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Investigating potential treatment strategies and underlying mechanisms in the Fragile X Syndrome mouse and rat models

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Declaration

I hereby certify that this thesis and its composition are entirely my own work, with the exception of the following:

1) The metabolic labelling experiment with simvastatin drug (5µM) was performed by Dr. Susana R. Louros.

2) Dr. Susana R. Louros contributed to metabolic labelling experiment with lovastatin drug.

3) Dr. Susana R. Louros contributed to metabolic labelling experiment with adult *Fmr1* KO rats.

4) Dr Susana R. Louros provided the processed hippocampal tissue I used for western blotting in Chapter4.

5) Dr. Sang Seo performed the statistical power analysis for the fear conditioning experiments

No part of the work contained in this thesis has been submitted for any other degree or professional qualification.

Signed …….. Date……..

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Abstract

Fragile X syndrome (FXS) is the most common heritable monogenic cause of intellectual disability (ID) and autism. Along with physical dysmorphic features, it is characterized by moderate to severe mental retardation, epilepsy and hyperactivity, learning disabilities, memory deficits and impairment in communication. Due to the prevalence of FXS in the general population and thanks to the availability of genetically engineered animal models, this disorder has been extensively studied for several decades and numerous treatment strategies evaluated. However, there are currently no targeted treatments and further research is essential.

FXS arises from loss of the FMRP protein, a negative regulator of protein synthesis which is highly expressed in neuronal cell bodies and synapses. FMRP regulates the translation of mRNAs downstream of group I metabotropic glutamate receptors (mGluRs). With its loss, the protein-synthesis dependent long-term effects of Gp1 mGluR activity are significantly exacerbated in the hippocampus of FXS mouse and rat models (*Fmr1* KO). Thus, the mGluR theory of FXS proposes the syndrome pathology arises from abnormally augmented responses to mGluR-driven protein synthesis. In support of this theory, inhibition of Gp1 mGluRs, in particular $mGluR_5$, can rescue multiple deleterious *Fmr1* KO phenotypes.

One of the signalling pathways linking Gp1 mGluRs to translation is the extracellular signal-regulated kinase (ERK1/2) pathway. ERK1/2 is a major regulator of protein synthesis and its inhibition has been proven beneficial for all major phenotypes of the *Fmr1* KO rodent models. This illustrates the promise of targeting over-translation for restoring function in FXS. This dissertation will describe work testing two potential treatment strategies following the idea that FXS can be ameliorated by diminishing the overtranslation of proteins resulting from the loss of FMRP, however not necessarily by targeting the ERK1/2 pathway. The two compounds are the statin simvastatin and the muscarinic receptor 4 positive allosteric modulator VU0152100 (M_4 PAM).

In the first part of this thesis, the investigation of statin drugs will be described. These compounds include lovastatin, which targets the mevalonate pathway upstream cholesterol synthesis, and consequently reduces the activation of the GTPase Ras that activates ERK1/2. Preliminary studies with lovastatin in rodents and humans have been encouraging, and new clinical trials with lovastatin are being considered. With respect to this, the newer statin simvastatin has been proposed as a better alternative due to its higher penetration to the brain, stronger effect on its target cholesterol, and larger availability for medical prescription worldwide.

The impact of simvastatin versus lovastatin was tested using biochemical measures of protein synthesis and signalling pathway activation in acute brain slices, and behavioural assays for audiogenic seizures. Unfortunately, results from this work suggest that simvastatin is ineffective at rescuing any of the *Fmr1* KO phenotypes tested. This might be due to results showing that, in contrast to lovastatin, simvastatin does not target the ERK1/2 pathway. This work was published in Muscas et al., 2019.

The second part of this thesis describes behavioural experiments in *Fmr1* KO mouse and rat models testing the potential of M_4 PAM as a therapeutic option for FXS. Recent work shows that M_4 is significantly overtranslated and overexpressed in the *Fmr1* KO hippocampus, and modulating it using an M_4 PAM corrects the most robust biochemical and physiological phenotypes of the *Fmr1* KO mouse, including excessive protein synthesis. Thus, the experiments detailed in this project assess the effects of M_4 PAM treatment on the seizure phenotype in juvenile *Fmr1* KO mice and a cognitive phenotype for novelty recognition in adult *Fmr1* KO rats, in both cases providing evidence that positive allosteric modulation of M_4 is an effective strategy. Some of this work was published in Thomson et al., 2017.

In the last part of this work, we tried to uncover the mechanisms by which this M_4 PAM ameliorates FXS phenotypes. However, more research is yet to be conducted for answering to this question. Together, the results described in this dissertation provide further evidence that targeting excessive translation is a valid strategy for treating FXS.

Lay abstract

Fragile X syndrome is a common neurodevelopmental disorder characterized by intellectual disability and autism spectrum disorder. It is caused by the loss of a protein which normally negatively regulates the translation of numerous targets. The availability of genetically engineered animal models, generated to lack that same protein affected in humans with Fragile X, has allowed for the categorization of the syndrome as a disorder of protein translation. Indeed, major phenotype of the Fragile X syndrome mouse model is the presence of elevated basal levels of protein in numerous brain areas.

Research aiming at identifying potential treatments for the syndrome has focused on manipulating protein synthesis regulatory pathways suggested to be involved in the disorder pathology. First part of this study shows that downregulating specific components of signalling cascades involved in protein synthesis can balance the overtranslation of protein characteristic of Fragile X. Compounds that miss these specific targets are shown ineffective at reducing the elevated protein synthesis and consequently fail at rescuing other pathological phenotypes.

Second part of this study shows how the increased protein synthesis can be reduced also via an alternative approach: augmenting the potency of signalling cascades already elevated in the syndrome. Although the mechanisms underlying the beneficial effects of this latter strategy are yet to be uncovered, the results described in this thesis provide further evidence that targeting excessive translation is a valid strategy for treating FXS, and support the notion that successfully rescuing the elevated translation is necessary to ameliorate other major biochemical, neurophysiological and behavioural phenotypes of the disorder.

