in FXS. Unfortunately, Novartis has announced in April 2014 that the company will be discontinuing its development program in Fragile X for its lead mGluR5 antagonist, Mavoglurant (AFQ056), following negative results in a large international clinical trial in adults (reported in the Fall of 2013) and most recently, in a trial in adolescents. In both placebo-controlled trials, patients taking Mavoglurant did not show improvement over placebo in any outcome measures.

6.2 GABA receptor theory

GABA receptors and the GABAergic system are known to be involved in anxiety, hyperactivity, epilepsy, autism spectrum disorder, insomnia, and learning and memory; these last processes are also disturbed in FXS. Various researchers are agreeing to argue that a dysfunction of the GABAergic system has neurophysiologic and functional consequences that might relate to the behavioral and neurological phenotype associated with FXS. Consequently, the GABA receptors might be novel targets for treatment of FXS. Thus, as described in the previous part, Chang et al., reported that *Fmr1*^{-/-} mutant drosophila die during development when reared on food containing increased levels of glutamate. This lethal phenotype also permitted to screen a chemically library of 2000 compounds and identified nine molecules that rescued lethality and among them three were involved in GABAergic inhibitory pathway. Thus, GABA treatment might rescue several known *Fmr1* mutant phenotypes in flies including mushroom bodies defects, excess Futsch translation and abnormal male courtship behavior.

The best known GABAergic drugs are the benzodiazepines (BZD) which are known to enhance GABAergic function, but their using exhibits undesirable side effects including sedation and ataxia and cessation of treatment can cause rebound of anxiety and insomnia (Nemeroff CB, 2003).

Therefore, another type of drugs known to act as allosteric modulators of the GABA_A receptor is the neuroactive steroids. Indeed, Ganaxolone (endogenous neurosteroid produced in the central nervous

system which modulates GABA through activation of GABA_A receptor) is in phase II clinical trials for promising treatment of catamenial epilepsy (Reddy DS, 2004). Use of Ganaxolone is now planned for treatment of audiogenically induced seizures in Fragile X mice.

For January 2014, Marinus and pharmaceuticals perform another study about Ganaxolone treatment and are currently recruiting participants. This Phase 2 proof-of-concept study is a double-blind, randomized, placebo-controlled, crossover study to investigate Ganaxolone treatment in children with FXS. Up to 60 subjects (ages 6-17 yrs) will be randomized to receive either ganaxolone or placebo for 6 weeks and then cross over to the opposite treatment for another 6 weeks. The aim of the study is to assess the safety, tolerability and efficacy of ganaxolone for treatment of anxiety and attention in subjects with FXS. The authors hypothesize that Ganaxolone treatment compared to placebo will improve anxiety and attention as measured by the several neuropsychological and psychometric tests.

The two strategies are summarized in the figure below.

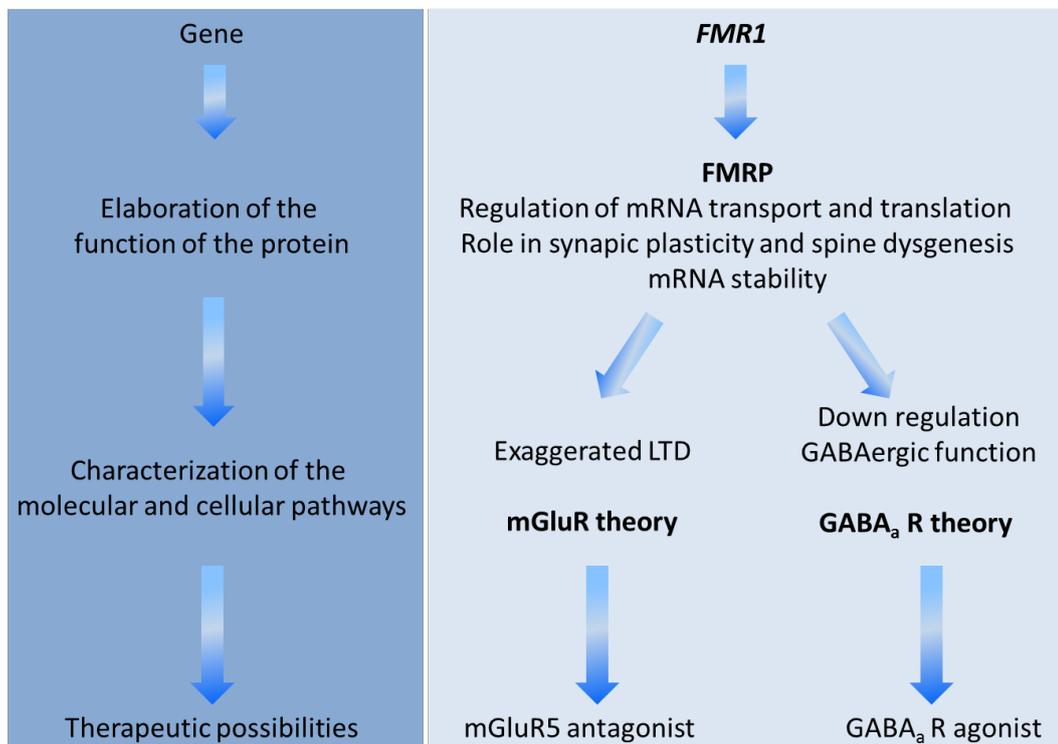


Figure 21: The mGluR theory and the GABA_A R theory strategies in FXS.

7 Specific goals and requirements

The goal of my work was to establish general tools and methods for neuronal reporter gene assays and evaluate their use for screening in Fragile X syndrome and others related pathologies. The various steps of this work are summarized in the scheme below.

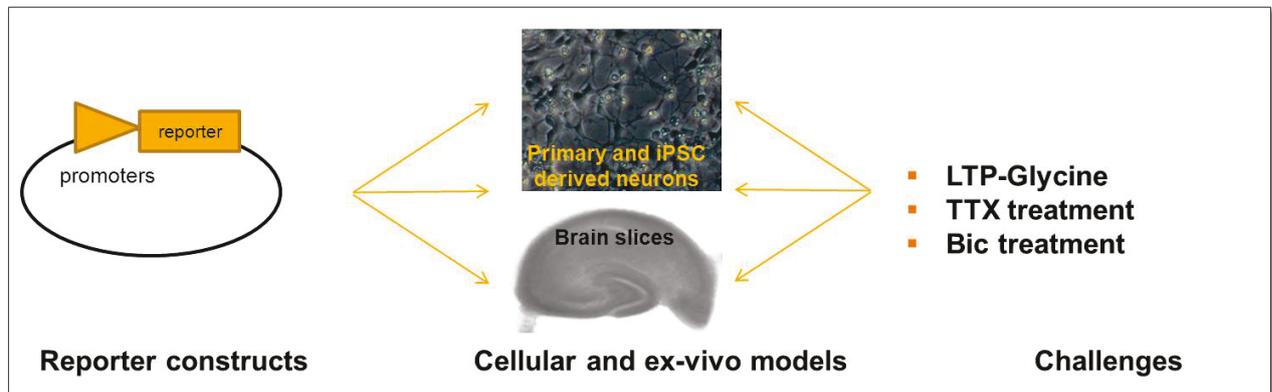


Figure 22: General scheme of the various steps of this work.

First we created various plasmids containing the wild type or mutated *FMR1* promoter to express various fluorescent reporter genes. Second, we transfected all the WTFMR1 plasmids in HeLa cells to test the plasmids constructs and the transfection protocol, then we transfected the WTFMR1-cytGFP promoter plasmids in rat cortical neurons to observe the plasmids expression efficiency in neuronal cells. All plasmids were also transfected in human neuronal precursors, rat cortical neurons and mouse hippocampal brain slices. We also tested various neuronal activity modulators like Bicuculline, TTX and Glycine in some experiments.

These experiments were aimed to better understand transcriptional regulation of *FMR1*. In the publication of Collins et al., it has been reported that three *FMR1* promoter mutations significantly reduce in vitro levels of *FMR1* expression in blood cells of patients with developmental delay who did not carry CGG expansion full mutations.

These three promoter mutations are localized near the three known transcriptional start sites (TSS) of the *FMR1* gene. The transfection of the mutated plasmids in brain slices permitted to evaluate the importance of each TSS in the initiation of the transcription of *FMR1* in defined neuronal cells and pointed to regulatory mechanisms that are involved in transcriptional regulation of *FMR1*.

In addition, we also wanted to know how neuronal activity could play a role on the neuronal *FMRI* expression. In order to answer these questions, the various transfected cultures were treated with neuronal activity modulators. These treatments are known to modulate the expression level of proteins involved in synaptic plasticity. Thus, we could ask how the neuronal activity and which signaling pathways play a role in the regulation of *FMRI* transcription? Is it in accordance with the mGluR theory suggested by Bear et al. or is it in accordance with the GABA receptor theory?

8 Materials and methods

8.1 Plasmid construction

The various pFMR1 (*FMR1* promoter gene) plasmids were generated by Gateway cloning to insert mCherry, GFP or tdTomato reporter gene with or without a nuclear localization signal in an expression clone containing the wild type or mutated *FMR1* promoter.

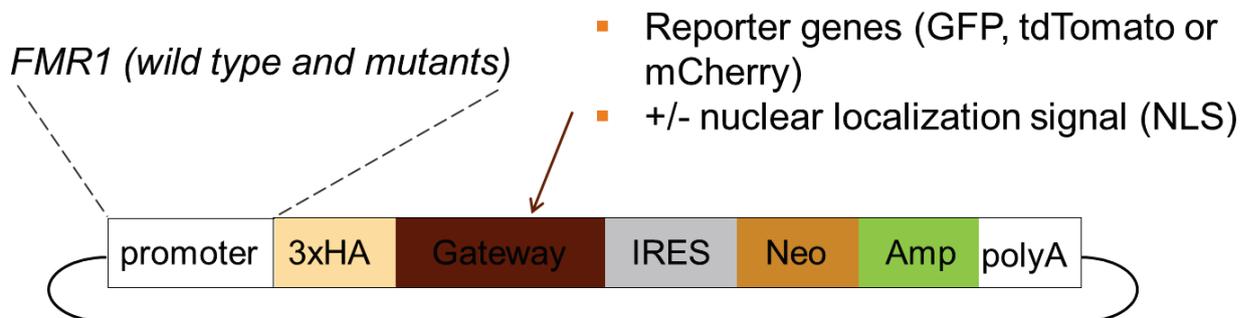


Figure 23: General plasmids construction map.

All the plasmids contain wild type or mutated versions of the *FMR1* promoter region, three HA-Tags which are used for the immunofluorescence staining, a Gateway cassette which permits introduction of the various reporter gene inserts using Gateway cloning (detailed below), an Internal Ribosome Entry Site (IRES) which are sequences where internal translational initiation of mRNA transcription can initiate, a Neomycin (Neo) resistance cassette, an Ampiciline (Amp) resistance cassette and a polyadenylation signal (polyA).

Gateway cloning protocol

Based on the well-characterized site-specific recombination system of phage lambda, the Gateway technology allows the cloning, combination and transfer of DNA segments into compatible plasmids while maintaining their orientation and the reading frame for fragments of interest.

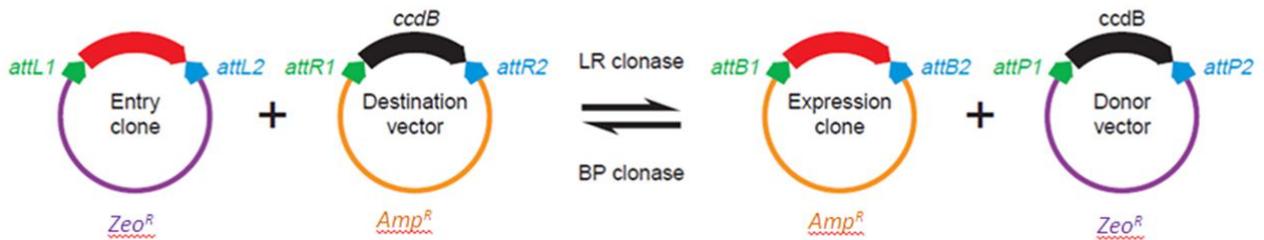


Figure 24: Schematic mechanism of gateway cloning.

The scheme shows the four types of plasmids and enzyme mixes involved in Gateway cloning reactions. Red curving arrows represent the fragment of interest.

The so called “entry clone” is at the center of the Gateway cloning process. It is a plasmid where the fragment of interest is flanked by attL sequences. It is obtained by combining a Donor Vector with the fragment of interest flanked by attB sites previously obtained by PCR. Once the entry clone is generated, the fragment of interest can be transferred to secondary plasmids called the destination vectors. The product of this reaction, called “LR reaction”, is the expression clone. This reaction is accomplished by mixing the entry clone (pDONR plasmid) and various destination vectors (here the pFMR1 plasmids WT, M1, M2 and M3) in the presence of LR clonase enzyme mix. An aliquot of these reactions is used to transform competent *E.coli* cells. In order to select only cells harboring the expression clone, two selection schemes are imposed: at first, the entry clone and the destination vector contain different antibiotic resistance genes (Zeocin and Ampicilline at 50mg/L and 100mg/L respectively), in second, the destination vector contains a counter selectable marker, the ccdB gene, which kills the cells by inducing gyrase mediated double stranded DNA breakage. Thus, only the expression clone will be carried in colonies selected by Ampicillin resistance.

In our study we had to create *FMRI* promoter plasmids containing the various nuclear and cytosolic reporter genes. We cloned the reporters into entry clones, obtained by standard cloning

procedure (restriction cloning) for the nuclear (destabilized) pFMR1 plasmids and by BP cloning for the cytosolic pFMR1 plasmids.

8.1.1 Generation of entry clones

First, we PCR amplified the GFP, tdTomato and mCherry reporter gene sequences from plasmids available in the lab. Two different modified PCRs were performed in order to amplify the cDNA of the reporters. One was for a restriction enzyme based cloning strategy for the nuclear entry clones containing the NLS and Pest sequences and another to get the cytosolic reporters that were used in BP reaction based cloning.

8.1.1.1 Nuclear entry clone production by enzymatic restriction cloning

The nuclear entry clones were created by combining the pDONR vector backbone amplified by inverse PCR and the cDNA of the fluorescent reporters by normal PCR using a restriction enzyme based strategy. In the resulting constructs the reporters with NLS and Pest sequences are flanked by the att sites allowing later transfer of the cDNAs into destination vectors via Gateway reactions.

8.1.1.1.1 Reporter gene sequence amplification step

The plasmid region containing the sequence of the reporter gene was amplified by PCR with a forward phosphorylated primer and a reverse primer containing an AgeI restriction site for the amplification of the nuclear mCherry and tdTomato reporter genes:

mCherry-tdTfPhos 5'-GGTACCATGGTGAGCAAGGG-3'

mCherry-tdTomato-R: 5'-CGTAACCGGTACTTGTACAG-3'

The destination vector sequence was amplified by inverse PCR from a plasmid containing the GFP reporter gene sequence. The PCR amplification was realized with a forward primer containing an AgeI site and a reverse phosphorylated primer:

Destabilized-V-F: 5'-GTTACCCGGTGTACAAGAAGC-3'

Destabilized-V-R Phos: 5'-GCTCACCATGGACACGTGTA-3'

The PCR are performed by using a 2X Phusion[®] Master Mix (Thermo Scientific[™]). The amplification protocol used was the following: a first denaturation step at 98°C for 30sec followed by 35 cycles containing one step at 98°C for 30 sec, one step at 68°C for 10 sec and an elongation step at 72°C for 40sec, then a final elongation step at 72°C for 7min.

8.1.1.1.2 Enzymatic restriction cloning

Then, the PCR products obtained were digested by AgeI. The PCR products have a blunt and sticky ends. In order to insert the PCR products in the entry clone, I used a T4 DNA ligase (a molar ratio of 3:1 insert vector is recommended for rapid ligation of DNA inserts to vectors to produce circular recombinant molecules).

After the ligation step, the plasmids obtained were transformed in competent cells One Shot[®] OmniMax 2T1 Chemically Competent *E.coli* cells (Invitrogen[™]). The transformations were seeded on agar gel with Zeocin (Zeo) antibiotic at 50mg/ml. After one night at 37°C, we got some colonies containing the plasmids. The colonies were screened by PCR and verified by enzymatic restriction. The colonies containing the good insert were seeded in a LB medium containing Zeo at 50mg/ml. After one night at 37°C with shaking, the plasmids were extracted using Qiagen Plasmid Plus Maxi Kit (Qiagen[®]) and verified by directed sequencing and restriction digestion.