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Abbreviations

Abbreviation	Definition
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer disease
ADHD	Attention deficit hyperactivity disorder
AGS	Audiogenic seizures
Akt	Serine/threonine protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	Ammonium persulfate
ASD	Autism spectrum disorder
BBB	Blood brain barrier
BFEC	Benign focal epilepsy of childhood
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element binding protein
CYFIP1	Cytoplasmic FMRP interacting protein 1
DHPG	3,5-Dihydroxyphenylglycine
eIF4E	Eukaryotic translation initiation factor 4E
ERK1/2	Extracellular signal-regulated kinase 1/2
FMR1	Fragile X mental retardation gene 1
FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
GABA	*gamma*-Aminobutyric acid
GPCR	G-protein-coupled receptors
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	Horseradish peroxidase
ID	Intellectual disability
KO	Knock-out
LTD	Long-term depression
LTP	Long-term potentiation
M1 - 5	Muscarinic receptor 1 to 5
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase

MEK1/2	mitogen/extracellular signal-regulated kinase
mGluR	Metabotropic glutamate receptor
MINK1	MAPK-interacting kinase 1
MPEP	2-Methyl-6-(phenylethynyl)pyridine
mTOR	Mammalian target of rapamycin
NF1	neurofibromatosis type1
NMDA	N-methyl-D-aspartate
NOR	Novel object recognition
OD	Object displacement
P70S6K or S6K1	P70S6 kinase or S6 kinase 1
PAM	Positive allosteric modulator
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIKE	PI3Kinase-enhancer
RSKs	P90 ribosomal S6 kinases
S6	Ribosomal protein 6
TSC1 - 2	Tuberous sclerosis complex 1 - 2
TRAP	Translating ribosome affinity purification
WR	Wild running
WT	Wild type

Chapter 1

Introduction

1.1 Intellectual disability and Fragile X Syndrome

Intellectual disability (ID) is a condition characterized by intellectual and adaptive impairment resulting in overall lower cognitive ability, with IQ scores <70 in mild cases to <50 in severe instances. Arising between childhood and adolescence, it has a worldwide prevalence of 1-3% (Roeleveld et al. 1997; Iwase et al. 2017). Affected individuals present with mild to severe social impairment and deficits in language, communication and daily living abilities, as well as higher rates of comorbidity with neurological or psychological disorders, particularly autism spectrum disorders (ASD)(Matson & Shoemaker 2009; Arvio & Sillanpää 2003; Vig & Jedrysek 1999; Wilkins & Matson 2009).

The significant gender bias in ID cases, with almost double the number of affected males over females, has implied a major role for the X chromosome in the development of the disorder, and has led to the identification of Fragile X syndrome (FXS) as the most common and heritable monogenic cause of ID worldwide (Lubs et al. 2012).

Fragile X syndrome is an X-linked neurodevelopmental disorder, affecting 1:4000 males and 1:8000 females (Lubs et al. 2012; Hagerman et al. 2017; Lozano et al. 2014). It was first reported as an X-linked inherited disability in 1943 (Martin & Bell 1943) and later correlated to a specific "constricted" site at the end of the X chromosome, called "marker of X chromosome" (Lubs 1969). It was only in 1991 that the *FMR1* gene was identified and FXS correlated to the partial or complete loss of this gene product: the fragile X mental retardation protein (FMRP) (Pieretti et al. 1991; Verkerk et al. 1991), a negative regulator of protein synthesis via mRNA and ribosome binding (Brown et al. 2001; Li et al. 2001; Laggerbauer et al. 2001).

1.1.1 Clinical manifestations of FXS

FXS presents with variable cognitive, neurological and behavioural deficits. Cognitively, it shows with varying ID and a range of learning disabilities from deficits

in working and short-term memory to visual-spatial processing and verbal comprehension (Munir et al. 2000; Wilding et al. 2002; Price et al. 2007; Cornish et al. 1999). Behavioural features include shyness, anxiety, aggressive behaviour, hypersensitivity to sensory stimuli, attention deficit hyperactivity disorder (ADHD) and ASD (Hagerman et al. 2009; Turk 1998; Reiss & Dant 2003; Kau et al. 2004; Baumgardner et al. 1995). Altered sleep patterns or disturbances can also be present (Gould et al. 2000).

FXS is also associated with a connective-tissue disorder resulting in dysmorphic features such as elongated face, large ears, macroorchidism, flat feet, scoliosis, hyperflexible joints and soft skin (Davids et al. 1990; Loesch et al. 2003). In addition, these connective-tissue anomalies are sometimes associated with otitis media and gastrointestinal problems (Kidd et al. 2014).

The severity of FXS symptoms is correlated to sex, age, environmental and genetic influences, and molecular variations in the mutation of the *FMR1* gene. Generally, for some deficits of the disorder more than for others, there seems to be negative correlation between the production of a functional FMRP and the clinical manifestations of the syndrome (Kaufmann et al. 1999; Dyer-Friedman et al. 2002; Loesch et al. 2004). For this reason, females, carrying a second copy of a functional X chromosome, tend to present ameliorated symptoms compared to affected males, with only between 25-50% of carriers showing borderline intellectual functioning IQ or <70 scores (Taylor et al. 1994; De Vries et al. 1996).

1.1.2 Autism and FXS

Although FXS is diagnosed genetically and autism only on the basis of behavioural observations, FXS patients very often present with the striking ASD features of repetitive behaviour, eye-gaze aversion, delayed or impaired speech and abnormal responses to sensory stimuli. Different studies have reported through the years varying data of ASD prevalence in FXS individuals, ranging from 25% to almost 50% (Hatton et al. 2006). Vice versa, among the ASD population, FXS is the most common single

gene cause of autism (Reddy 2005; Hagerman et al. 2008). Corroborating this overlap of phenotypic spectrum between FXS and autism is the finding that up to 28 genes for FMRP targets are known candidates for ASD development. These include *NLGN3*, *NRXN1*, *TSC2*, *PTEN* and *SHANK3* (Darnell et al. 2011; Jansen et al. 2017).

However, since not all individuals with FXS have ASD, the link between loss FMRP and autism is still unclear. The study of monogenic syndromes within the ASD spectrum such as FXS is therefore crucial for the understating of key mechanisms underlying autism pathology.

1.1.3 Epilepsy in ASD and FXS

The presence of ASD in FXS patients increases the chances of CNS associated secondary medical diagnosis, primarily seizures (García-Nonell et al. 2008). Incidence of seizures in FXS is estimated by different studies between 10-20% (Musumeci et al. 1999; Berry-Kravis 2002), with one study indicating a 12% incidence in individuals with FXS only and 28% in FXS+ASD patients (García-Nonell et al. 2008). The most common form of seizure are complex partial seizures of mild to moderate severity; generalized tonic-clonic seizures and simple partial seizures also occur but less often. Overall, they are usually well-controlled with treatment and generally resolve at the end of childhood (Berry-Kravis et al. 2010; Musumeci et al. 1999). However, along with ASD, FXS individuals reported to have seizures are found to be more often associated with severe cognitive deficits than FXS individuals with no epilepsy (Berry-Kravis et al. 2010). It is still unclear whether this overlap between autism and epilepsy in FXS is due to seizures causing autism development after CNS damage or autism-linked mutations associated with FXS predisposing to seizure occurrence (García-Nonell et al. 2008).

EEG abnormalities are another characteristic of FXS. A study from 2002 found abnormal EEGs to be more highly present in FXS patients with seizures than without, with 10 out of 13 patients with seizures having abnormal EEG patterns and only 5 out of 22 patients without seizures also showing EEG abnormalities. In both groups of

patients, most of these EEG abnormalities were categorized as centrotemporal spikes that disappeared after childhood ages (Berry-Kravis 2002). This type of EEG pattern, although not always associated with seizures, is the most common in FXS. Similarly, as previously said, the most common form of epilepsy in FXS are complex partial seizures that also resolve within childhood. The combination of this type of epilepsy and the presence of centrotemporal spikes resembles a benign seizure syndrome with centrotemporal spikes termed benign focal epilepsy of childhood (BFEC). Indeed, all patients from this 2002 study with centrotemporal spikes had remission of seizures within childhood ages. For this reason, it was thought at that time that EEG patterns of patients could be used to deliver prognostic on the severity and duration of seizures in FXS (Berry-Kravis 2002).

However, there still are several controversies in this matter. A more recent study failed to find an association between seizures and EEG abnormalities in FXS patients (Heard et al. 2014). Although some EEG abnormalities might be indicative of seizures, there is also the case of abnormal EEGs occurring irrespective of seizures and vice versa. More research is necessary to conclude whether EEGs could become a biomarker for some type of epilepsy in the future.

1.1.4 Genetics of FXS

The *FMR1* gene (Xq27.3 locus) of FXS individuals carrying a full mutation was found to have an expanded exon region of variable lengths at the 5' UTR (untranslated region). This expansion carried an abnormally high number of >200 CGG repeats and was associated to a CpG island resulting hypermethylated (Verkerk et al. 1991; Pieretti et al. 1991). Complete methylation caused silencing of the gene and loss of FMRP production, with some levels of *FMR1* mRNA present in patients with incomplete methylation (Pieretti et al. 1991). The expanded and hence hypermethylated FMR1 gene appears under the microscope as an elongated and constricted site at the end of the long (q) arm of the X chromosome, hence the name “fragile site” (Lubs 1969).

Not affected individuals carry a varying number of 6 to 54 CGG repeats, whereas premutation carriers have been found to have between 52 and 193 repeats (Fu et al. 1991). Contrary to normal length alleles, premutation alleles are very unstable and tend to expand at varying magnitudes from parent to progeny. Although generally clinically nonpenetrant, premutation carries can sometime present cognitive and physical deficits, believed to be associated with decreased FMRP production at varying levels (Tassone et al. 2000). Females can develop early menopause and between 10-20% have fragile X-associated primary ovarian insufficiency (Sullivan et al. 2005). Both affected sexes can also present with fragile X-associated tremor ataxia syndrome, consisting in intention tremor, autonomic dysfunction, Parksinsonism and cerebellar ataxia (Jacquemont et al. 2003).

Whereas a CGG expansion with >200 units in the *FMR1* gene is by far the primary cause of FXS, few cases of the syndrome have been reported with *FMR1* gene deletion (Coffee et al. 2008) or missense mutations thought to affect FMRP activity. A missense mutation in G266E was reported in a patient with FXS facial features, severe intellectual impairment, ADHD and autism. This mutant G266E FMRP was found specifically unable to carry out its functional association with polyribosomes and binding to known mRNA targets (Myrick et al. 2014). Similarly, a I304N point mutation found in a second patient was found located in one of the two RNA-binding domains that FMRP possess. When modelled in a genetically engineered mouse, this I304N mutant FMRP still maintained some functionality and expression, but its inability to bind RNA resulted in the development of several behavioural and electrophysiological phenotypes of FXS (De Boulle et al. 1993; Zang et al. 2009). Point mutations interfering with FMRP role as protein synthesis regulator therefore result in the development of FXS associated symptoms similarly to when FMRP expression is partially or entirely lost.

1.1.5 FXS animal models

The identification of the *FMR1* gene and its protein FMRP as mutated and lost in FXS led to the generation of genetically modified animal models, allowing for comparative biochemical, electrophysiological and behavioural studies between wild-type (WT) and *Fmr1* knock-out (KO) animals. The used of these models enabled for great progress in the unravelling of the biochemical and neurological changes caused by FMRP loss in FXS, starting with the role of FMRP are protein synthesis regulator at synapses.

From drosophila to zebrafish, the most widely studied is the conventional *Fmr1* KO mouse model, which allowed for the study of the syndrome within a mammalian system from the early 1994. The *Fmr1* KO mouse was generated by inserting a neomycin cassette in the *FMR1* coding region (exon5), causing complete loss of FMRP expression (Bakker et al. 1994). Although different from *FMR1* silencing in humans, where transcription of the CGG-expanded gene is prevented by methylation, both mechanisms result in complete absence of FMRP protein.

Later, to study the different roles of FMRP activity during either young or adult ages and within a specific tissue of the body, a second *Fmr1* KO mouse was generated. This model, called *Fmr1* KO2, was created by adjoining the *FMR1* promoter and exon1 with loxP sites for Cre recombination, to allow for regulation of temporal and spatial expression of FMRP within the rodent body (Mientjes et al. 2006).

Differently, the more recently developed *Fmr1* KO rat was generated via zinc finger nuclease technology (Hamilton et al. 2014).