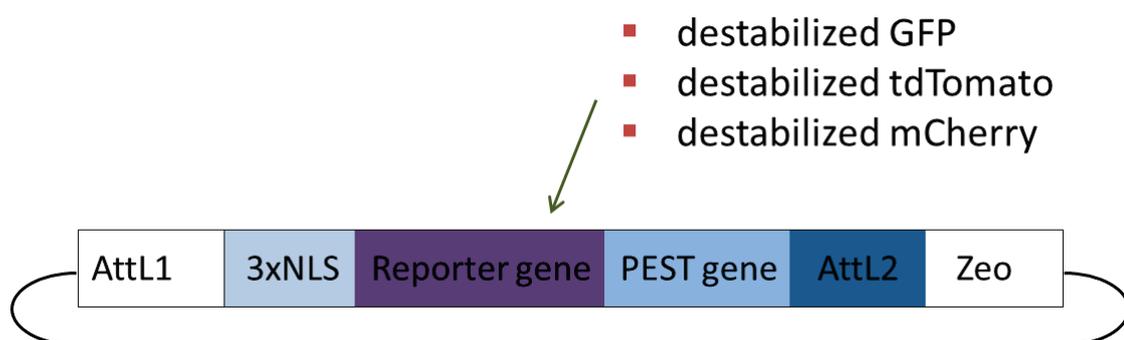


Figure 25: General scheme of destabilized entry clones

8.1.1.2 Cytosolic entry clones production by BP cloning

8.1.1.2.1 Reporter gene sequence amplification step

I first amplified by PCR the mCherry and tdTomato reporter genes sequences, from plasmids kindly provided by the laboratory, by using two modified primers containing the attB sites necessary for the BP cloning allowing inserting the reporter gene sequences PCR products in the pDONR plasmid in order to get the cytosolic entry clones containing the reporter gene sequences.

mCherry-Tomato-F 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGGTGAGCAAGGGCGAG-3'
mCherry-Tomato-R 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTACTACTTGTACAGCTCGTCCATGCC-3'

The PCR were done by using the 2X Phusion[®] Master Mix (Thermo Scientific[™]) with a first denaturation step at 98°C for 30sec follows by 35 cycles containing one step at 98°C for 30 sec, one step at 68°C for 10 sec and an elongation step at 72°C for 40sec, then a final elongation step at 72°C for 7min. The PCR products were isolated by cutting the band after agarose gel electrophoresis followed by purification with a PCR purification kit from Qiagen[®].

8.1.1.2.2 BP cloning reaction

The purified PCR product is mixed with the pDONR vector in presence of a BP clonase enzyme in order to create the entry clones containing the fragment of interest. See below for an overview of the set-up. For more detailed information, refer to the manual.

1. Add the following components to a 1.5 ml tube at room temperature and mix:
attB-PCR product (=10 ng/μl; final amount ~15-150 ng) 1-7 μl
Donor vector (150 ng/μl) 1 μl
TE buffer, pH 8.0 to 8 μl
2. Thaw on ice the BP Clonase[™] II enzyme mix for about 2 minutes. Vortex the BP Clonase[™] II enzyme mix briefly twice (2 seconds each time).
3. To each sample (Step 1, above), add 2 μl of BP Clonase[™] II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
4. Return BP Clonase[™] II enzyme mix to -20°C or -80°C storage.

5. Incubate reactions at 25°C for 1 hour.
6. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Transformation

1. Transform 1 µl of each BP reaction into 50 µl of One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells (Catalog no. C8540-03). Incubate on ice for 30 minutes. Heat-shock cells by incubating at 42°C for 30 seconds. Add 250 µl of S.O.C. Medium and incubate at 37°C for 1 hour with shaking. Plate 20 µl and 100 µl of each transformation onto selective plates. Note: Any competent cells with a transformation efficiency of $>1.0 \times 10^8$ transformants/µg may be used.
2. Transform 1 µl of pUC19 DNA (10 ng/ml) into 50 µl of One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells as described above. Plate 20 µl and 100 µl on LB plates containing 100 µg/ml kanamycin, or the appropriate selection marker for your donor vector.

The transformations are plated on LB agar gel with Zeocin at 50mg/ml.

Expected Results

An efficient BP recombination reaction will produce >1500 colonies.

After one night at 37°C on LB agar plate, colonies that grown are selected and replated in 50 ml of liquid LB agar containing Zeocin at 50mg/ml. The plasmids are then extracted by Midi Prep extraction kit (Qiagen®). The entry clones obtained containing the fragment of interest in the good open reading frame is selected for the LR clonase reaction.

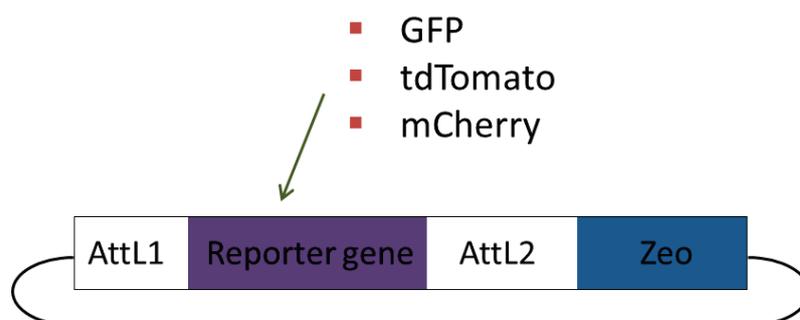


Figure 26: general scheme of cytosolic entry clones

8.1.2 Generation of expression clones by LR reaction

Through the attL sites contained in the entry clones, we introduced the reporter gene sequences in various destination vectors containing the pFMR1 wild type or mutated promoter by LR cloning reaction.

In order to insert the mCherry and tdTomato reporter gene sequences in the destination vectors containing the *FMRI* wild type and mutated promoter, we carried out LR reactions. Thus, we obtained the various expression clones. See below for an overview of the set-up. For more detailed information, refer to the manual.

1. Add the following components to a 1.5 ml tube at room temperature and mix:
 - Entry clone (50-150 ng) 1-7 μ l
 - Destination vector (150 ng/ μ l) 1 μ l
 - TE buffer, pH 8.0 to 8 μ l
2. Thaw on ice the LR Clonase™ II enzyme mix for about 2 minutes. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
3. To each sample (Step 1, above), add 2 μ l of LR Clonase™II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
4. Return LR Clonase™ II enzyme mix to -20°C or -80°C storage.
5. Incubate reactions at 25°C for 1 hour.
6. Add 1 μ l of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Transformation

1. Transform 1 μ l of each LR reaction into 50 μ l of One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells (Catalog no. C8540-03). Incubate on ice for 30 minutes. Heat-shock cells by incubating at 42°C for 30 seconds. Add 250 μ l of S.O.C. Medium and incubate at 37°C for 1 hour with shaking. Plate 20 μ l and 100 μ l of each transformation onto selective plates. Note: Any competent cells with a transformation efficiency of $>1.0 \times 10^8$ transformants/ μ g may be used.
2. Transform 1 μ l of pUC19 DNA (10 ng/ml) into 50 μ l of One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells as described above. Plate 20 μ l and 100 μ l on LB plates containing 100 μ g/ml kanamycin, or the appropriate selection marker for your donor vector.

The transformations are plated on LB agar gel with Ampicilline at 100mg/ml.

Expected Results

An efficient LR recombination reaction will produce >5000 colonies.

After one night at 37°C on LB agar plate, colonies that grown were selected and reseeded in 50 ml of liquid LB medium containing Zeocin at 50mg/ml. The plasmids were then extracted by Qiagen Plasmid Plus Midi kit (Qiagen®).

The plasmids obtained are called expression vectors and can be transfected in various cell types in order to express the various reporter genes under the control of the various *FMRI* promoters.

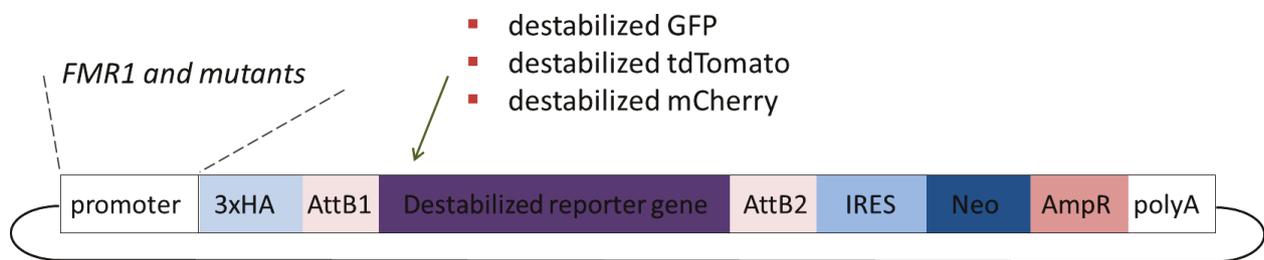


Figure 27: General scheme of nuclear expression clones

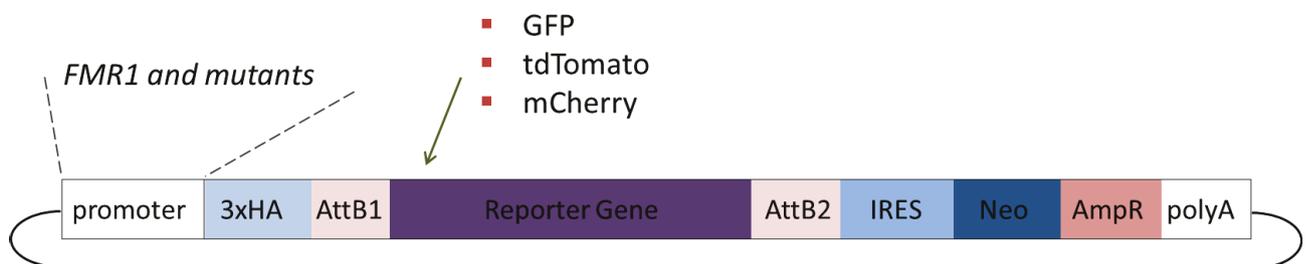


Figure 28: General scheme of cytosolic expression clones

The plasmids were then extracted in using the Plasmid Plus midi Prep extraction kit(Qiagen®) (See annex 1).

8.1.3 Verification of the plasmids integrity control step

In order to validate the obtained clones, plasmids were sequenced and digested based on Vector NTI® maps. We selected restriction enzymes that have, ideally, three restrictions sites in the plasmids with at least one in the fragment of interest.

8.2 Lipofectamine[®] 2000 transfection in HeLa cells, rat cortical neurons and in human neuronal precursors

“Lipofectamine[®] 2000 is a cationic liposome formulation that functions by mixing with nucleic acid molecules, allowing them to overcome and to be taken up by the cell. This cationic liposome based reagent that provides high transfection efficiency and high levels of transgene expression in a range of mammalian cell types in vitro using a simple protocol.

The positive charge on the surface of the liposome generates an electrostatic interaction with nucleic acids and facilitates contact with the negatively charged cell membrane. The neutral co-lipid mediates fusion of the liposome with the cell membrane effecting entry of the nucleic acid. To achieve expression of the transgene, DNA must reach the nucleus of the cell and become accessible to the transcriptional machinery.”

(Dalby B. and al., 2004).

Because the transfection of primary cells is problematic and very low transfection efficiencies are often reported, first, I tested my plasmids in HeLa cells. The enhanced transfection efficiencies would facilitate study of neurogenesis, neurophysiology and pathology for neurons which are an important class of primary cells.

8.2.1 HeLa cell transfection

The HeLa cells come from an immortal cell line derived from cervical cancer. The cells were culture at 37°C with 5%CO₂ in DMEM with 10% Fetal Bovine Serum (FBS). Twenty four hours before transfection, 1x10⁶ were plated in 2 ml of media in each well of six well cell culture dishes. After three days in culture, cells were trypsinized and replated in eight well chambers slides coated with 0.5mg/ml Poly-d-lysine. Each well in the chamber slices contained 150 000 cells. After one day in culture, the cell density was 90%. At this point, cells were transfected using Lipofectamine[®] 2000 (Invitrogen) complexes with the plasmids encoding WTFMR1-cyt_mCh and WTFMR1-cyt_tdt. Transfections were carried out in Opti-MEM (Invitrogen), using 10µl of Lipofectamine 2000 and 1µg of each plasmid in a 10:1 ratio. Four hours after transfection, the media was replaced with fresh DMEM with 10%FBS. Forty-eight hours after transfection, slices were fixed, stained

and analyzed following the protocols described below in the immunohistochemistry and microscopy paragraphs.

8.2.2 Rat cortical neurons transfection

Cortical neurons from E18 embryos (Annex 2) were transfected as following. First, cortical neurons were cultured in Neurobasal medium [DMEM/10%FCS/Glutamine medium (Gibco®)] in eight well chambers coated with 0.5mg/ml Poly-d-lysine containing with a density of 150 000 neurons pro well. Half of the culture medium was changed one time during 15 days of culturing. At 15 days *in vitro* 250 ml of media was removed and located in an eight well chambers slide. 8µl of Lipofectamine® 2000 was diluted in 100µl OptiMEM® I and incubated at room temperature for 5 min. In an Eppendorf tube, 1.5µg of WTFMR1_cytGFP and WTFMR1_nucGFP DNA plasmids were combined with 100µl OptiMEM® I and incubated at room temperature for 5 min. The diluted DNA and Lipofectamine® 2000 (LF2000) were mixed at room temperature for 20min. The removed 250 µl of media reserved was replaced in each well with 400µl of fresh Neurobasal/B27/L-glutamine medium and 100 µl of OptiMEM®I containing DNA and LF2000 transfection complexes was added to each well. After one week at 37°C, slices were fixed, stained and analyzed following the protocols described below in the immunohistochemistry and microscopy paragraphs.

8.2.3 Human neuronal precursors transfection

The human neuronal progenitors are differentiated from frozen samples of induced pluripotent stem (IPS) cells (Annex 3). They were cultured in the proliferation medium (PM) containing DMEM /F12/GlutaMax medium [Invitrogen Gibco®, cat 31331-028], N2 supplement [Invitrogen™, cat 17502048] (1/100), B27 supplement [Invitrogen™, cat 17504044] (1/50), PEN/Strep (1/100). Two Growth factors are added extemporaneously in the PM medium, Basic human Fibroblast Growth Factor (bhFGF) 100 µg/ml stock to 10ng/ml (1:10 000) and Human Epidermal Growth Factor (hEGF) 100 µg/ml stock to 10ng/ml (1:10 000). The cells are plated in eight well chambers slides containing Matrigel® with 150 000 cells by well in 0.5ml PM medium. 1µM of Rock inhibitor is added in the PM medium. After one day in culture at 37°C, the medium is changed to remove the Rock inhibitor. Transfections are then performed by adding 4 µl/well Lipofectamine® 2000 to

100µl of DMEM (Gibco®). In an Eppendorf tube, 1.5 µg of each plasmid encoding WTFMR1-cytGFP, WTFMR1-nucGFP, M1FMR1-cytGFP, M1FMR1-nucGFP, WTFMR1-cyt_mCh, M1FMR1-cyt_mCh, WTFMR1-cyt_tdT and M1FMR1-cyt_tdT are mixed with 100µl of DMEM (Gibco®) and growth factors; and incubated at room temperature for 5 min. The diluted DNAs and Lipofectamine® 2000 are mixed at room temperature for 20min. 25 µl/well of the mix is then added in 250µl of fresh DMEM (Gibco®) + growth factors medium. After one night at 37°C, slices were fixed, stained and analyzed following the protocols described below in the immunohistochemistry and microscopy paragraphs.

8.3 Genegun experiment in hippocampal brain slices

8.3.1 Preparation of organotypic hippocampal brain slices

The experiments are performed on hippocampal slices coming from pups at postnatal day 6- 9. The cultures of hippocampal slices are based on the interface method, which does not require special equipment. Hippocampal slices are isolated at postnatal day 6-9, and can be cultured up to 6 months *in vitro*. The preserved architecture facilitates the analysis of defined hippocampal synapses, cells and entire projections and allows the identification and manipulation of defined neurons. The axonal projections can be monitored in the slices in their original state and establish identifiable presynaptic terminals onto excitatory and inhibitory neurons in the hilus, CA3, CA2 and CA1 regions. Three-dimensional organizations are preserved by a long-term thickness of 100-150µm. This preparation can also allow studying brain architectures from mice of genetic backgrounds for which the postnatal viability is poor. The protocol of preparation of organotypic hippocampal slice cultures is detailed in the publication from Gogolla N. and Galimberti I., in Nature Protocols (2006) (Annex 4).

8.3.2 Gene gun protocol or biolistic transfection protocol

The biolistic transfection is a physical method of transfecting cells by bombarding living tissue like mouse hippocampal slices with high velocity DNA coated gold particles. Gene gun transfection is an efficient and easy method of transfecting neurons and is especially useful for fluorescently labelling a small subset of cells in tissue slice. The protocol is composed of three major steps:

coating microcarriers with DNA, transferring the microcarriers into a cartridge to make a bullet and firing the DNA coated microcarriers into cells using a pulse of helium gas to use a hand-held gene-gun (O'Brien J. and Lummis S., 2006; Woods G. and Zito K., 2008).

Brain slices were transfected 6 hours after preparation with plasmids encoding WTFMR1-cytGFP and M1FMR1-cytGFP using Helios gene gun system (Bio-Rad Laboratories, # 165-2431) as previously described (Galimberti, 2006; and Gogolla, 2006). Briefly, 8 µg of plasmid DNA was coated on 4 mg of 1.6 µm diameter gold particles in 20 µl spermidine (250 mM), 27 µl Nupherin (3 mg/ml) and 8 µg DANN. Then the coated DNA was precipitated with 1 mM CaCl₂, and washed three times in pure ethanol. The gold particles were coated onto PVC tubing, dried using pure N₂ gas, and stored at 4°C in desiccant. DNA-coated particles were delivered with a standard pressure of 180 psi (pound per square) and distance of 2 cm from the brain slice tissue. Finally, slices were fixed, stained and analyzed following the protocols described below in the immunohistochemistry and microscopy paragraphs.

8.4 Drug treatments and Immunofluorescence

8.4.1 Drug treatments

After biolistic transfection, the hippocampal slices were treated with three neuronal activity modulators to visualize effects on expression levels of our various plasmids.

- Glycine Chemical LTP: Preincubation for 10 min in extracellular solution ECS (150 mM NaCl, 2 mM CaCl₂, 5 mM KCl, 10 mM HEPES (pH 7.4), 30 mM Glucose, 0.5 µM TTX, 1 µM strychnine, 20 µM bicuculline methiodide). Let stand 200µM glycine (1M) 15min in ECS.
- 10 µM Bicuculline (1M) by well overnight at 37°C.
- 1 µM Tetrodotoxin (1M) by well overnight at 37°C.

8.4.2 Immunofluorescence

8.4.2.1 *HeLa cells, cortical neurons and precursors neurons slices preparation*

After the transfections, the slices were fixed in adding 0.5ml/well 4% PFA incubate for 20 min at room temperature. Then, the slides were washed 3 times with PBS. At this step, the slices could be leaved in the last PBS solution and stored at 4°C for one month. The slices were permeabilized with 500µl/well PBS +0.1% Triton 100 for 5 min and were washed in PBS 2 times 5 min. At the end, the slides were blocked by adding 500µl/well 2.5%BSA in PBS + 0.1% Tween 10 for 30min. The slices can be conserved one month at 4°C in blocking solution.

8.4.2.2 *Hippocampal slices preparation*

After biolistic transfection and compound treatment, the slices cultures were fixed with 1ml of prewarmed at 37°C 4% PFA solution added above and 1 ml beneath insert membrane for 5 minutes at room temperature. After washing the slices in PBS, they were permeabilized with 1 ml of permeabilization solution (0.5% Triton X-100 in PBS) above and 1 ml beneath the insert membrane. Then the slices were incubated overnight, or for at least 12 h, at 4°C. Slices can be kept in the permeabilization solution for up to 18 h. The following day, the permeabilization solution was removed and a blocking solution (20% BSA in PBS) was added. At this point, the slices can be left for 4 h at room temperature, or overnight at 4°C. Sections can be kept in the blocking solution at 4 °C for at least 2–3 days. To reduce the volume of antibodies solutions needed, the slices are cut off from the membranes of the culture plates inserts. Then, the culture plates inserts are placed on a plastic cover (preferably a transparent plastic cover lying on a dark background to make the tissue easily visible). Thus, forceps and scalpel are used to carefully cut the membrane piece together with the hippocampal slice out of the surrounding membrane. It is useful to keep 1–2 mm of distance to the tissue to avoid damage. (Optional: the slices can already be cut off just after the fixation to further limit the amounts of permeabilization and blocking solutions required). At the end, it is important to always keep the top side of the membrane facing up, and do not flip it around.

8.4.3 Staining

8.4.3.1 *Hela cells*

- *Incubation with primary antibody*

- Anti HA-Tag mouse (Anti-HA tag antibody [HA.C5] (ab18181), Abcam[®]): dilution 1/2000 in BSA solution.

Incubate overnight in humid chamber at 4°C. Wash 3 times in PBS/0.1% Tween for 10min.

- *Incubation with secondary antibody*

Alexa fluor[®] 647 anti-mouse (red) (Invitrogen[®]) was diluted in 1/1000 μ l in BSA solution. 250 μ l of this solution was added to each well for 1 hour at room temperature in dark humid chamber. Wash three times in PBS and mount the slides with special medium including DAPI (Fluoroshield[™] with DAPI, F6057 Sigma Aldrich[®]). Keep the slides in the dark at room temperature overnight in dark chamber.

8.4.3.2 *Cortical neurons staining*

- *Incubation with primary antibodies*

- Anti GFP chicken (Anti-GFP antibody (ab13970), Abcam[®]): dilution 1/2000 in BSA solution.
- Anti HA-Tag mouse (Anti-HA tag antibody [HA.C5] (ab18181), Abcam[®]): dilution 1/2000 in BSA solution.

Incubate overnight in humid chamber at 4°C. Wash 3 times in PBS/0.1% Tween for 10min.

- *Incubation with secondary antibody*

Alexa fluor[®] 488 anti-chicken (green) (Invitrogen[®]) and Alexa fluor[®] 647 anti-mouse (red) (Invitrogen[®]) were diluted 1/1000 in BSA solution and 250 μ l/well of this mix was added for 1 hour at room temperature in dark humid chamber. Slides were washed three times in PBS and were mounted with special medium including DAPI (Fluoroshield[™] with DAPI, F6057 Sigma Aldrich[®]). Keep the slides overnight in a dark chamber at room temperature.

8.4.3.3 *Neuronal precursors staining*

- *Incubation with primary antibodies*

- Anti-NeuN mouse (MS X Neuronal Nuclei, ref: MAB377, Millipore®): dilution 1/1000 in BSA solution.
- Anti HA-Tag rat (Anti-HA tag antibody [3F10] (ref: 12158167001), Roche®): dilution 1/2000 in BSA solution.

Incubate overnight in humid chamber at 4°C. Wash 3 times in PBS/0.1% Tween for 10min.

- *Incubation with secondary antibody*

Alexa fluor® 555 anti-mouse (yellow) (Invitrogen®) and Alexa fluor® 488 anti-rat (green) (Invitrogen®) were diluted 1/1000 in BSA solution and 250µl/well of this mix was added for 1 hour at room temperature in dark humid chamber. Slides were washed three times in PBS and were mounted with special medium including DAPI (Fluoroshield™ with DAPI, F6057 Sigma Aldrich®). Keep the slides overnight in a dark chamber at room temperature.

8.4.3.4 *Hippocampal slices staining*

- *Incubation with primary antibodies*

- Anti-NeuN mouse (MS X Neuronal Nuclei Millipore® ref: MAB377): dilution 1/400, incubation 66h at 4°C into a wet chamber with shaking.
- Anti-GFP chicken (Anti-GFP antibody (ab13970), Abcam®): dilution 1/2000 incubation 66h at 4°C into a wet chamber with shaking.

Wash 3 times in PBS shake 10min between each one.

- *Incubation with secondary antibody*

- Alexa fluor® 647 anti-Mouse (red) (Invitrogen®) and Alexa fluor® 488 anti-chicken (green) (Invitrogen®) were diluted 1/500 in PBS + 0.3% triton, 160µl/well was added 2h at room temperature. Slides were washed with PBS with 1/1000 DAPI. After a last wash with PBS, slices were mounted using ProLong®(Life technologies).

8.5 Endogenous FMRP signal

Dissociated cortical neurons were produced from rat embryos at E18. Cortical neurons are plated in Neurobasal medium for 14 days. 8 on the 24 wells chambers of 14 days old cultures were treated with 20 μ M bicuculline, 8 others wells were treated with 1 μ M TTX and the 8 staying were untreated. The slices are permeabilized with 500 μ l/well PBS +0.1% Triton 100 for 5 min and washed in PBS 2 times 5 min. At the end, the slices are blocked by adding 500 μ l/well 2.5%BSA in PBS + 0.1% Tween 10 for 30min. The slices can be conserved one month at 4°C in blocking solution.

I performed on them a confocal microscopy study in order to visualize if the treatment can modulate or not the endogenous signal level in brain. This study permits to better understand the role of FMRP, its distribution and its localization in wild type rat brain.

Endogenous FMRP expression level was analyzed after treatments by immunofluorescence using an anti-FMRP mouse antibody (FMRP Antibody (H-120): sc-28739, Santa Cruz®) and an anti-MAP2 rabbit antibody (Anti-MAP2 antibody (ab32454), Abcam®). The anti-FMRP together with the anti-MAP2 antibody allowed us to detect FMRP within dendritic processes.

The first antibodies were diluted in BSA medium overnight. After washing in PBS three times, second antibodies: such as anti-mouse Alexa fluor® 488 and anti-rabbit Alexa fluor® 647 (Invitrogen™) were diluted in 1/1000 in BSA for 1 hour at room temperature.

8.6 Imaging, quantification and data analysis

Images were acquired using a 40x objective on a confocal laser scanning microscopy (LSM) 710 (Carl Zeiss®, Thornwood, NY). Lsm images were quantified to with the ImageJ® software. Signal intensities for reporter genes expression were analyzed within a defined region of interest traced all around the cell body of defined neurons and the background signal adjacent of each region of interest was substracted. To normalize the reporter gene signals expression, mean IF intensities of cell body WT neurons were determined and compared to mutated neurons. Quantitative analysis of the IF percentages were represented using Microsoft Excel software (Microsoft®, Redmond, WA).

9 Results

9.1 Evaluation of the transcriptional effects of three disease associated mutations in the *FMR1* promoter

9.1.1 Wild type and mutated *FMR1* promoter plasmid constructs

To generate the various constructs with the mutated or the wild type *FMR1* gene promoter, we introduced the mCherry and tdTomato reporter genes sequences in a pDONR vector by BP cloning, enzymatic digestion and ligation for the nuclear variants. We obtained four pDONR plasmids containing respectively nuc_tdTomato, nuc_mCherry, cyt_tdTomato and cyt_mCherry gene reporters. The pDONR plasmids obtained were verified by enzymatic digestion and sequencing. These verification steps validated the pDONR plasmids constructions and allowed their use for the LR reactions.

The reporter genes in the pDONR constructs are flanked by AttL sites which allow LR reaction facilitated transfer of the insert in destination vectors. With such approach we obtained plasmids containing the wild type or one of the three mutant (WTFMR1, M1FMR1, M2FMR1 and M3FMR1) *FMR1* promoters respectively with or without the nuclear localization and destabilization (Pest) sequences. The constructs were verified by restriction digestion and sequencing comparing the raw data with the corresponding Vector NTI[®] maps.

9.1.2 Cytosolic plasmids construction

The various cytosolic plasmids constructs are resumed in the figure below.

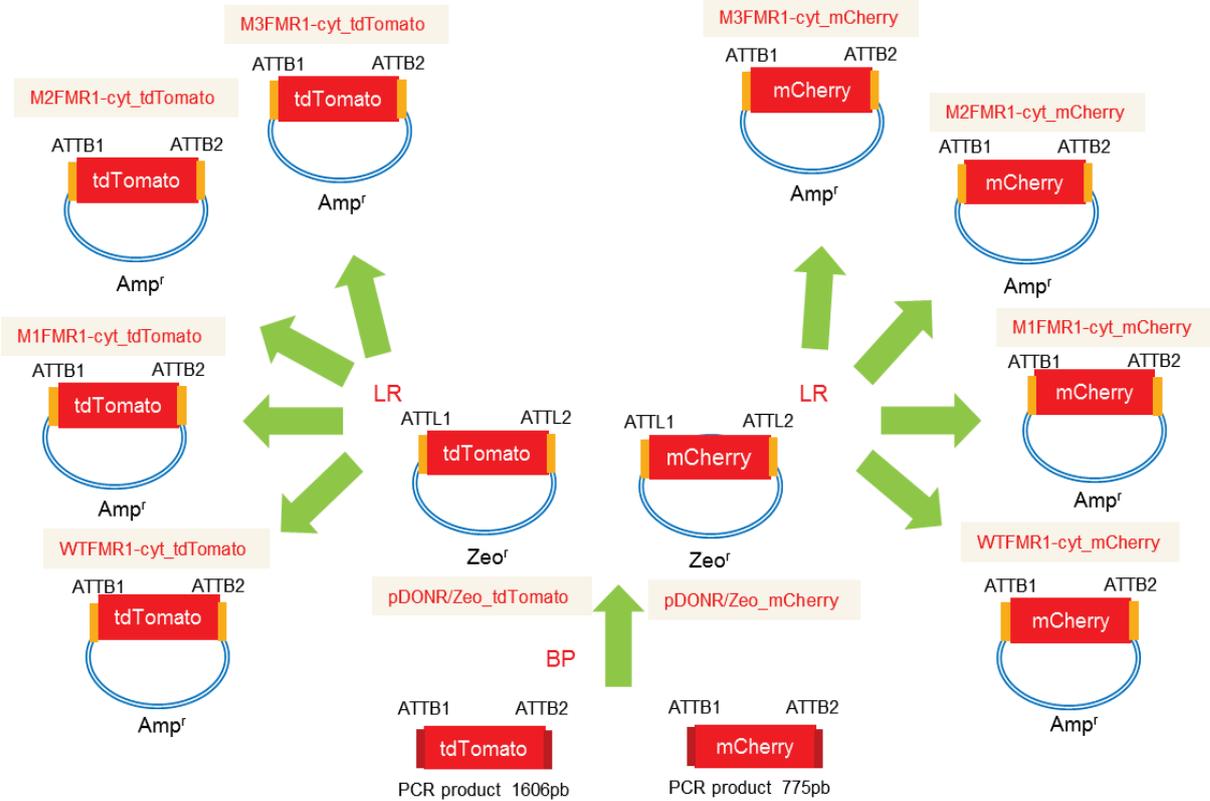
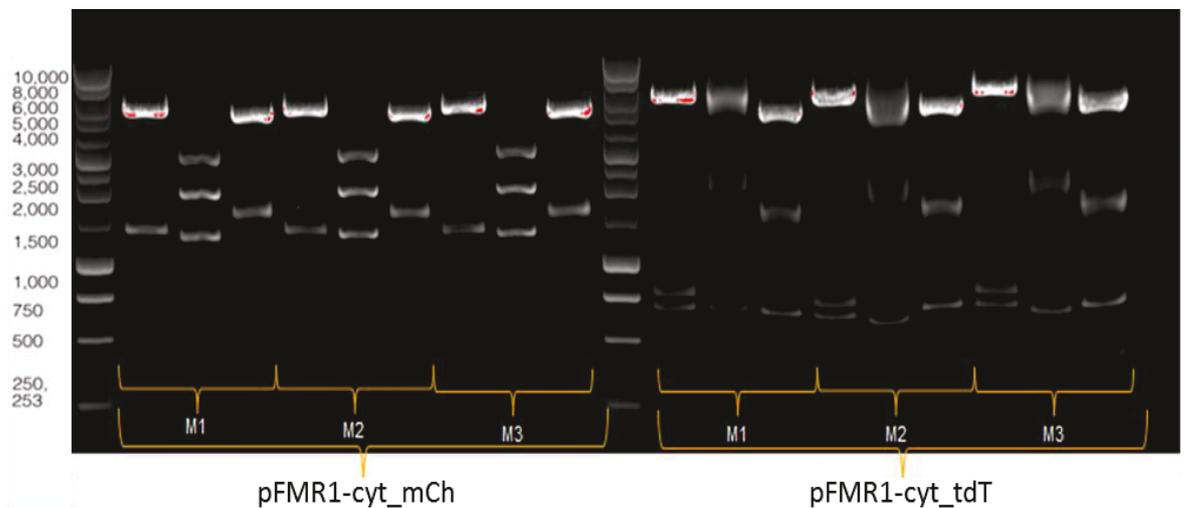


Figure 29: cytosolic plasmids constructions.

The constructions were verified by enzymatic restrictions and sequencing.



Pist	Enzyme name	Company	Buffer	BSA	[U/ μ l]	T [$^{\circ}$ C]	mCherry Expected sizes (bp)	Results		
								M1	M2	M3
1	MscI	Neb	4	/	3	37	1292/4578	OK	OK	OK
2	AvaII	Gibco	Gibco	/	5	37	1262/1863/2742	OK	OK	OK
3	NcoI	Neb	3	/	10	37	435/1458/4196	OK	OK	OK

Pist	Enzyme name	Company	Buffer	BSA	[U/ μ l]	T [$^{\circ}$ C]	tdTomato Expected sizes (bp)	Results		
								M1	M2	M3
1	MscI	Neb	4	/	3	37	726/767/5097	OK	OK	OK
2	EagI	Neb	3	/	5	37	2606/4203	-	-	-
3	NcoI	Neb	3	/	10	37	423/728/1464/4196	OK	OK	OK

Figure 30: Raw data of the enzymatic restrictions of the cytosolic pFMR1 expression clones (from Lab Book).