The recent availability of both mouse and rat *Fmr1* KO models has allowed for the comparison of core biochemical, electrophysiological and behavioural FXS phenotypes between mammalian species (Hamilton et al. 2014; Kazdoba et al. 2014; Till et al. 2015), giving new insights on potentially conserved neuropathology of the disease between rodent models and humans, hence giving information on the potential translation of successful drug treatments in the former to the latter.

1.1.6 Structure of FMRP

After identification of the *FMR1* gene, sequence analyses the human and mouse gene revealed the presence of three different RNA-binding domains. Two evolutionarily conserved K Homology (KH) domains were identified in a middle region of FMRP not involved in isoform splicing, whereas an arginine and glycine rich domain termed the RGG box was found at its carboxyl end (Ashley Jr. et al. 1993; Siomi et al. 1993). These three motifs had been previously found in several RNA-binding proteins and ribonucleoprotein (RNP) families, and a single I304N missense mutation in the second KH motif was reported to cause FXS (De Boulle et al. 1993; Zang et al. 2009), validating the identification of FMRP as an RNA-binding protein. The KH2 motif was later shown to specifically bind the so called RNA "kissing complex" (Darnell et al. 2005). Differently, the RGG box recognizes intramolecular high-affinity G-quartets RNAs (Darnell et al. 2001). Specific arginines (533 and 538) of the RGG box were found necessary for FMRP association with polyribosomes and more arginines were shown to selectively bind different mRNA targets (Blackwell et al. 2010).

FMRP is mainly expressed in the cytoplasm but has also been found in the nucleus (Verheij et al. 1993; Devys et al. 1993). Indeed, it presents a nuclear localization signal (NLS) at the N-terminal and a nuclear export signal (NES) at the C-terminal (Eberhart et al. 1996). The presence of an NLS signal has been hypothesized to indicate the ability of FMRP to bind mRNA in the nucleus and export it out to the cytoplasm (Eberhart et al. 1996), however, major known FMRP targets have been found correctly located at their synapse site even in the absence of FMRP, suggesting there might be a second RNA-binding protein involved in their nuclear export (Steward et al. 1998).

Figure 1.1 Structure of FMRP
Nuclear localization signal to the N-terminal and nuclear export signal to the C-terminal. In between, the two KH domains for RNA binding, and the RGG box at the very end of the C-terminus.

1.1.7 Functional role of FMRP

Although widely expressed throughout the body at early stages of development, in later stages and adulthood FMRP is most strongly expressed in testes and brain, particularly at parykaria and in granules at synapses, but not in glia (Antar et al. 2004; Devys et al. 1993; Hinds et al. 1993). Equipped with its three different RNA-binding domains, different studies in human and mouse tissue found FMRP to co-fractionate or co-localize with poly(A) RNA and actively translating polysomes in the cytosol and the dendritic tree of neurons (Feng et al. 1997a; Feng et al. 1997b; Khandjian et al. 1996). FMRP association with polyribosomes also appeared to be RNA-dependent (Eberhart et al. 1996). Further studies corroborated the idea of FMRP as negative regulator of protein synthesis by showing FMRP dose-dependent repression of mRNA translation. Only mRNAs that bind FMRP are suppressed and when deprived of their FMRP-binding sequences mRNA translation is no longer inhibited. FMRP therefore works as negative regulator of protein synthesis from specific mRNA targets (Laggerbauer et al. 2001; Li et al. 2001), and as FMRP selectively binds to roughly 4% of total human RNA, the effects of its loss in FXS are striking and severe (Ashley Jr. et al. 1993; Brown et al. 2001).

The mechanisms via which FMRP exerts its translational inhibition are still debated, but two main options have been supported by different experimental results (Bhakar et al. 2012). On one hand, FMRP is thought to block translation at the elongation stage by stalling ribosomes when bound to its target mRNAs (Darnell et al. 2011). On the other end, FMRP has been proposed to inhibit translation at the initiation stage by blocking cap-dependent initiation via interaction with the cytoplasmic FMRP-interacting protein 1 (CYFIP1) and forming a complex with the eukaryotic translation initiation factor 4E (eIF4E) (Napoli et al. 2008).

1.2 FMRP role at the synapse

Polyribosomes were early found to cluster at the base of dendritic spines, confirming the local synthesis of proteins designated for the support of synaptic structure, maintenance and plasticity (Steward & Levy 1982; Weiler et al. 1997). The finding of FMRP neuronal expression, its co-localization with polyribosomes and its repression of translation suggested a prominent role of this protein in regulation of synaptic signalling and plasticity. In support of this regard, FMRP-target mRNAs in the mouse brain were found to strongly overlap with over 30% of the presynaptic and postsynaptic proteomes respectively, including NMDA and metabotropic glutamate receptor (mGluR) complex proteins, components of intracellular and synaptic signalling pathways involved in long-term potentiation (LTP) and depression (LTD), glutamate and GABA receptor signalling and CREB signalling (Darnell et al., 2011). Consistently, FXS individuals and FMRP lacking animal models present abnormal dendritic spine morphologies and densities (Grossman et al. 2006; Comery et al. 1997; Irwin et al. 2001; Rudelli et al. 1985).

1.2.1 FMRP activity downstream metabotropic glutamate receptors

Glutamate synapses have received particular attention over the past thirty years of research as they were shown to trigger postsynaptic protein synthesis via mGluRs. mGluRs are transmembrane receptors part of the G-protein-coupled receptor (GPCR) family. The binding of extracellular ligands triggers a receptor conformational change and activation of the associated G protein, of which subunits exert regulatory effects on downstream target molecules such as enzymes, other receptors, transcription and translation factors (Niswender & Conn 2010). Group1 (Gp1) mGluRs are largely expressed throughout the CNS and localized within the postsynaptic localization of excitatory synapses (Nicoletti et al. 2011). They include $mGluR_1$ and $mGluR_5$, both coupled to Gq/G11 G proteins and most selectively activated by the agonist 3,5-dihydroxyphenylglycine (DHPG) (Ferraguti & Shingemoto 2006).

Activation of Gp1 mGluRs triggers a complex phosphorylation signalling cascade culminating in quick, specific association of synaptic mRNAs and their translation (Weiler & Greenough 1993). This cascade comprises different signalling pathways including the extracellular signal-regulated kinase1/2 (ERK1/2) and mammalian target of rapamycin (mTOR) pathways (Page et al. 2006; Niswender & Conn 2010; Ferraguti et al. 2008).