The enzymatic digestions gave good results and the sequencing was in agreement with these results.

9.1.3 Nuclear plasmids constructions

The plasmids with nuclear reporters were also generated by LR cloning and were verified as described above for the cytosolic variants.

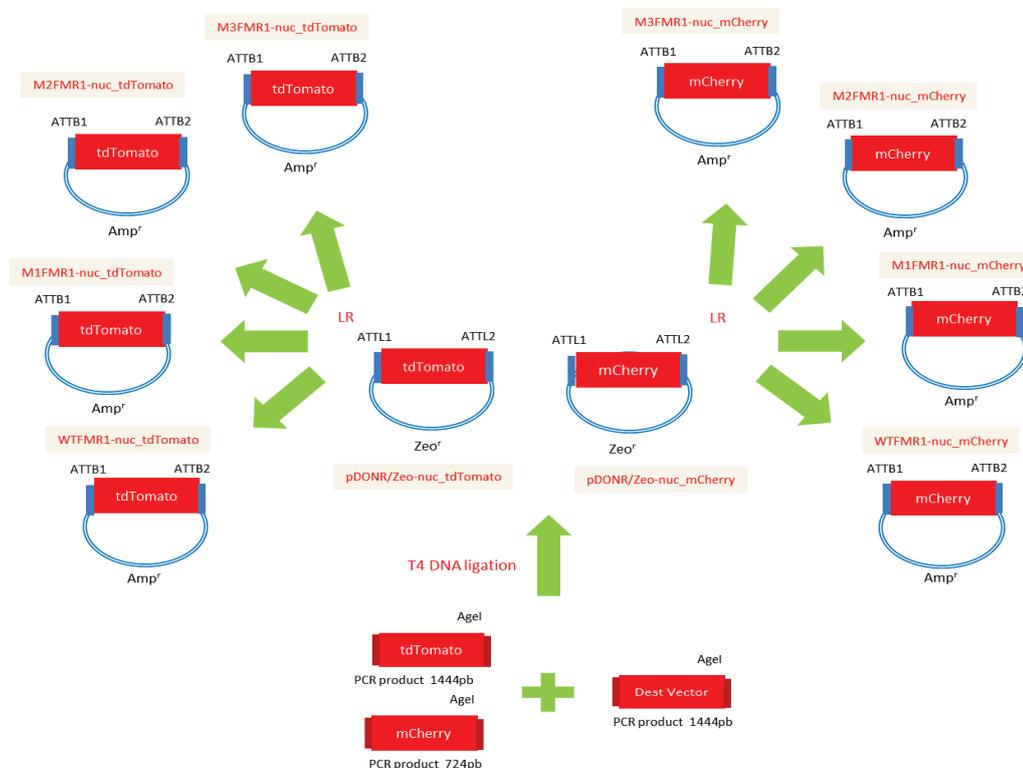
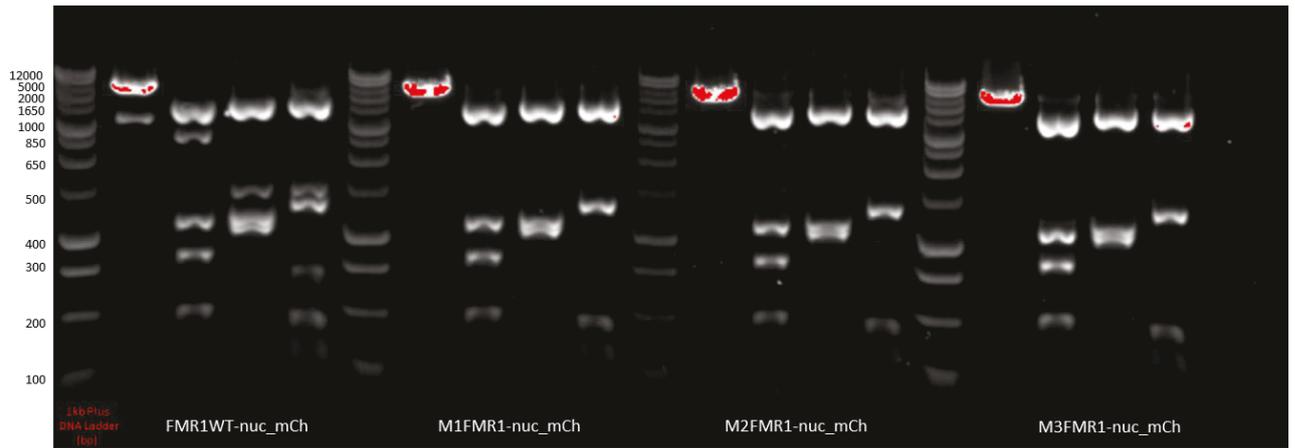
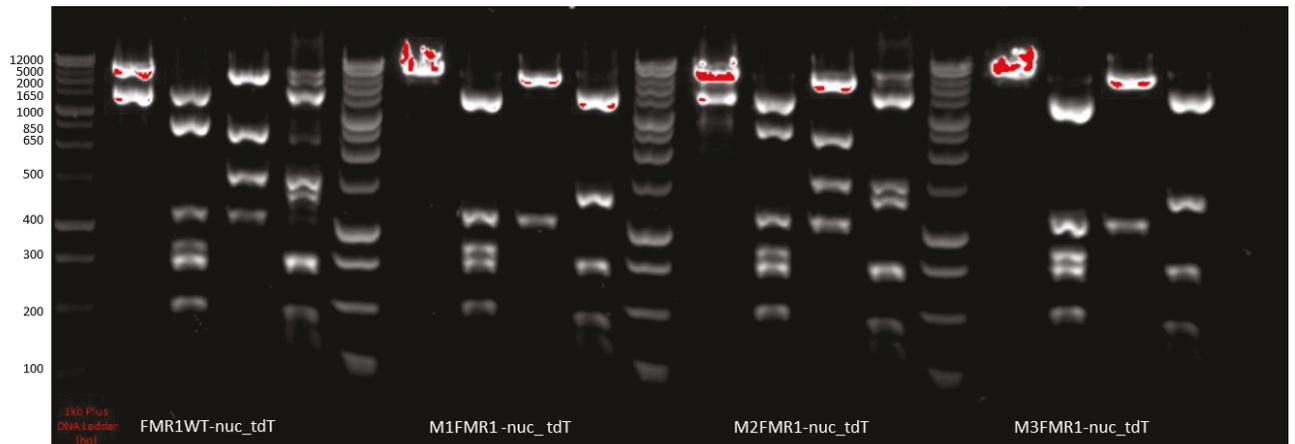


Figure 31: destabilized plasmids construction



Pist	Enzyme name	Company	Buffer	BSA	[U/ μ l]	T [$^{\circ}$ C]	mCherry Expected sizes (bp)	Results			
								WT	M1	M2	M3
1	AgeI	Roche	1	/	3	37	6230	-	OK	OK	OK
2	PstI	Neb	H	/	10	37	465/832/996/3937	-	OK	OK	OK
3	PvuII	Roche	M	/	10	37	1069/1127/4034	-	OK	OK	OK
4	NCOI	Roche	3	/	10	50	297/1301/4632	-	OK	OK	OK



Pist	Enzyme name	Company	Buffer	BSA	[U/ μ l]	T [$^{\circ}$ C]	tdTomato Expected sizes (bp)	Results			
								WT	M1	M2	M3
1	AgeI	Roche	1	/	3	37	6950	-	OK	-	OK
2	PstI	Neb	H	/	10	37	458/726/832/983/3937	-	OK	-	OK
3	PvuII	Roche	M	/	10	37	1069/5867	-	OK	-	OK
4	NCOI	Roche	3	/	10	50	290/423/726/1301/4196	-	OK	-	OK

Figure 32: Raw data of the enzymatic restrictions of the nuclear pFMR1 expression clones (from Lab Book).

This cloning was not as efficient as the generation of the cytosolic plasmids. I could only generate M1FMR1-nuc_mCh, M2FMR1-nuc_mCh and M3FMR1nuc_mCh and the M1FMR1-nuc_tdTomato and the M3FMR1-nuc_tdTomato expression clones during my work. I sequenced all the plasmids and the results were in agreement with the enzymatic digestions.

The obtained expression constructs were further verified by introducing them into HeLa cells, human neuronal precursors or rat cortical neurons by lipofectamine®2000 transfection or mouse hippocampal slices by Genegun.

9.1.4 Expression of the reporter constructs in HeLa cells

To test the generated constructs, I transfected the pFMR1_mCh and pFMR1_tdTomato constructs into HeLa cells. Both of these plasmids carry the wild type promoter with cytoplasmic reporters.

The plasmids and staining tested are summarized in the following table:

Plasmids used	Staining	Interpretation
WTFMR1-cyt_tdT	HA-tag(red): reporter expression	No cell transfected
WTFMR1-cyt_mCh	from pFMR1 Dapi (blue): nuclear staining	No cell transfected

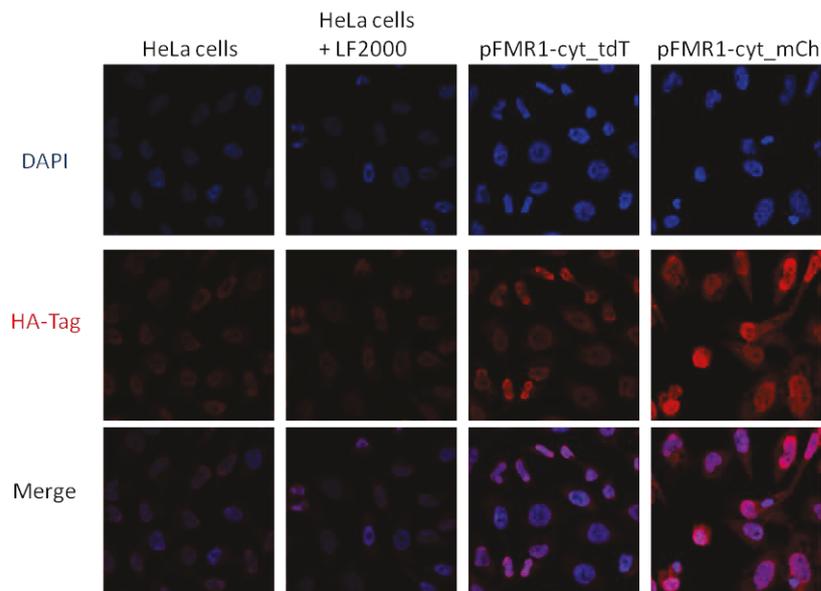


Figure 33: HeLa cells transfections with WTFMR1-cyt_mCh or WTFMR1-cyt_tdT expressing cytosolic mCherry or tdTomato respectively from a wild type *FMR1* promoter. The fluorescent proteins are fused with an HA-tag, which was used to enhance the signal by indirect immunofluorescence staining using an anti-HA antibody. The two first columns showing the HeLa cells alone and the HeLa cells with just Lipofectamine 2000 were used for controlling the transfection efficiency.

There was no significant difference in the intensity of the signal level between un-transfected controls and plasmid transfected wells. Thus, we cannot conclude from these experiments that these plasmids are working. The negative results may be due to technical issues with the transfection, or that these constructs do not express in HeLa cells or at a very low level because of the *FMRI* regulatory sequences. It is worth nothing, that beside the *FMRI* promoter our constructs also uses the 5'UTR of *FMRI* which does not have the consensus Kozak translational initiation motif and may contribute to the low expression of the proteins. Although *FMRI* is ubiquitously expressed in humans, there are marked differences between different tissues, testis and brain showing the highest expression levels. In order to test this hypothesis, I cloned tdTomato reporter gene in pcDNA-DEST40 Gateway vector by gateway cloning. This pcDNA-DEST40 Gateway® vector has cytomegalovirus (CMV) promoter for high level mammalian expression and a C-terminal V5 6x His Tag for easy detection (Invitrogen™). The plasmids obtained were then transfected in HeLa cells by Lipofectamine 2000 (figure 34).

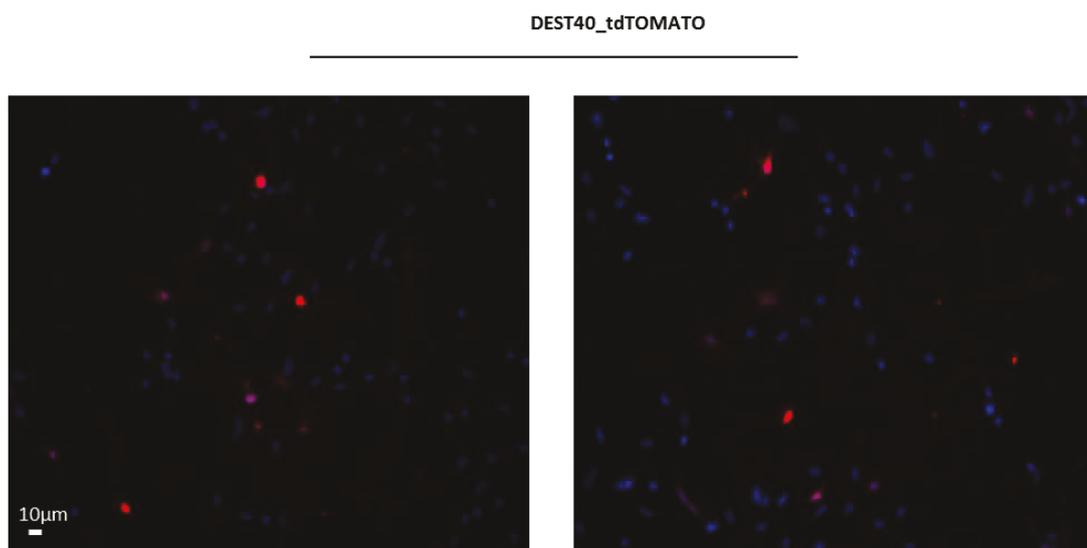


Figure 34: Expression of pcDNA-DEST40_tdTomato in HeLa cells using Lipofectamine® 2000 transfection.

Apparently the purple cells expressed the tdTomato reporter gene under the CMV promoter, thus leading to the conclusion that the tdTomato reporter gene construct is correct. It seems likely that the low expression from the *FMRI* promoter in HeLa cells was the reason for the negative results in the previous experiment. This finding suggests that the tdTomato reporter gene fragment flanked by attL sites can be used for gateway cloning with others destination vectors plasmids. It also seems likely that the expression of the tdTomato reporter gene under the control of *FMRI* promoter would be more efficient in neuronal cells.

9.1.5 Evaluation of the expression level of wild type *FMRI* promoter plasmids in rat cortical neurons

To understand whether our plasmids are expressing GFP in neurons, we expressed and acquired images of wild type nuclear and cytosolic pFMRI-GFP in rat cortical neurons at 15 DIV.

Plasmids used	Staining	Interpretation
WTFMR1-nucGFP	- Dapi (blue): nuclear staining - eGFP (green) : endogenous eGFP fluorescence	Transfected cells: 1/field Nucleus signal localization: ok
WTFMR1-cytGFP	- HA-tag (red): immune-detection of tagged eGFP with anti-HA antibody	Transfected cells: 1/field Nucleus signal localization: ok Cytosolic signal localization: ok

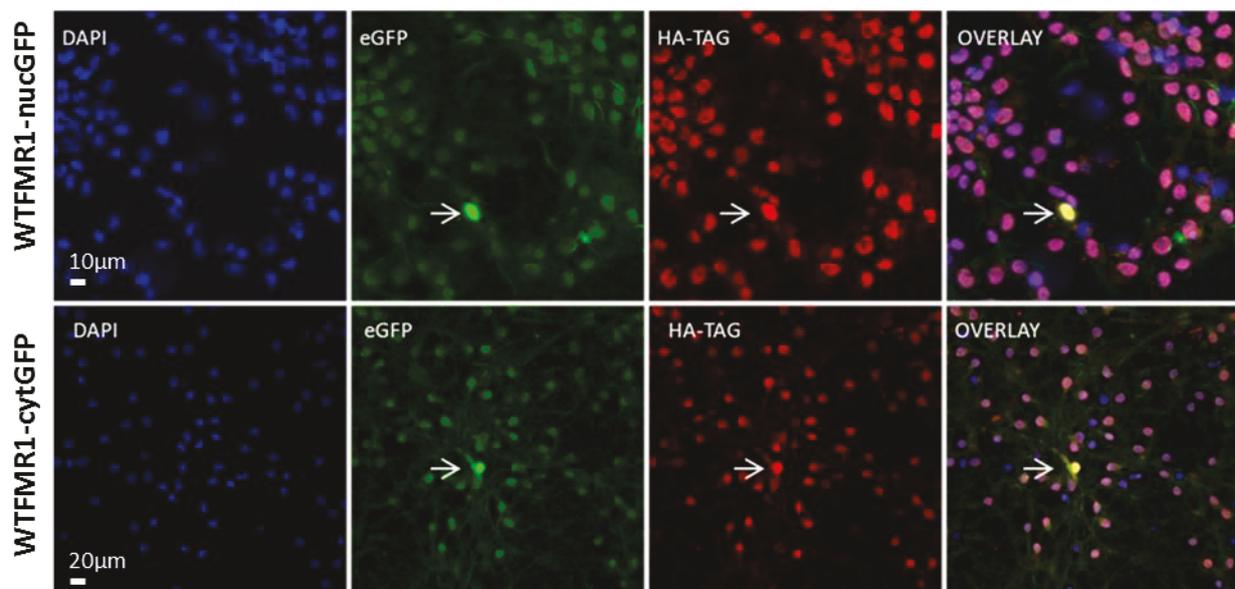


Figure 35: Representative images of transfected cortical neurons with nuclear and cytosolic WTFMR1-GFP plasmids in rat cortical neurons. Nuclei were stained with Dapi. Endogenous fluorescence of GFP or immunostaining of the GFP fused HA-tag was used to monitor reporter expression. Cortical neurons expressing either nuclear GFP or cytoplasmic GFP are shown with white arrows.

Although the transfection was high only in a few neurons were expressing GFP. Strong GFP positive neurons from the WTFMR1-nucGFP or WTFMR1-cytGFP constructs were found to have signal in the nucleus and in the cytosol. We could conclude that the *FMRI* promoter was transcriptionally active and that the constructs with the PEST destabilization signal were expressed at a similar level as the version without a PEST signal. In contrast to the transfection in HeLa cells,

in cortical neurons we were able to show that the *FMRI* promoter is transcriptionally active. Our results confirm that our constructs are selectively expressed in neuronal cells and can be used to address how neuronal activity influences the *FMRI* promoter expression.

9.2 Regulatory mechanisms of the *FMRI* gene expression

9.2.1 Comparison of the expression level of wild type and mutant (M1*FMRI*) *FMRI* promoter in human neuronal precursors

To evaluate whether the (A254G) mutation reduces the transcription of the *FMRI* promoter activity, we expressed cytosolic GFP from wild type or mutated *FMRI* promoter (M1) in human neuronal precursors.

	WTFMR1- cytGFP	M1FMRI- cytGFP	WTFMR1- nucGFP	M1FMRI- nucGFP	WTFMR1- cyt_mCh	M1FMRI- cyt_mCh	WTFMR1- cyt_tdT	M1FMRI- cyt_tdT
Staining	HA-tag (green): p <i>FMRI</i> expression reporter NeuN (yellow): neuronal cell marker							
Number of observed cells	2	3	2	6	10	12	5	5
Normalized mean intensity/area	4.6	2.1	2.5	0.9	1.6	0.8	3.0	0.9

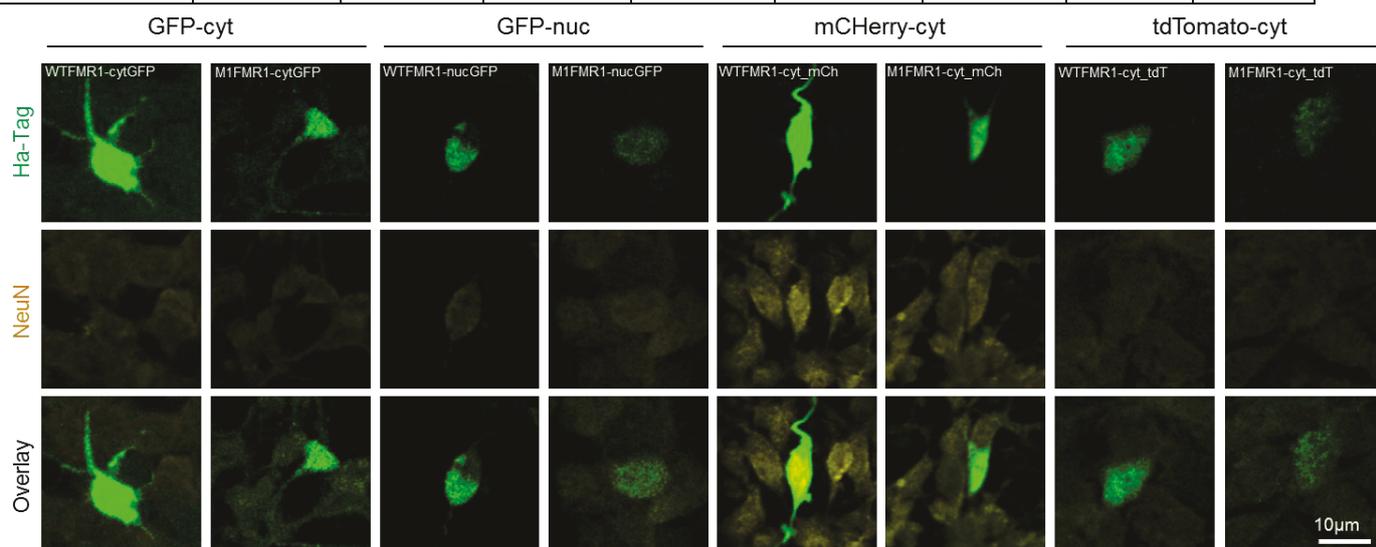


Figure 36: Representative images of transfected human neuronal precursors with nuclear and/or cytosolic GFP, mCherry and tdTomato reporter genes under the control of the WTFMR1 and M1FMRI promoters. Nuclei were stained with NeuN and human neuronal precursors expressing the various reporter genes were stained for the HA-Tag.

We used NeuN staining to distinguish neuronal cells from the non-neuronal cells and we found 20% of the cells being neuronal were transfected with our constructs. Indeed, in the transfected neuronal cells we can clearly distinguish a green fluorescence which colocalizes with the NeuN yellow staining indicating that transfected neuronal cells express our plasmids. In addition, our results clearly showed a significant decrease of the Ha-tag expression level with the M1 mutation (A254G) compared to the wild type *FMR1* promoter. M1FMR1 could significantly reduce the transcription of the *FMR1* gene.

To quantify these data, we analyzed the signal intensity level of each cells using ImageJ. This software allows the quantification of the mean signal intensity in a defined area. Our results are represented in the graphs below.

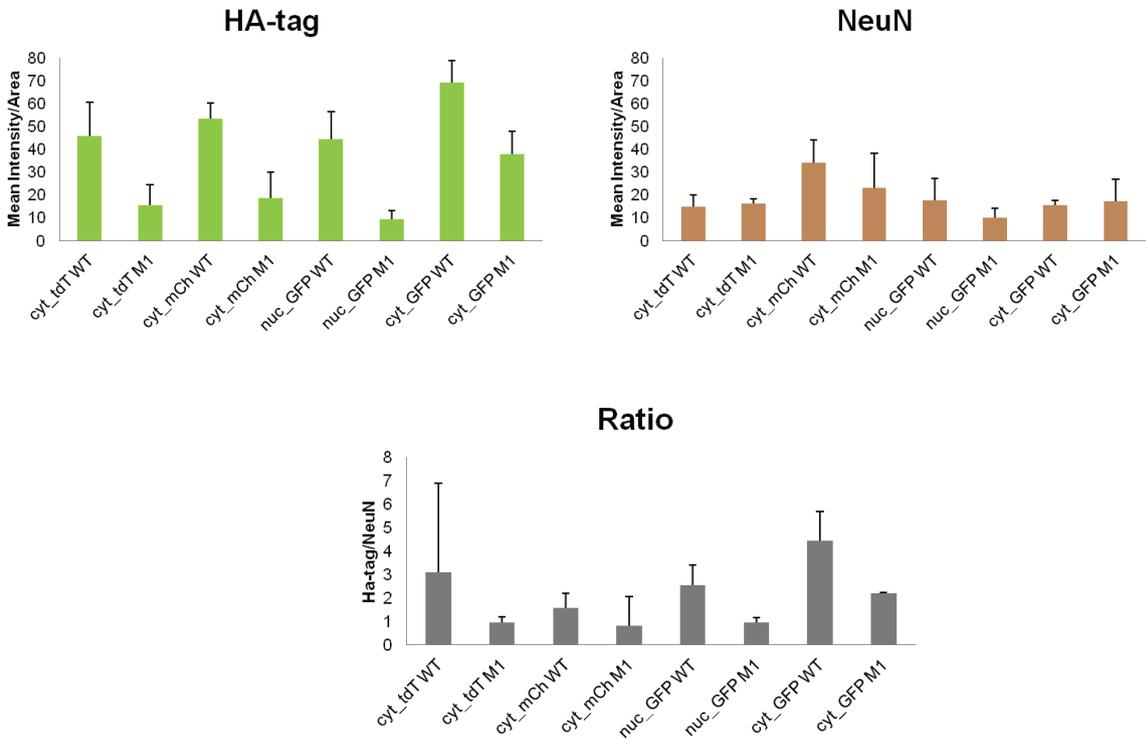


Figure 37: Quantitative analysis of the signal intensity of the various WTFMR1 plasmids compare to the M1FMR1 plasmids in human neuronal precursors. The HA-tag graph represents the mean intensity/area of the HA-tag intensity level in various cells transfected by the WTFMR1-cyt-tdT, M1FMR1-cyt_tdt, WTFMR1-cyt_mCh, M1FMR1-cyt-mCH, WTFMR1-nucGFP, M1FMR1-nucGFP, WTFMR1-cytGFP and the M1FMR1-cytGFP. The NeuN graph represents the mean intensity/area of the NeuN expression level in various neuronal cells. The Ratio graph normalizes the activity of the M1FMR1 promoter expression to the WTFMR1 promoter expression in neuronal cells.

All the various WTFMR1 and M1FMR1 plasmids were expressed in human neuronal precursors. We found a significant decrease between the various WTFMR1 and M1FMR1 plasmids suggesting that the M1 mutation (A254G) is reducing the *FMR1*-dependent expression in human neuronal

precursors. Table I below summarizes the percentages of the reduced M1FMR1 expression level compare to WTFMR1 in human neuronal precursor.

	M1FMR1nuc_GFP	M1FMR1cyt_GFP	M1FMR1cyt_mCherry	M1FMR1cyt_tdTomato	AVERAGE
M1FMR1 expression level compare to the WTFMR1 expression level	37,40%	49,20%	50,90%	30,80%	42,08%

Table I: Decrease intensity of M1FMR1 expression level compare to the WTFMR1 expression level. The decrease intensity is depicted as the ratio of the various reporter genes expression level under the control of the M1FMR1 promoter gene to the corresponding reporter genes expression level under the control of the WTFMR1 promoter gene.

We found that the M1FMR1 expression level between the various reporter genes was decreased to 42.08% compare to the WTFMR1, indicating that the M1 mutation (A254G) was having an effect independent from the reporter dye. This experiment allowed us to validate our plasmids constructs in human neuronal cells.

9.3 Neuronal activity regulates *FMRI* expression in hippocampal brain slices

FMRI expression is likely activated by the neuronal activity. To reveal how the neuronal activity influences M1FMR1 expression, we biolistically transfected hippocampal brain slices with WTFMR1-GFP and M1FMR1-GFP plasmids and we treated the transfected slices with modulators of synaptic plasticity.

9.3.1 M1FMR1 decreases *FMRI* expression in CA1 pyramidal neurons

To understand how neuronal activity affects M1FMR1 expression, we treated with Bicuculline, TTX and Glycine hippocampal slices that were biolistically transfected with M1FMR1-GFP and WTFMR1-GFP. We found a clear decrease of M1FMR1-GFP compared to WTFMR1-GFP levels, suggesting that glutamatergic activity enhances *FMRI* expression.

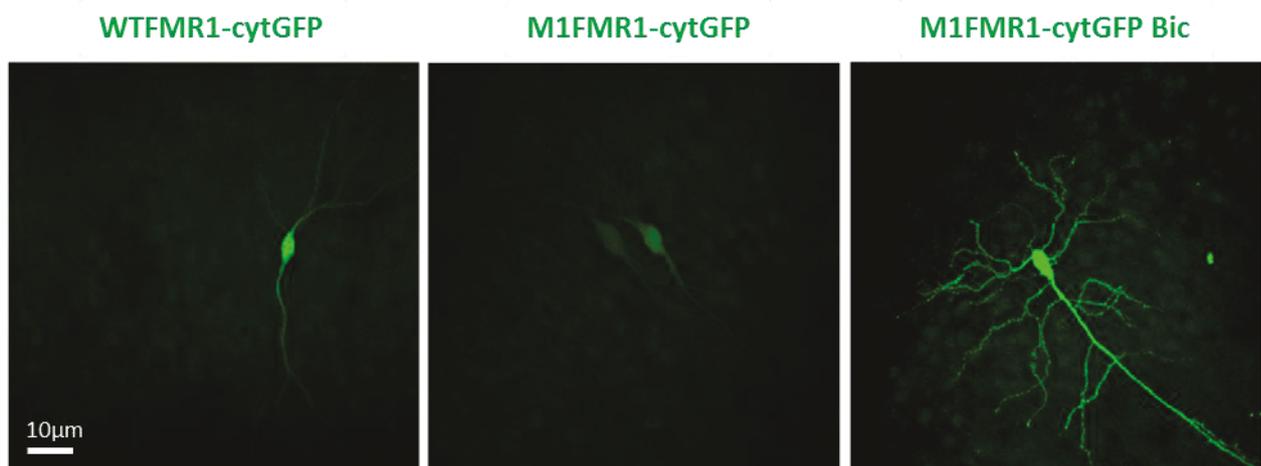


Figure 38: Examples of biolistic transfected CA1 pyramidal neurons in hippocampal slices at DIV14.

9.3.2 Glutamatergic activity likely involved in increasing *FMRI* expression

To quantify WTFMR1-cytGFP and M1FMR1-cytGFP expression, we measured the GFP signal intensity in the soma of CA1 pyramidal neurons and normalized it to the NeuN staining intensity. In the table latter against, we are summarizing the plasmids transfected and the staining used, moreover, we are adding the results of the image J analysis corresponding to the normalized GFP signal intensity level measured in the various observed cells.

Plasmids used	WTFMR1-cytGFP				M1FMR1-cytGFP			
	CTRL	TTX	Bic	LTP-Gly	CTRL	TTX	Bic	LTP-Gly
Staining	GFP (green): pFMRI-GFP expression staining NeuN (red): neuronal cells marker							
Number of observed cells	14	8	3	15	4	3	5	3
Normalized mean intensity/ area	1.5	1.9	3.5	4.0	0.7	0.7	3.5	2.4

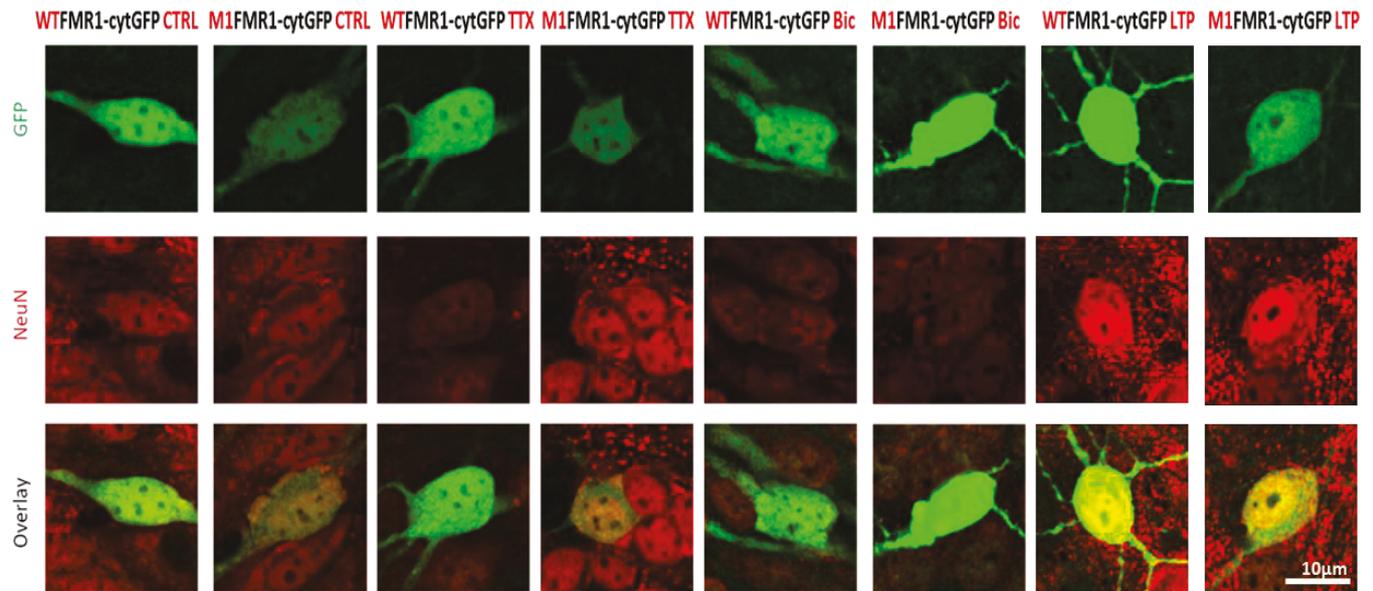


Figure 39: Gallery of images for the different experiments with pFMR1_GFP wild type or M1 mutated plasmids in hippocampal slices treated or not with Bic, TTX or LTP. In the first line of pictures, I observe the GFP expression signal variability between the cells transfected with the WTFMR1 plasmid and the cells transfected with the M1FMR1 plasmids with and without treatment. In the second line of pictures, I observe the NeuN staining in the various slices treated or not. This staining permits to insure that the cells observed are neuronal cells. The last line of pictures permits to visualize the overlay.