Because the major effects of FMRP loss in FXS are associated with deficits in cognition due to impaired synaptic formation and plasticity, several research studies have focused on brain areas known to be involved in learning and memory, such as the well-defined structure of the hippocampus.

In synaptoneurosomes from rat hippocampal neurons, within 1-2 minutes from selective Gp1 mGluRs activation, screening of the mRNAs associated with polyribosomes showed rapid association of FMRP mRNA to the translational machinery and consequent increase in FMRP expression. Moreover, FMRP protein was stained associated to polyribosomes in the same synaptoneurosome fraction (Weiler et al. 1997). Hence, FMRP seems to modulate the *de novo* synthesis of synaptic proteins via regulating translation of specific mRNAs downstream of mGluRs activation. Surprisingly, among the FMRP mRNA targets, FMRP mRNA was later found to be one itself (Ceman et al. 1999; Schaeffer et al. 2001). This suggests a negative-feedback loop mechanism that regulates FMRP expression and its inhibitory activity on translation.

1.2.2 mGluR-dependent LTD is exaggerated in *Fmr1* KO mice

Although both Gp1 mGluR1 and mGluR$_5$ are present in the hippocampus, only mGluR$_5$ immunoreactivity is present in all hippocampal areas, being the only Gp1 receptor present in the CA1 region (Shigemoto et al. 1997). In the CA1 region of the hippocampus, stimulation of Gp1 mGluR$_5$ with DHPG results in acute depression of synaptic transmission with subsequent reduced baseline, a form of LTD mechanistically different from the well-known NMDA-dependent LTD. This mGluR-

LTD was proven to be dependent on *de novo* protein synthesis from per-existing mRNAs within the dendritic compartment (Huber et al. 2000; Huber et al. 2001), and it was shown to be coincident with internalization of surface AMPA receptors (Snyder et al. 2001; Carroll et al. 1999). Consistent with the idea of FMRP as a negative regulator of protein synthesis, hippocampal mGluR-LTD of *Fmr1* KO mice is enhanced by 10-15% compared to WT controls and presents with excessive AMPA receptor internalization (Huber et al. 2002; Nakamoto et al. 2007).

In the *Fmr1* KO, $mGluR_5$ expression in the hippocampus was found comparable to WT controls (Huber et al. 2002; Giuffrida et al. 2005), and the exaggerated mGluR-LTD was found no longer dependent of protein synthesis (Nosyreva & Huber 2006). This proves normal constitutive signal from $mGluR_5$ in *Fmr1* KO mice and correlates the exaggerated LTD to elevated basal levels of protein synthesis due to absence of negative regulation from FMRP. These elevated protein levels at basal states are hypothesized to be sufficient for the support of LTD in *Fmr1* KO mice, making *de novo* protein synthesis obsolete (Nosyreva & Huber 2006).

1.2.3 Basal protein synthesis is exaggerated in the *Fmr1* KO hippocampus

Besides enhancing the protein-synthesis dependent synaptic modifications downstream $mGluR_5$ activity, loss of FMRP protein in FXS results in elevated basal protein synthesis levels in several brain areas. An *in vivo* study found a significant increase in protein synthesis levels in the cortex, hippocampus, thalamus and hypothalamus of *Fmr1* KO mice compared to WT controls at 4 and 6 months of age (Qin et al. 2005). *In vitro*, an assay to measure the incorporation of ^{35}S-methionine/cysteine amino acids into newly synthesized proteins in hippocampal slices showed a similar 10% increase in basal protein levels of *Fmr1* KO mice versus WT littermates (Dölen et al. 2007). Autoradiographs of obtained lysates showed increased translation of proteins at all molecular weights, indicating FMRP as repressor of translation of a wide range of targets. IP experiments to analyse the expression of known FMRP targets versus known non-targets proved this increased basal translation of proteins in *Fmr1* KOs to be due to loss of FMRP (Osterweil et al.

2010). These studies have shown hippocampal slices to reliably recapitulate the *in vivo* findings and present the advantage of potential pharmacological manipulation.

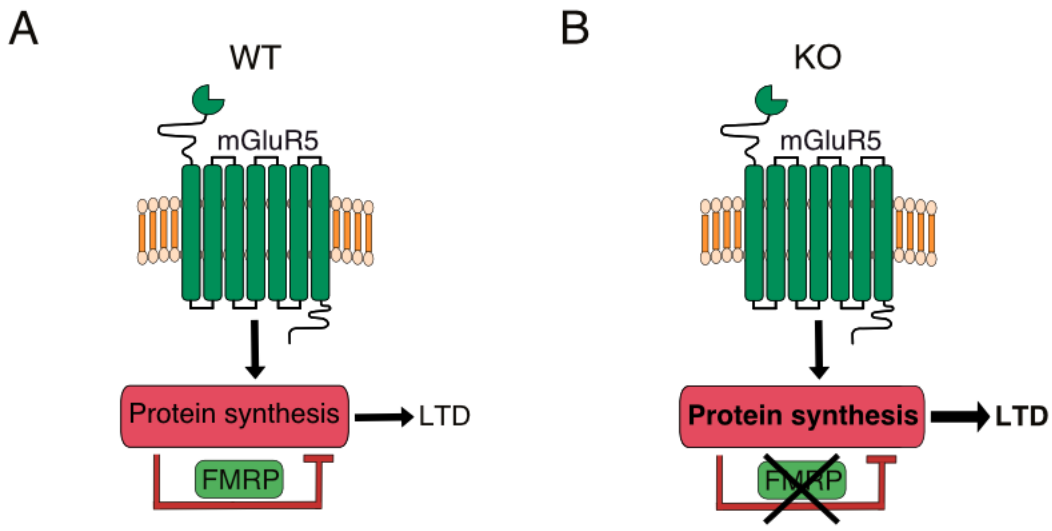

Figure 1.2 FMRP loss in *Fmr1* KO rodents and mGluR-dependent LTD
(**A**) In WT controls, protein synthesis downstream mGluR5 is inhibited by FMRP negative regulation. These newly synthesized proteins support the long-term effects of mGluR5 activation, such as LTD. (**B**) In *Fmr1* KO rodent models, protein synthesis downstream mGluR5 is not inhibited by FMRP and is significantly increased at basal levels compared to WT. The consequent mGluR5-dependent LTD is also exaggerated and no longer protein synthesis-dependent.

1.2.4 The mGluR theory of FXS

The findings that FMRP synthesis is triggered and regulated by Gp1 mGluRs activation, that basal protein synthesis of several brain areas is exaggerated in the absence of FMRP, and that this increase in protein levels results in exaggerated mGluR-LTD, perhaps slowing normal synaptic grow and causing seizures, led to the formulation of the mGluR theory. The theory postulates many protein-synthesis dependent effects downstream mGluRs activation to be exaggerated in the absence of FMRP, and to be the trigging cause of main FXS cognitive and behavioural

phenotypes, such as: epilepsy, intellectual deficit, memory and learning disability, thinning and increased number of dendritic spines (Bear et al. 2004).

For instance, dendritic spines were found to significantly elongate after DPHG stimulation of mGluR5 in cultured hippocampal slices, their growth being protein-synthesis dependent (Vanderklish & Edelman 2002). Moreover, mGluR5 stimulation with DHPG increased WT protein synthesis levels but failed to further exaggerate the elevated *Fmr1* KO protein synthesis, this because mRNA translation downstream mGluR5 basal signalling is perhaps already saturated due to loss of FMRP negative regulation (Osterweil et al. 2010).

Differently, Gp1 mGluR5-specific antagonists MPEP (2-methyl-6-phenylethynyl pyridine hydrochloride) and SIB 1893 have anticonvulsant properties and can block seizures in mice (Chapman et al. 2000), which could be linked to the *Fmr1* KO seizures phenotype; similarly, neuronal firing for circadian rhythmicity requires Gp1 mGluRs signalling (Park et al. 2003a), and altered night-day cycle with disrupted sleep is another characteristic of FXS patients (Gould et al. 2000).

Hence, the theory proposes the investigation of therapies targeting the activation of mGluR5 and its downstream signalling pathways to correct core FXS deficits. In support of it, genetically engineered *Fmr1* KO mice with only 50% WT levels of mGluR5 expression showed correction of most *Fmr1* KO mice phenotypes, including exaggerated LTD, elevated hippocampal protein synthesis, defective ocular dominance plasticity, exaggerated inhibitory avoidance extinction, increased spine density, and increased sensitivity to audiogenic seizures (AGS) (Dölen et al. 2007). Pharmacological inhibition of mGluR5 signalling with the antagonists MPEP and CTEP produced similar effects (Michalon et al. 2014; Osterweil et al. 2010), also rescuing increased AMPA receptor internalization (Nakamoto et al. 2007), increased open field entry and exploration at both juvenile and young adult ages (Yan et al. 2005), and prolonged epileptiform discharges in the CA3 hippocampal area (Chuang et al. 2005).

1.2.5 mRNA translation downstream mGluRs

Gp1 mGluRs are coupled to mRNA translation via the ERK1/2 and mTOR pathways, both of which exert regulatory activities on components of 5' cap-dependent translation. Initiation is the rate-limiting step of 5' cap-dependent translation and the most targeted by regulatory signalling from extracellular stimuli. This step starts with the binding of the eucaryotic initiation factor (eIF) 4F protein complex to the 5' cap. The heterotrimeric eIF4F complex comprises several proteins, including the cap-binding eIF4E, the ribosome scaffolding protein eIF4G, and the RNA helicase eIF4A (Gingras et al. 1999). Both ERK1/2 and mTOR pathways control translation by modulating, directly or indirectly, the activity of eIF4E. Moreover, both pathways can regulate translation of a mRNA family with an oligopyramidine track to their 5'cap (TOP mRNA) by phosphorylating p70S6 Kinase (p70S6K or S6K1) and ribosomal S6 protein (S6) (Dufner & Thomas 1999; Antion et al. 2008).

1.2.6 The mTOR pathway downstream mGluRs

The mTOR pathway is an intracellular signalling pathway crucial for the regulation of cell cycle, proliferation and apoptosis. mTOR is a serine/threonine kinase that serves as core component of two protein complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2) (Laplante & Sabatini 2009). In the hippocampus, Gp1 mGluR signalling to the mTOR pathway is mediated by long Homer proteins, scaffolding proteins that bind the mGluRs intracellular tail and activate downstream effectors. Upstream mTOR is the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), which is activated via the mGluR-Homer coupling to the small GTPase PI3Kinase-enhancer (PIKE) (Rong et al. 2003). At the cell membrane, PI3K transforms phosphatidylinositol-4,5-bisphosphate (PIP2) lipids to phosphatidylinositol-3,4,5-trisphosphate (PIP3), with which the 3-phosphoinositide-dependent kinase-1 (PDK1) interacts to activate the seine/threonine protein kinase B (PKB or Akt) through phosphorylation at Thr308, along with PDK2 (mTORC2) phosphorylation at Ser473 (Liao & Hung 2010). Active Akt then phosphorylates the tuberous sclerosis complex-2 (TSC2), in complex with

TSC1, blocking their inhibition of the small GTPase Rheb (Ras homolog enriched in brain), resulting in mTORC1 activation (Huang & Manning 2009). Active mTORC1 promotes 5' cap-dependent translation by phosphorylating the eIF-4E binding proteins (4E-BPs) and disrupting their inhibitory binding to eIF4E (Beretta et al. 1996)

1.2.7 The ERK1/2 pathway downstream mGluRs

The ERK1/2 pathway is one of the mitogen-activated protein kinase (MAPK) signalling pathways, important for complex signalling cascades involved in processes such cellular proliferation, development and differentiation (Zhang & Liu 2002). Upstream ERK1/2 is the small GTPase Ras, which once GTP-bound activates its effector Raf kinase. Raf phosphorylates and activates the mitogen/extracellular signal-regulated kinase (MEK1/2), which in turn activates ERK1/2 (Molina & Adjei 2006). Several receptors can stimulate the ERK1/2 pathway, including GPCRs. Downstream mGluRs, ERK1/2 activity is believed to get promoted via protein kinase C (PKC), Ras and Raf activation by mGluRs Gq subunit (Hawes et al. 1995).