The number of transfected cells was very low. On average, we observed only one transfected CA1 pyramidal neuron by field. However, the GFP expression variability under the control of the WTFMR1 or the M1FMR1 promoter is interesting. At first, we controlled the mutational effect in wild type cells and we visually observed significant decrease GFP signal intensity in cells transfected by M1FMR1 promoter plasmid compare to cells transfected with WTFMR1 promoter plasmid. With TTX and LTP-Gly treatments, the GFP signal intensity was also decreasing in the M1FMR1 transfected cells compare to the WTFMR1 transfected cells.

On the other side, in M1FMR1 cells treated with bicuculline, the observed GFP signal intensity level seemed to be rescue and similar to the WTFMR1 GFP signal intensity level compare to the non-treated M1FMR1 transfected cells.

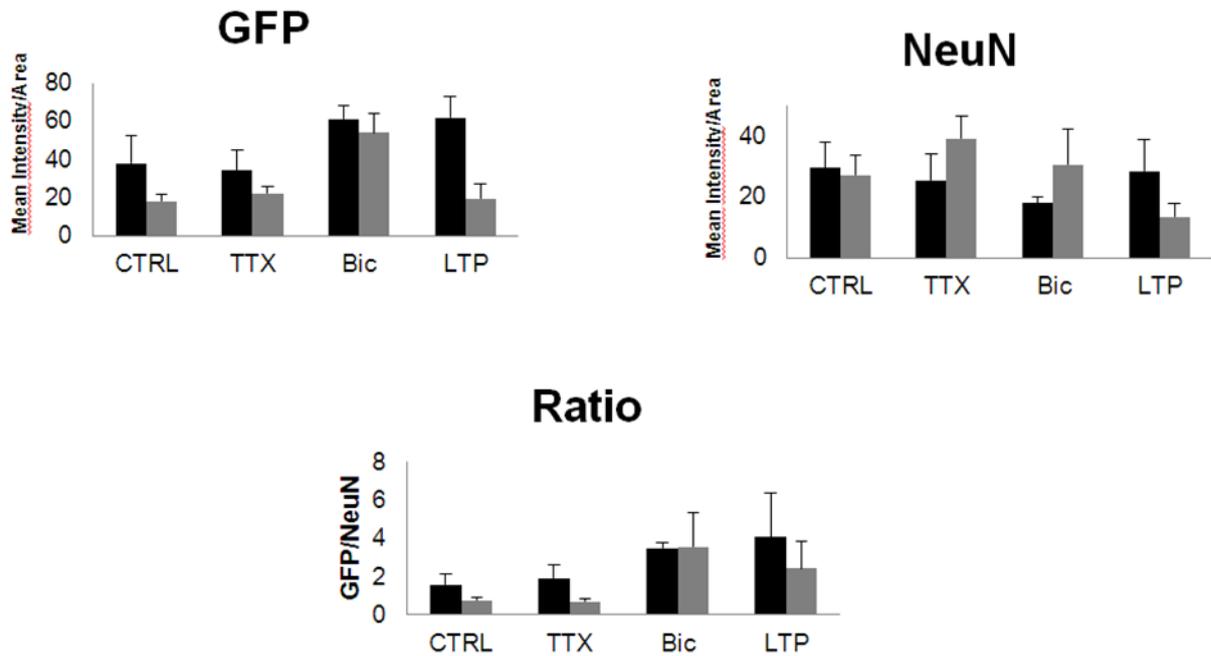


Figure 40: Quantitative analysis of the signal intensity of the various WTFMR1 plasmids compare to the M1FMR1 plasmids in CA1 pyramidal neurons. The black sticks indicate the WTFMR1-cytGFP expression and the grey sticks indicate the M1FMR1-cytGFP expression. The GFP graph represents the mean intensity/area of the GFP intensity level in transfected cells none treated and treated with Bicuculline, TTX or Glycine. The Ratio graph allows normalizing the activity of the M1FMR1 promoter expression compare to the WTFMR1 promoter expression in neuronal cells in comparing the GFP staining results to the NeuN staining results.

The Image J quantitative analysis of the signal intensity of the various WTFMR1 compare to the M1FMR1 plasmids was exclusively observed in CA1 pyramidal cells of the hippocampal brain slices. We found that the signal is more intense in cells treated by the bicuculline compare to the non-treated cells. Moreover, this signal increasing with bicuculline treatment can be visualized in the cells transfected by the M1FMR1 plasmid as well as in the cells transfected by WTFMR1 plasmids. . With the TTX treatment, the GFP signal intensity variability is similar to the one observed without treatment. The TTX treatment did not influence the *FMRI* expression. For the slices treated with glycine, the GFP signal variability between the WT and the M1FMR1 promoter plasmid follows the same result as in cells without treatment. In addition, the WTFMR1 promoter expression is increased with glycine compare to the WTFMR1 promoter expression visualized in control cells. However, the M1FMR1 promoter expression is similar to this one observed in control cells, this finding suggests that the glycine did not influence the M1FMR1 promoter expression.

	Neuronal activity modulators			
	CTRL	TTX	BIC	LTP-Gly
M1FMR1 expression level compare to the WTFMR1 expression level	45,70%	34,57%	99,70%	59,40%

Table II: Percentages of the values of the M1FMR1 signal level compare to the WTFMR1 signal level in hippocampal slices treated or not by TTX, BIC or Glycine. The values were calculated in dividing each M1FMR1 ratio values by the corresponding WTFMR1 ratio values.

In the table II, we calculated the ratio between GFP and NeuN expression level. Without treatment, the M1FMR1 promoter expression level compare to the WTFMR1 promoter expression level is decreased to 45.7%. For TTX, we observed a low effect on the M1FMR1 expression with a decreasing signal estimated to 34.6%. With LTP-glycine, the M1FMR1 expression is slightly increased compare to the control cells. Glycine is known to induce LTP by activating the NR1 subunit of the NMDA receptors. However this activation is not sufficient to rescue the M1FMR1 promoter expression level. Upon bicuculline treatment, M1FMR1transfected cells expression is rescued to 99,7%. Bicuculline is known to be an antagonist of the inhibitory action of GABA.

This experiment in hippocampal slices allowed highlighting the role of the glutamatergic activity in regulating *FMRI* expression in CA1 pyramidal neuronal cells. It permitted to validate the effect of the M1 mutation previously described in the human precursor neurons and to test various treatments in order to highlight the mechanism of the M1 mutation.

9.4 Neuronal activity influences endogenous FMRP level

In the last part of this study, we analyzed endogenous FMRP level in dissociated cortical neurons upon treatments with bicuculline and TTX.

9.4.1 Endogenous FMRP distribution is modulated by neuronal activity

The table below summarizes the values of the endogenous FMRP signal intensity level normalized by the endogenous MAP2 signal intensity level in 9 fields no treated, 9 fields treated with TTX and 9 fields treated with Bic.

Observed wells	WT FMRP	TTX FMRP	Bic FMRP
Staining	FMRP (green) / MAP2 (red)		
Number of observed fields	9	9	9
Normalized mean intensity/ area	0.36	0.33	0.40

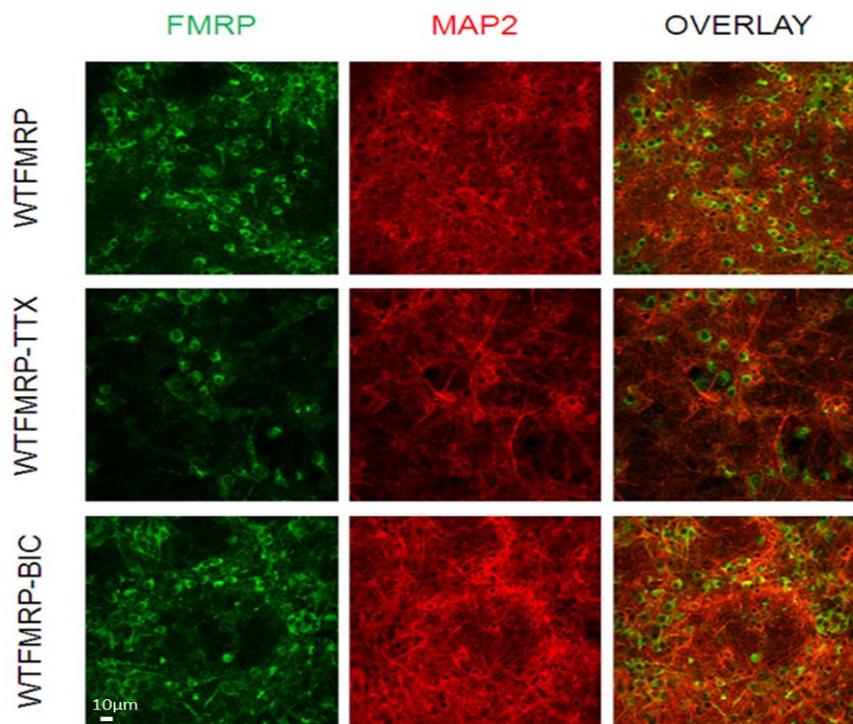


Figure 41: Representative images for endogenous FMRP expression in rat cortical neurons. MAP2 stained the microtubules of neuronal cells. FMRP antibody stained the *FMR1* gene expression in the cytosol of neuronal cells. The overlay allows highlighting the colocalization of the FMRP and MAP2 in the cytosol of neuronal cells.

As shown in the figure 41, endogenous FMRP was observed in the soma and in the cytosol of the cortical neurons. Moreover, we found a different pattern for the FMRP distribution between bicuculline and TTX treatment conditions. FMRP distribution was increased upon bicuculline whereas TTX lead to a decreased of the distribution suggesting that neuronal activation and in particular glutamatergic signaling modulates FMRP distribution in cortical neurons.

9.4.2 The glutamatergic receptor can be a potential target to rescue FXS phenotypes

To quantify our imaging results, we measured the mean intensity signal of the endogenous FMRP.

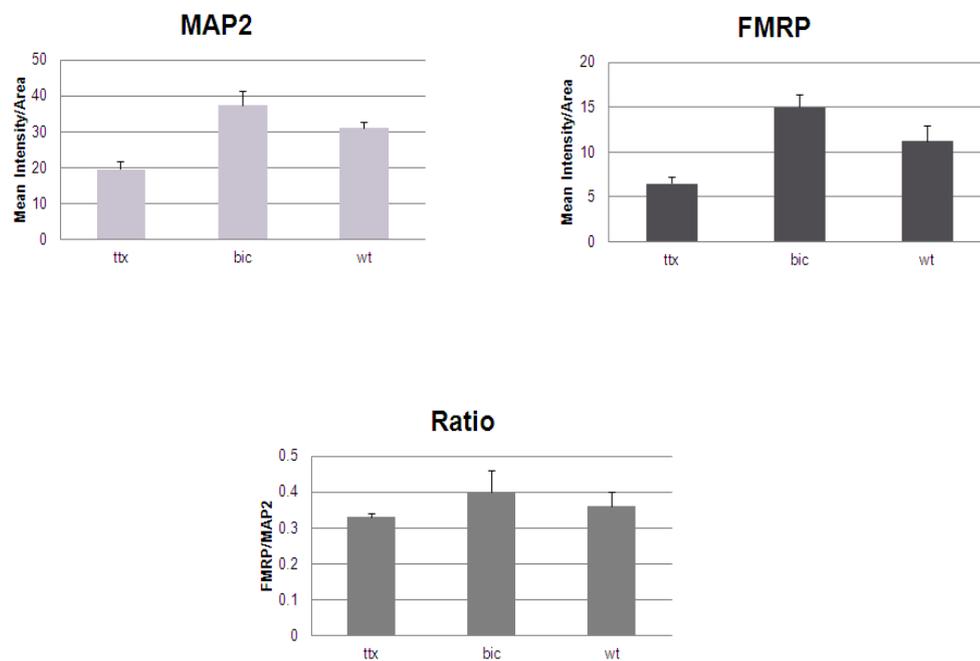


Figure 42: Quantitative analysis of FMRP and MAP2 signal intensity levels in rat cortical neurons.

The MAP2 antibody is used to stain the neuronal cells. The signal of FMRP and MAP2 is increased with bicuculline treatment and decreased with TTX in all the cells observed. Like in hippocampal brain slices, the FMRP expression is increased in cells treated with bicuculline and decreased in cells treated with TTX. The neuronal activity modulates the endogenous FMRP distribution underlying the probable involvement of the glutamatergic receptors function, which are sensitive to the bicuculline treatment antagonist to the gabaergic receptors, in the therapeutic effect.

10 Discussion

FXS is caused by an expansion of a CGG repeat in the 5'UTR region of the *FMRI* gene (Mandel et al., 1991). However, through the use of pooled template massively parallel sequencing in 2004, Collins et al., found three novel variants in promoter region of *FMRI* gene in a clinical population composed with patients who had been tested negative for *FMRI* repeat expansion at the Emory Genetics Laboratory over a 5 year span. This sequencing was performed only in males in which a variant would be hemizygous and more likely to be penetrant. These three novel variants were the first to be identified in conserved defined elements of the *FMRI* promoter gene. These three promoter mutations: c.-254A>G, c.-293T>C and c.-332G>C (also called M1, M2 and M3), are localized respectively within an Inr-like sequence near the primary TSS, at the TSS II with both Inr-like sequence and TATA like sequence; and within the overlapping binding sites for SP1 and AP-2 α transcription factors. Using a luciferase assay, the authors proved that all these three mutations independently reduced the activity of the *FMRI* promoter respectively to 36,2% , 29,2% and 5,9% in blood cells.

In this study, we assessed how neuronal activity may have an effect on *FMRI* wild type or mutated promoter expression using various plasmids expressing GFP under the control of the *FMRI* promoter in human neuronal precursors, rat cortical neurons and mouse hippocampal brain slices treated with three neuronal modulators.

10.1 Importance of regulatory elements in the *FMRI* promoter

To evaluate the impact of transcriptional regulatory elements for *FMRI* expression in defined neuronal cells, we have constructed various plasmids containing the wild type or mutated *FMRI* promoter regions driving reporters with nuclear or cytosolic localization. The *FMRI* promoter was coupled to three reporter genes: mCherry, tdTomato and GFP and the various plasmids were transfected in various cell types of the neuronal lineage including human neuronal precursors, rat cortical neurons and mouse hippocampal slices.

The plasmid constructs were generated using two different approaches: restriction mediated cloning and Gateway cloning. For the first approach we amplified the fragment of interest and the vector by PCR using modified primers containing phosphorylated end and restriction sites necessary for

enzymatic digestions. The two PCR products were then digested by the corresponding enzymes and ligated using a T4 DNA ligase. This approach is often less efficient than Gateway cloning and may require more time if specific parameters need to be optimized.

The second approach was the gateway cloning. This technology requires amplification of the fragment of interest using modified primers containing special sites called att sites. The PCR products do not need to be digested and can be cloned directly after purification with various vectors containing complementary att sites in presence of special cloning enzymes. Thus, this technology allows standardized generation of multiple plasmids in parallel very quickly in two cloning steps by just designing primers containing att sequences. During primer design, it is very important to ensure that the reading frame of the cDNA matches the reading frame of the destination vector.

After plasmid production, we transfected them in various cell types. First, we tried to transfect them in HeLa cells. These cells were used in the luciferase assay from Collin's. We tried several protocols but we did not observe reporter expression from our constructs. At this point, we could not exclude technical issues. In order to rule out mistakes during the design or cloning process of the pDONR construct, we transfected the tdTomato reporter gene in another plasmid containing a CMV promoter known to be highly expressed in HeLa cells. After transfection, we could detect the tdTomato reporter gene expression with good signal intensity. Thus we concluded that our pDONR constructs were correct and that the reason for the lack of expression of the reporters in the *FMRI* promoter constructs was likely due to the low expression levels of the *FMRI* promoter in HeLa cells.

Next, we transfected our GFP plasmids in rat cortical neurons. This experiment allowed testing the transcriptional activity of the *FMRI* promoter in neuronal cells. In contrast to the transfection in HeLa cells, in cortical neurons, we were able to show that the *FMRI* promoter is transcriptionally active in neuronal cells. We observed that few cells were transfected efficiently with the M1FMRI plasmids (A254G mutant). However, no transfected cells were observed using the M2FMRI (T293C mutant) and the M3FMRI (G332C mutant) plasmid. Indeed, the signal intensity of the cells transfected by M1FMRI plasmid was very low and it was very probable that the signal intensity of the others mutants was too weak and did not permit visualization of the transfected cells. These results can be explained either by the low transfection efficiency or by the low expression efficiency of the *FMRI* promoter. Moreover, we used the 5'UTR of the *FMRI* gene which does not have the

consensus Kozak translational initiation motif and may contribute to the low expression of the proteins.

Then, the M1FMR1 GFP, mCherry and tdTomato promoter plasmids were transfected in human neuronal precursors to visualize the effect of the M1 mutation (c.-254A>G) in this cell type. The M1FMR1 transfected cells showed a reduced signal compared to WTFMR1 transfected cells (on average the signal is decrease to 42.08% with M1FMR1 plasmids compare to the WTFMR1 plasmids) indicating that this mutation has an effect independent of the reporter gene. These results suggested that this mutation reduces *FMRI* expression most likely by affecting the binding of transcription factors.

The M1 variant is located within an Inr-like sequence near the primary transcription start site (Kumari and Usdin, 2001). Binding of Inr binding proteins to the Inr elements will facilitate the polymerase II complex formation either via interaction with TBP and/or other transcription factors or by interacting with the CTD of RNA polII (Pugh and Tjian, 1991). USFs transcription factors, which appear to be major regulatory factors involved in activation of the *FMRI* gene in brain and testis extracts, have been reported to be Inr element binding proteins suggesting that these factors play a role in the regulation of *FMRI* transcription (Beilina et al., 2004). In addition, deletion of the binding site for these factors reduces *FMRI* transcriptional activity in neuronal cell lines (Kumari and Usdin, 2001). This finding suggests that the M1 mutation could prevent the binding of USFs to the Inr element resulting in a reduced the *FMRI* transcriptional activity. Moreover, in 2004 Smith et al. demonstrated that USFs are required for normal central nervous system development and may be relevant given the role of *FMRI* in learning and memory. These findings could suggest that M1 mutation cause learning and memory impairments in FXS in affected individuals.

This is the first time that a defined analysis of the c.-254A>G mutations effect on *FMRI* expression has been studied in human neuronal precursors. Thus, this c.-254A>G mutation is likely to cause phenotypes that overlap with at least with some Fragile X syndrome phenotypes. Given that the M1 mutation is located near the primary transcription start site of the *FMRI* gene and this site is critical for the *FMRI* expression, c.-254A>G represents a clear risk to develop mental retardation due to the loss of FMRP. Our results demonstrate the importance of certain regulatory elements in the *FMRI* promoter for proper FMRP expression. To understand how the neuronal activity modulates the wild type or the M1 mutated *FMRI* promoter expression, we tested the effect of three neuronal modulators on mouse hippocampal brain slices biolistically transfected by our WTFMR1-GFP and M1FMR1-GFP plasmids.

10.2 Glutamatergic signaling influences neuronal *FMRI* expression

The transfected mouse hippocampal brain slices were treated by TTX that blocks the action potential discharges and consequently neuronal activity, Bic that serves to increase the glutamate release by inhibition of the GABA receptors and glycine that serves to induce NMDA-dependent LTP (chemical LTP).

The treatment with glycine allowed increasing the WTFMR1 gene expression but not the M1FMR1 gene expression. The NMDA-dependent LTP visualized in this experiment allowed to highlight that after glycine application the NMDA-dependent LTP is increased in normal cells likely in triggering a massive calcium entry which could permit to recruit transcription factors required for *FMRI* gene transcription. However, due to the presence of the M1 mutation in *FMRI* promoter region, the transcription factors could likely not bind to the *FMRI* promoter region preventing the *FMRI* gene transcription. This experiment allowed simply to highlight the fact that *FMRI* gene is also involved in the NMDA-dependent LTP probably by modulating the protein synthesis involved in this facilitation.

In contrast, we found a significant increase of the M1FMR1-GFP signal intensity in CA1 pyramidal neurons upon bicuculline treatment in organotypic hippocampal slices. Indeed without the bicuculline treatment the signal expression of the M1FMR1-GFP was decreased to 45.7%. The bicuculline treatment allowed rescuing the M1FMR1-GFP signal expression level to 99.7%.

On the other hand, treatment with TTX was decreasing the GFP signal in M1FMR1 transfected cells to 34.6%, suggesting that action potentials are critical to *FMRI* expression most likely by influencing glutamate release and signaling cascade influencing the calcium entry which is required for activating transcription factors such as USF1/2 required for *FMRI* gene transcription.

These two neuronal activity modulators triggered opposite reaction in the *FMRI* transcription in triggering or not the activation of transcription factors necessary for the *FMRI* gene transcription initiation.

Taken together, our results suggest a role for neuronal activity in controlling *FMRI* expression in defined neuronal populations such as glutamatergic CA1 pyramidal neurons.

This likely reflects transcriptional upregulation of *FMRI* gene in the CA1 pyramidal neurons which are exclusively excitatory neurons.

Bicuculline is an antagonist to the inhibitory action of GABAergic pathway and TTX blocks the action potential discharge by blocking voltage gated sodium channels (VGSCs). These channels play a critical role in neuronal function. In blocking these channels, TTX prevents the action potentials to depolarize the cell membrane of excitatory neurons and consequently also prevents the *FMRI* gene transcription by preventing the neuronal activity to recruit the transcriptional factors.

Bicuculline is known to increase neuronal synaptic activity and to cause both massive epileptiform activity and synaptic release of glutamate. This glutamate releasing also triggers mGluR5 and AMPARs activation. These activations allow triggering the activation of transcription factors required for the *FMRI* gene transcription.

In addition, inhibition of inhibitory action of GABAR caused by the bicuculline would lead to increase the excitatory glutamatergic pathway in CA1 pyramidal neurons.

By blocking GABA inhibition, the glutamate release activates the GluA1 subunit of the AMPA receptors triggering an increase of *FMRI* gene transcription which is necessary for the maintaining of the LTD protein synthesis balance.

AMPARs mediate the majority of fast excitatory neurotransmission in the central nervous system and are important for almost aspects of brain function such as learning, memory and cognition. They are composed of combinations of four separate subunits (GluA1-4) which form ligand-gated channels. Increases in the number and changes in the composition of synaptic AMPARs mediate LTP and LTD. AMPARs are assembled in the endoplasmic reticulum. First they are assembled as dimers, which then come together to form dimers of dimers to make a tetramer (Ayalon, 2001). In rat hippocampal neurons, synaptic AMPARs are predominantly combinations of GluA1 and GluA2 subunits. Each of AMPAR subunits are regulated by phosphorylation. In LTP, activity-dependent phosphorylation of GluA1 seems to deliver AMPARs to synapse. In contrast, GluA1 dephosphorylation is a signal for AMPAR internalization and LTD. Dephosphorylation at the c-terminal GluA1 residue Thr840 occurs in response to NMDA stimulation (Delgado, 2007) suggesting a potential role in LTD.

These results suggested that the glutamatergic receptor function antagonist to the gabaergic receptors has a therapeutic effect. In blocking the inhibition action of GABA receptors, a sufficient increase of excitatory glutamatergic activity triggered by the glutamate release and the AMPARs

activation, would allow to modulate *FMRI* expression and to rescue the loss of FMRP product observed in FXS patients.

In order to verify if glutamatergic receptor function is affecting endogenous *FMRI* expression, we analyzed the endogenous FMRP distribution in dissociated rat cortical neurons. FMRP distribution was increased upon bicuculline whereas TTX lead to a decreased of the FMRP distribution supporting the fact that neuronal activation and in particular glutamatergic signaling modulates FMRP distribution in neurons.

Upon the bicuculline treatment, we highlight the synaptic activity functioning on the *FMRI* gene transcription. The *FMRI* gene transcription is triggered by the activation of the Gp1-mGluR excitatory receptors by glutamate release. This activation could activate the transcription factors required for the *FMRI* gene transcription by triggering the entry of calcium which is important for LTD. Indeed, *FMRI* transcription is required for controlling the LTD protein synthesis and avoided an exaggerated LTD preventing excessive internalization of AMPA receptor.

At the end all these experiments allowed to conclude that the glutamatergic receptor function can be a potential therapeutic target to rescue FXS phenotypes and in particular the learning and memory impairments. According to the mGluR theory, the inhibition of the GABA receptors could increase a glutamate release which could trigger activation of the excitatory ionotropic NMDA and AMPA glutamatergic receptors which are known to be involved in neuronal development and cognition.

11 Conclusion and perspectives

This work was carried out in the team of Ivan Galimberti and Barna Fodor in the Developmental Molecular Pathways (DMP) department at the Novartis Institute for Biomedical Research (NIBR) at Basel in Switzerland. All the experiments of this thesis were performed in collaborating with various scientists and technicians; the time of their contribution and the time repartition of this work are summarized in the annex 5.

The Developmental and Molecular Pathways (DMP) department utilizes core signaling pathways as an organizing principle for drug discovery. This platform combines chemical genetics, molecular genetics and molecular profiling to identify novel therapeutic targets to a variety of genetic diseases.

The team is working on the uncovering of the various molecular pathways involved in Autism Spectrum Disorder (ASD) like Fragile X Syndrom (FXS) disease.

The main objective of this thesis was to analyze the role of synaptic activity on the *FMRI* gene transcription to highlight a potential therapeutic target that would restore the level of expression of the FMRP protein often greatly reduced or absent in FXS patients.

We based our work on Collin's article describing novel mutations found in FXS patients upon massively parallel sequencing. We started this study by the cloning of three *FMRI* promoter variants potentially involved in the pathomechanism of FXS.

To highlight the molecular mechanism by which these mutations modulate the expression of the *FMRI* gene, we first cloned these various mutated *FMRI* promoters in expression vectors coupled to three reporter genes. The Gateway cloning allowed us to obtain rapidly twenty plasmids. This first step allowed me to accustom with the cloning basics including the subtleties of the various cloning steps and the use of various analysis software.

These plasmids were then transfected into various neuronal cells cultures to demonstrate the effects of these mutations on the FMRP expression in the cerebral cortex. Thus, I enriched my knowledge in cell culture with the help of technicians and scientists working with the cell cultures. Moreover, I could follow the technicians at the animal facility and thus could closely observe various animal experiments. In addition, I learnt to work with iPSC based human neuronal precursor cells.

Then, I analyzed all the cell culture results with confocal microscopy. The confocal microscopy allowed me to visualize the architecture of the hippocampal brain structure and to better understand how the cerebral cortex functions. The results were then statistically analyzed using the ImageJ software.

In parallel, I performed an important bibliographic work to understand the basics of FXS and the action mechanisms of synaptic activity on the genes involved in numerous deleterious biologic processes implied in ASD. Various hypotheses are published to explain the cellular and molecular mechanisms of FXS. Two were mainly studied, the GABAergic hypothesis and the mGluR hypothesis involving the most important glutamate receptors at the postsynaptic synapses in the brain.

This thesis presented preliminary data of the study of the effects of three mutations located in the *FMRI* promoter region. As a result of these experiments, we could highlight the importance of the glutamatergic receptor as a therapeutic target. This conclusion is in agreement with the mGluR theory previously described by Bear et al.

However, these experiments would need to be confirmed with additional high quality data. First, it would be very interesting to carry out transfection experiments with the other mutants to evaluate the importance of the other regulatory elements in the *FMRI* promoter in neuronal cultures and to compare it to the signal intensity levels in HeLa cells. Then, it would be interesting to quantify FMRP RNA by quantitative PCR on cortical neuronal cells. This additional experiment would allow quantifying the effect of TTX and Bic on endogenous FMRP expression. In addition, it would be interesting to test in animal models the direct effects of these variants on learning and memory –e.g. by using the novel genome editing tools (CRISPR Cas9) to engineer animal models with these mutations.

In the coming years, the FXS pathogenesis mechanisms hopefully will be understood better. Currently, many aspects of the molecular and cellular mechanisms of this syndrome are unknown. Emerging new technologies like NGS or iPSCs allow novel approaches to identify mutations and study consequent molecular mechanisms as exemplified by the three mutations in the *FMRI* promoter that we focused on this study. These approaches hopefully accelerate identification of new therapeutic targets for FXS.

12 APPENDICES

12.1 Annex 1: Plasmids Midi Prep extractions

The detailed protocol below comes from Qiagen Handbook plasmids Midi Prep.

- 1- Resuspend pelleted bacteria in 2 ml or 4 ml Buffer P1. Ensure that RNase A has been added to Buffer P1. For efficient lysis, it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
- 2- Add 2 ml or 4 ml Buffer P2, gently mix by inverting, and incubate at room temperature for 3 min. The lysate should appear viscous. Do not allow lysis to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO₂ in the air. If Lyse Blue has been added to Buffer P1, the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.
- 3- Add 2 ml or 4 ml Buffer S3 to the lysate and mix immediately by inverting 4–6 times. Proceed directly to step 4. Do not incubate the lysate on ice. After addition of Buffer S3, a fluffy white precipitate containing genomic DNA, proteins, cell debris, and SDS becomes visible. It is important to transfer the lysate into the QIAfilter cartridge immediately in order to prevent later disruption of the precipitate layer. If the mixture still appears viscous and brownish, more mixing is required to completely neutralize the solution. If Lyse Blue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.
- 4- Transfer the lysate to the QIAfilter cartridge and incubate at room temperature for 10 min. If working with low-copy plasmid culture volumes, it may be beneficial to centrifuge the lysate before clarifying through QIAfilter cartridge. The centrifugation is performed instead of the 10 min incubation and leads to convenient filtration without clogging. The centrifugation can be performed directly in the same vessel which is used for alkaline lysis if this vessel is appropriate for centrifugation. Centrifugation should be performed for 5 min at approx. 4500 x g. Minor amounts of the precipitated material will be on the surface of the

supernatant and can easily be removed with a pipette tip while pouring the supernatant into the QIAfilter cartridge. Important: Room-temperature incubation is essential for optimal performance of the QIAfilter cartridge. Do not agitate the QIAfilter cartridge during this time. A precipitate containing proteins, genomic DNA, and detergent will float and form a layer on top of the solution. This ensures convenient filtration without clogging. If, after the incubation, the precipitate has not floated to the top of the solution, carefully run a sterile pipet tip around the walls of the cartridge to dislodge it.

- 5- During incubation, prepare the vacuum manifold and the QIAGEN Plasmid Plus Midi spin columns.
- 6- Gently insert the plunger into the QIAfilter cartridge and filter the cell lysate into a new tube, allowing space for the addition of Buffer BB. Filter until all of the lysate has passed through the QIAfilter cartridge, but do not apply extreme force.
- 7- Add 2 ml Buffer BB to the cleared lysate and mix by inverting 4–6 times.
- 8- Transfer lysate to a QIAGEN Plasmid Plus Midi spin column with a tube extender attached on the QIAvac 24 Plus.
- 9- Switch on the vacuum source, apply approx. –300 mbar, and draw the solution through the QIAGEN Plasmid Plus Midi spin column. After the liquid has been drawn through all columns, switch off the vacuum source. Closing VacValves after liquid has drawn through the first columns ensures that the vacuum pressure remains constant and stable for the remaining samples.
- 10- To wash the DNA, add 0.7 ml Buffer ETR and proceed with one of the following two steps:
Washing using a vacuum: After adding 0.7 ml Buffer ETR, switch on the vacuum source. After the liquid has been drawn through all columns, switch off the vacuum source.
Washing using a microcentrifuge: Discard the tube extenders and place the QIAGEN Plasmid Plus Midi spin column into the 2 ml collection tube provided. Wash the column by adding 0.7 ml Buffer ETR and centrifuging for 1 min at 10,000 x g. Discard the flow-through.
- 11- To further wash the DNA, add 0.7 ml Buffer PE and proceed with one of the following two steps: Washing using a vacuum: After adding 0.7 ml Buffer PE, switch on the vacuum source. After the liquid has been drawn through all columns, switch off the vacuum source.
Washing using a microcentrifuge: Place the QIAGEN Plasmid Plus Midi spin column in the empty collection tube from step 12. Wash the DNA by adding 0.7 ml Buffer PE and centrifuging for 1 min at 10,000 x g. Discard the flow-through.