Active ERK1/2 is known to modulate cap-dependent translation via two separate mechanisms. First, by phosphorylating the MAPK-interacting kinase1 (MNK1), which in turn phosphorylates eIF4E on its Ser209 residue, increasing mRNA translation (Banko et al. 2004); second, by activating p90 ribosomal S6 kinases (RSKs), required for S6 phosphorylation at Ser235/236, promoting its association to the initiation complex (Roux et al. 2007).

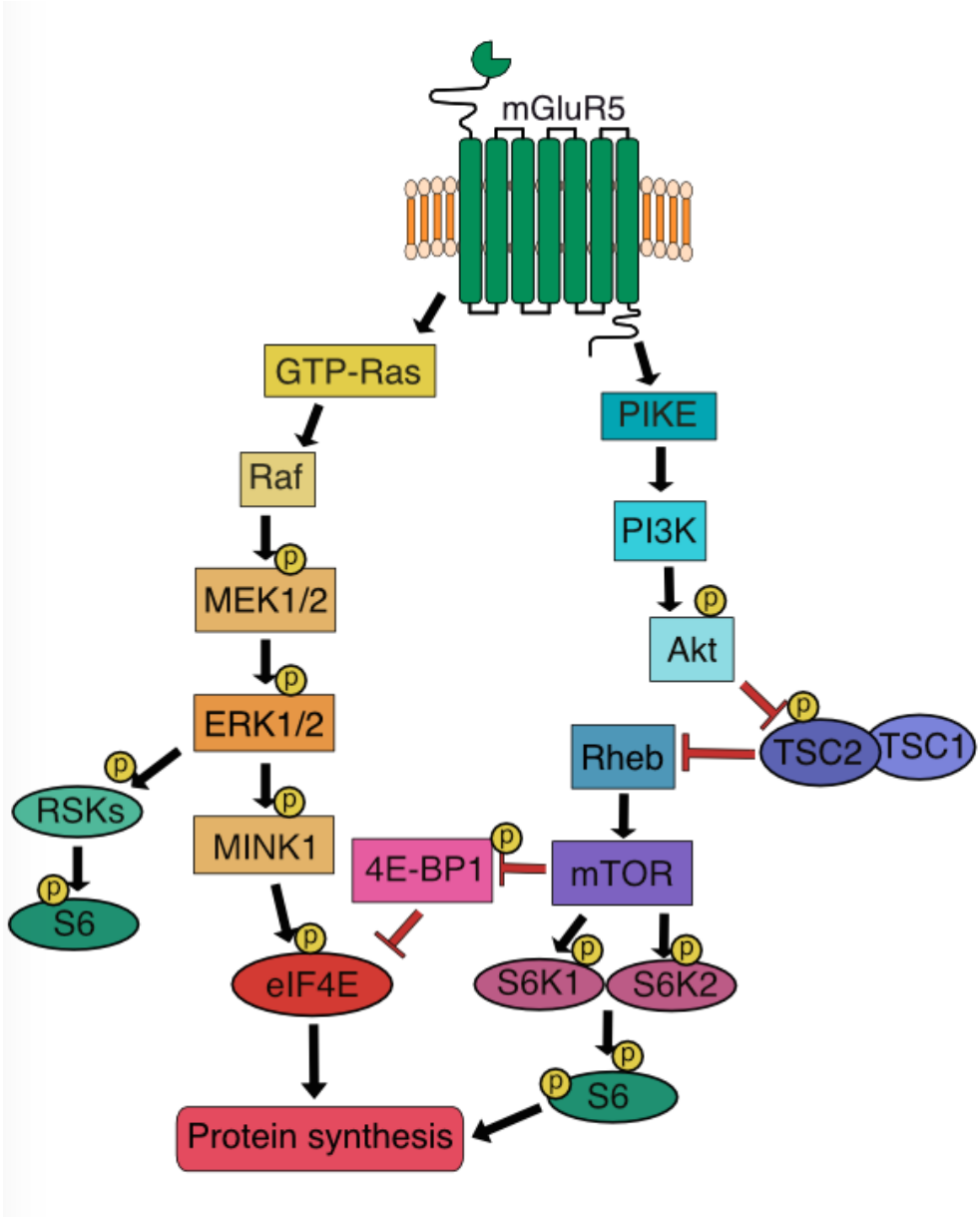

Figure 1.3 Regulation of 5' cap-dependent translation by ERK1/2 and mTOR pathways

Simplified schematics of ERK1/2 and mTOR pathways downstream mGluR5 activation, both pathways promote 5' cap-dependent translation via both independent and convergent signalling.

1.2.8 The mTOR pathway in *Fmr1* KO mice

Different studies showed mGluR-LTD to require signalling from the mTOR pathway. In WT hippocampus, mGluR-LTD and PI3K/mTOR activation were blocked by

inhibition of mGluR-Homer interactions (Ronesi & Huber 2008) and pharmacological application of PI3K inhibitors and rapamycin (Hou & Klann 2004). However, in *Fmr1* KO mice, long Homer scaffolding proteins were shown to associate remarkably less with the mGluR5 intracellular C-terminal tail (Giuffrida et al. 2005), mTOR was found inactive and mGluR-LTD was proven unaffected by Homer interaction disruption (Ronesi & Huber 2008). According to this, mGluR-LTD is blocked by rapamycin treatment in WT mice but rapamycin-insensitive in the *Fmr1* KO (Sharma et al. 2010). This suggests a disconnection of mTOR signalling from mGluRs activation in FXS. In support of these findings, Akt and p70S6K basal activation were found unchanged between *Fmr1* KO and WT mice in different studies (Muscas et al. 2019; Osterweil et al. 2010), and even though the mTOR pathway is known for promoting basal protein synthesis in WT tissues, inhibition of mTOR activation with rapamycin had no effect on the elevated hippocampal protein synthesis of *Fmr1* KO mice (Osterweil et al. 2010). Furthermore, Akt activation was also found unchanged after DHPG stimulation of hippocampal tissue (Osterweil et al. 2010).