- 12- To completely remove the residual wash buffer, centrifuge the column for 1 min at 10,000 x g in a microcentrifuge.
- 13- Place the QIAGEN Plasmid Plus Midi spin column into a clean 1.5 ml microcentrifuge tube. To elute the DNA, add 200 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAGEN Plasmid Plus Midi spin column, let it stand for at least 1 min, and centrifuge for 1 min.

12.2 Annex 2: Primary neuron culture protocol

Steps

- extract the organs you need in cold PBS
- dissect in PBS/BSA/Glucose solution.
- dissociate in Trypsin/DNase solution
- plate the cells in a DMEM/10%FCS/Glutamine medium
- start the culture in the complete medium
- change ½ of the medium every 2 days

Complete Medium

For 400ml of Complete Medium :

In a 50ml Falcon : 1 g BSA (Sigma A-9418), +4°C
 2 mg Transferrin (Sigma T-5761), +4°C
 1.6 mg Insulin (Sigma I-5500), -20°C

Dissolve in ~35ml DMEM (Gibco 21969-035), Vortex

Add 4 µl of the ethanolic solutions (15 to 21)

 400 µl of the solutions 1 to 11, 13 and 14

 64 µl of Katalase solution (12)

 evt 2ml Pen/Strep. 1000U/ml

Complete to 50 ml with DMEM

Add the 50 ml to 350 ml DMEM and filter the solution

#		Order Nr. by Sigma	Stock sol. [mg/ml]	□l of stock sol. /100 ml Medium	Storage of the substance	Storage of the solution
1	L-Alanin	A-7627	2	100	RT	-20°C
2	Biotin	B-4501	0.1	100	0-5°C	-20°C

3	L-Carnitin	C-0283	2	100	RT	-20°C
4	Ethanolamin	E-9508	1	100	RT	-20°C
5	D ⁺ -Galactose	G-0625	15	100	RT	-20°C
6	L-Prolin	P-0380	7.76	100	RT	-20°C
7	Putrescin	P-7505	16.1	100	RT	-20°C
8	Na-Pyruvat	P-5280	25	100	0-5°C	-20°C
9	Na-Selenit	S-1382	0.016	100	RT	-20°C
10	Vit. B12	V-2876	0.34	100	0-5°C	-20°C
11	Zink-Sufat	Z-4750	0.194	100	RT	-20°C
12	Katalase	C-40	16	16	-20°C	-20°C
13	Glutathion	G-6013	1	100	0-5°C	-20°C
14	SOD	S-2515	2.5	100	-20°C	-20°C
	Pen./Strep.	Gibco	1000U/ml	500	-20°C	-20°C
	Ethanollic solutions					
15	Linoleic acid	L-1376	100	1	-20°C	-20°C
16	Linolensäure	L-2376	100	1	-20°C	-20°C
17	Progesteron	P-8783	0.63	1	-20°C	-20°C
18	All trans Retinol	R-7632	10	1	-20°C	-20°C
19	Retinylacetat	R-7882	10	1	-20°C	-20°C
20	Tocopherol	T-3251	100	1	0-5°C	-20°C
21	Tocopherolacetat	T-3001	100	1	0-5°C	-20°C

PBS/BSA/Glucose

For 500ml: 500 mg BSA

5 ml 1M Glucose or 0.9 g D-Glucose (MW = 180.2, Sigma G-5400)

Dissolve in PBS, sterile filter, aliquot in 50ml Falcon

Store at -20°C

Poly-D-Ornithine, 0.5mg/ml

Dissolve 25 mg poly-D-Ornithine (Sigma P-8638) in 50ml Borate buffer 150mM pH 8.4.

Sterile filter

Add to the wells: 0.5ml/well for a 24 well-plate

Incubate the plates at least 60min at 37°C (could be several days)

Wash the plates 3x with PBS before use, do not let them dry

Store the 0.5mg/ml Poly-D-Ornithine at 4°C

Detailed protocol

1. Store dissecting instruments in EtOH prior to preparation (then before using the forcep on the brains, let the ethanol evaporate)
2. Kill pregnant rat E18 with CO₂ and remove embryos
3. Wash embryos on 70% EtOH (rarely do this)
4. Place the embryos in cold PBS, remove from embryonic sac and transfer them to a new plate with cold PBS.
5. Extract brains and place half and half in a 6cm dish with DM (PBS/BSA/Glucose), one of these dishes keep in the fridge while we do the dissection of the first half
6. Dissect cortex / hippocampus and put in 3 ml or more of DM, in a 6-well plate that is placed on an ice block
7. When finished with the dissection, transfer the brain parts to a falcon and pipette off excess of DM. Add 6-10 ml of Trypsin to 10-12 cortices and DNase at 1:1000 concentration. HC and ST, 4ml of Trypsin is enough.
8. Gently invert the tube up and down and place in the 37C **water bath** for **no longer than 15 minutes**. During this 15 min time I invert the tube up and down a few times.
9. Remove all trypsin with pipette (don't use vacuum: too strong). Add 6ml plating medium (note: plating medium is left at room temp, along with trypsin, before starting dissection).
10. Spin down 800 rpm for 5 min (tabletop centrifuge for 15ml tubes).
11. Add 1,2 ml of PM that should be @ 4°C.
12. Start dissociation, ~12 times with each Pasteur pipette size (starting from the bigger to the smaller, use 2-3 sizes), try to avoid air bubbles.
13. Add 6 ml of PM (could be more but not less) pipette up and down and pass it through a cell strainer (40um) then, pass another 6 ml of PM (could be more but not less) through the cell strainer to make sure that all cells come down. For HC and ST I, use 6ml total, consider to not use the cell strainer.
14. When doing more than one brain region, I keep the tubes on ice while plating one type of neurons.
15. Count cells pipetting 10ul of cell suspension into 10 ml of Casy buffer.
16. Plate neurons in PM, when on glass, I previously coat the dishes in PDL and then add laminin at the time of plating

17. Both in PM as well as the next day Complete medium I use Primocin instead of common Pen/Strep as it's less toxic for the cells.

PBS/BSA/Glucose

500mg BSA
0.9g d-Glucose
500ml PBS

Sterile filter and freeze at -20C in 50ml aliquots

Plating Medium

DMEM + Glutamax (Cat# 32430) or other DMEM supplemented with 1X Glutamax or Glutamine
10% FBS
1X Primocine

For Plating on Glass Coverslips

1. The night before plating, place coverslips in appropriate sized wells and cover with 0.5mg/ml Poly-d-lysine (in water, sterile filtered). Leave in CO₂ incubator at 37°C.
2. Before plating wash plates 1-2x with excess water (at least half of well volume).
3. Leave in hood under UV light for >15min before plating neurons. Laminin may be included in plating buffer.

12.3 Annex 3: Neuronal progenitor culture protocol

<u>Medium: PM+</u>	500 ml
DMEM/F12/GlutaMax medium [Invitrogen Gibco, cat 31331-028]	480 ml
N2 supplement [Invitrogen, cat 17502048] (1/100)	5 ml
B27 supplement [Invitrogen, cat 17504044] (1/50)	10 ml
Pen/Strep (1/100)	5 ml

Add fresh (keep maximum 10 days)

Basic human Fibroblast Growth Factor (bhFGF) 100 ug/ml stock to 10ng/ml (1:10 000)	5 ul
Human Epidermal Growth Factor (hEGF) 100 ug/ml stock to 10ng/ml (1:10 000)	5 ul

Splitting

- Aspirate medium
- Wash cells with PBS
- Add 0.5 ml Tryple, incubate 5 min at 37 C
- Add 0.5 ml PM+ medium,
- Pipette up and down with the P1000 pipette to destroy the monolayer and bring the cells into suspension
- Apply on a 40 um filter placed on a 50 ml falcon to obtain single cell suspension
- Dilute the cells by adding 5 ml PM+ medium
- Take 10ul aliquot for cell counting – add to 10 ml of CASY solution in a CASY container
- Count the cells by CASY, using program: cells
- Centrifuge the cells (1000 rpm for 5min)
- Prepare matrigel coated dishes: aspirate metrigel medium and wash 1x by PBS
- After centrifugation aspirate the medium, leave 100ul at the bottom of the tube and break the cell pellet by finger flicking
- Resuspend the pellet in appropriate amount of PM+ and distribute to dishes
- Plate: 5 000 000 in 4 ml PM+ for a 6cm dish and 200 000-500 000 in 0.5 ml PM+ for a 24-well
- Add ROCK inhibitor to 1 uM concentration – use a 10mM stock – dilute 1:1000 (4 ul in 4 ml)
- Change medium next day to remove the ROCK

Change medium every 2-3 days: Mon-Wed-Fri

Matrigel

- Aliquot the stock according to the data sheet – given volume x 1.5
- Keep aliquots at -20 C
- Thaw aliquot for 30 min at 4 C
- Prepare 12 ml of cold ex-vivo medium (directly from the fridge) in a falcon tube
- Keep the aliquot of matrigel on ice, transfer the aliquot volume to the ex-vivo by a P1000 with a cooled tip
- Resuspend matrigel in ex-vivo medium by pipetting up and down few times (10 ml pipette)
- Distribute to dishes: 3 ml for 6cm dish, 0.5 ml for 24-well
- Transfer directly to the fridge –let the gel polymerase over night
- Use the dishes within 10 days
- Wash with PBS before plating cells

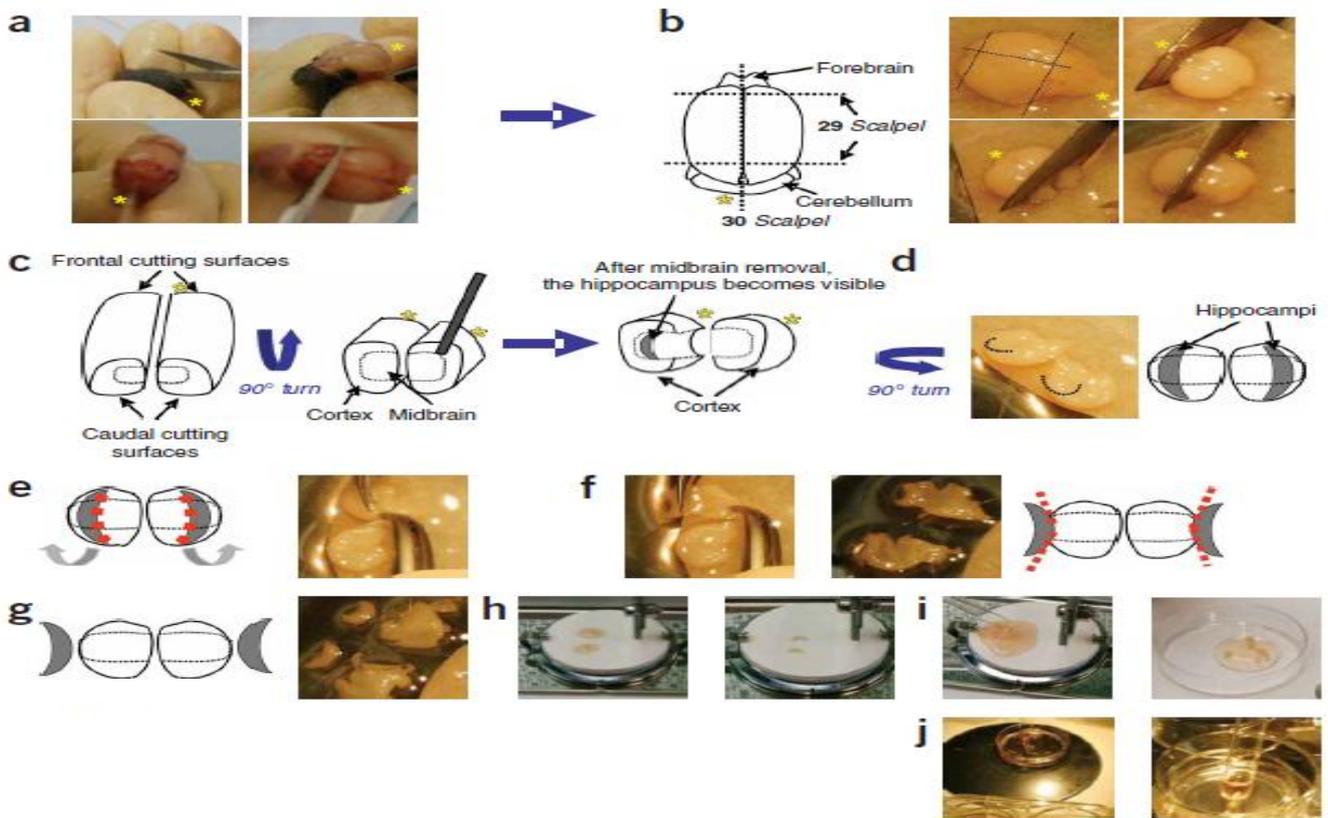
Thawing

- Thaw the vial of frozen cells at 37 C
- Prepare a 15 ml falcon tube with 10 ml warm PM+ medium
- When the suspension is nearly thawed and just a little bit of ice is left gently transfer the thawed cells to the falcon
- Centrifuge the cells (1000 rpm for 5min)
- Prepare matrigel coated dishes: aspirate metrigel medium and wash 1x by PBS
- After centrifugation aspirate the medium, leave 100ul at the bottom of the tube and break the cell pellet by finger flicking
- Resuspend the pellet in 4 ml of PM+ and plate on a 6 cm dish (if 5×10^6 were frozen)
- Add ROCK inhibitor to 1 uM concentration – use a 10mM stock – dilute 1:1000 (4 ul in 4 ml)
- Change medium next day to remove the ROCK

12.4 Annex 4: Hippocampal slices preparation protocol

1- Mouse hippocampal brain slices preparation

- Pups: postnatal day 6-9
- Illustration of hippocampal slice preparation protocol



These pictures and schemes describe the mouse hippocampal slices preparation, for legends see Gogolla N, 2006.

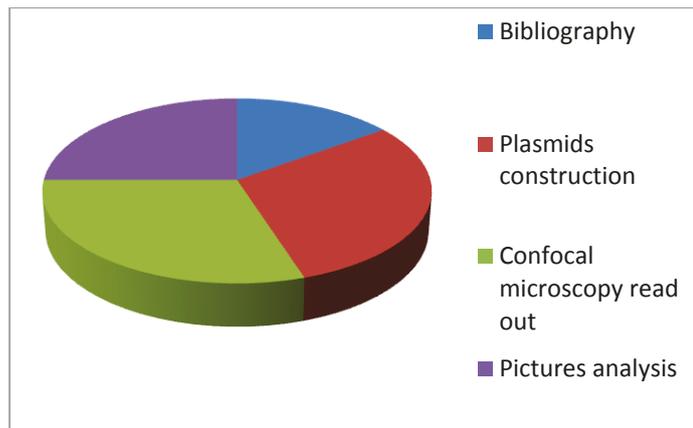
2- Slices preparation

- Culture medium: 50ml MEM (Modified Eagle Medium: containing amino acids and proteins) + 1ml penicillin/streptomycin + 120mg tris(hydroxymethyl)aminomethan + 910ul NaHCO₃(7.5%) + 50ml horse serum inactivated by heat + 50ml HBSS (Hank's Buffered Salt Solution) + 200ml ddH₂O.
- Insert 1ml culture medium by well and insert with 4 hippocampal slices by well in a plate with 6 inserts.
- Incubate at 35°C
- 5%CO₂
- Change culture medium /3days

12.5 Annex 5: Estimation of the time distribution of this work

Time distribution

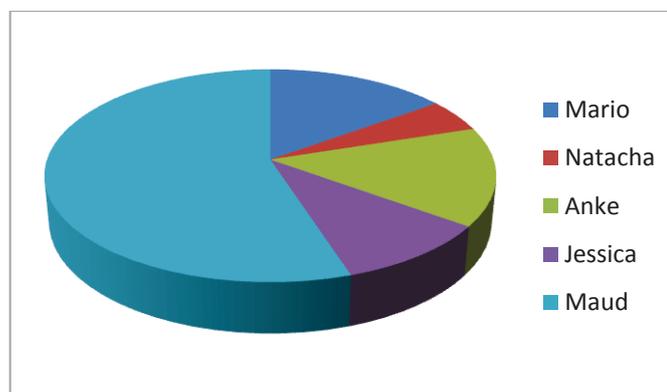
	Time repartition (%)
Bibliography	15
Plasmids constructions	30
Confocal microscopy read out	30
Pictures analysis	25



Collaborations

This work was realized with the help of various scientists and associates which are working in the Fodor and Galimberti labs.

	Time (%)	Techniques
Mario	15	hippocampal brain slices preparations
Natacha	5	cortical neurons preparations
Anke	15	GFP plasmids constructions
Jessica	10	neuronal precursors preparations
Maud	55	plasmids/transfections/cells cultures



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