However, a study on differently processed hippocampal tissue found increased PI3K an mTOR activation in *Fmr1* KO mice (Sharma et al. 2010); while a second found increased PI3K and Akt expression in cortical synaptoneurosomes, along with elevated protein synthesis that could be rescued with PI3K inhibition (Gross et al. 2010). This hyperactivation of mTOR pathway components could result from overexpression of the FMRP-target PI3K catalytic subunit and PI3K enhancer PIKE in *Fmr1* KO mice (Gross et al. 2010; Sharma et al. 2010). These differences between experimental studies could arise from analyses of different brain tissues, significantly different tissue processing techniques, mouse background strain, age and colony. Overall, Gp1 mGluRs seem to modulate mTOR signalling through long Homer proteins, with which interaction appears disrupted in FXS. Further studies are necessary to better elucidate the role of mTOR signalling in *Fmr1* KO cortical and hippocampal tissues.

1.2.9 The ERK1/2 pathway in *Fmr1* KO mice

Besides mTOR signalling, mGluR-LTD also requires activation of the ERK1/2 pathway. In WT animals, upon hippocampal stimulation with DHPG, ERK1/2 pathway activation via phosphorylation was shown to rapidly increase in several studies (Gallagher et al. 2004; Osterweil et al. 2010; Banko et al. 2006), and mGluR-LTD, along with ERK1/2 activation, was significantly inhibited by the MEK1/2/ERK1/2 inhibitor U0126 (Gallagher et al. 2004).

In *Fmr1* KO mice, some research found increased basal levels of phosphorylated ERK1/2 (Hou et al. 2006; Wang et al. 2012), with sustained activation upon DHPG stimulation (Hou et al. 2006). This argues for an exaggerated response of the ERK1/2 pathway to constitutive mGluR signalling in FXS. Others found no hyperactivation of ERK1/2 at basal states (Osterweil et al. 2010; Muscas et al. 2019) but showed a marked activation upon DHPG stimulation similar to WT controls. This suggests constitutive activation of mGluRs and ERK1/2 in FXS, but a hypersensitive response of the downstream protein synthesis machinery normally regulated by FMRP. These differences found in ERK1/2 basal activation levels could be related to the type of tissue examined, the processing of the tissue, the animal background strain, age and colony.

Regardless, the ERK1/2 pathway has been shown to positively respond to pharmacological and genetic manipulation for the treatment of core FXS deficits. ERK1/2 inhibition with the compound U0126 corrected the elevated protein synthesis in the hippocampus of *Fmr1* KO mice (Osterweil et al. 2010), whereas the MEK1/2 SL 327 inhibitor rescued the characteristic increased susceptibility to AGS (Wang et al. 2012). Crossing of *Fmr1* KO mice with a mutant line with genetic reduction of eIF4E phosphorylation downstream ERK rescued numerous of the core FXS phenotypes investigated, including elevated hippocampal protein synthesis, exaggerated LTD, impaired preference for social novelty, aggressive and hyperactive behaviours, and AGS (Gkogkas et al. 2014). Moreover, in the CA3 area of the *Fmr1* KO hippocampus, the MEK inhibitor PD98059 rescued the prolonged epileptiform discharges (Chuang et al. 2005).

1.3 Hyperexcitability phenotypes of *Fmr1* KO mouse model

In the *Fmr1* KO mouse, the loss of FMRP, a negative regulator of protein synthesis, causes a dysregulation of mRNA translation, which leads to core biochemical and electrophysiological phenotypes also conserved in the *Fmr1* KO rat model. Some of these phenotypes include exaggerated protein synthesis, abnormal development of synapses and irregular synaptic plasticity and maintenance, ultimately resulting in altered neuronal connections and signalling throughout the brain (Qin et al. 2005; Dölen et al. 2007; Till et al. 2015; Huber et al. 2002; Grossman et al. 2006; Comery et al. 1997).

Numerous studies on the physiology and biochemistry of the *Fmr1* KO mouse brain have highlighted different forms of increased neuronal excitability and hypersensitivity of sensory circuits, which parallel very well with behavioural and cognitive abnormalities of FXS patients (Contractor et al. 2015). One example is the presence of persistent activity states, depolarized firing states also called UP states, which in the *Fmr1* KO mouse somatosensory cortex were found to occur spontaneously both during sleep and awake times, their duration being about 59% longer than in WT mice (Hays et al. 2011; Gibson et al. 2008). This increase in UP states occurrence and duration in the *Fmr1* KO mouse is one phenotype that suggests changes in cortical excitation in FXS. Another example is prolonged epileptiform discharges in the CA3 hippocampal region. In WT rodents, after the induction of epileptiform bursts with the $GABA_A$ antagonist picrotoxin, stimulation of Gp1 mGluRs with DHPG transforms these bursts into prolonged epileptiform discharges that are protein synthesis-dependent (Merlin et al. 1998). In the *Fmr1* KO mouse, lack of FMRP allows for these CA3 epileptiform discharges to occur spontaneously after induction of increased neuronal activity with $GABA_A$ antagonism, without requirement for DHPG stimulation of Gp1 mGluRs (Chuang et al. 2005). This finding corroborates the hypothesis of hypersensitive responses occurring downstream mGluR-ERK1/2 signalling in FXS (Osterweil et al. 2010). Indeed, the induction and maintenance of these discharges is prevented by antagonist inhibition of ERK1/2 signalling or Gp1 mGluRs respectively (Chuang et al. 2005).

These excitability characteristics of the *Fmr1* KO neuronal network allow for investigation of mechanisms and potential targeted treatments.

1.3.1 Increased susceptibility to audiogenic seizures in *Fmr1* KO mice

FXS presents with about 20% incidence of seizures that generally stop within the end of childhood (Musumeci et al. 1999; Berry-Kravis et al. 2010; Berry-Kravis 2002). This characteristic form of epilepsy is well modelled in the *Fmr1* KO mouse by a phenotypic increased susceptibility to AGS at juvenile and young adult ages.

Generally, naive mice are exposed to a repetitive sound with high frequency range and >120 dB for 2 minutes. Seizures present with three recognized stages of severity: 1) wild running (WR; prominent, uncontrolled running and flailing), 2) clonic seizure (loss of balance and intense convulsions), 3) tonic seizure (cessation of movement and extension and stiffening of limbs and tail) (Musumeci et al. 2000; Yan et al. 2005).

This *Fmr1* KO behavioural phenotype is well conserved across mice background lines and has incidence of about 60-70% in *Fmr1* KO males compared to 0-20% in WT (Musumeci et al. 2000; Yan et al. 2005; Dölen et al. 2007). Moreover, it is the only FXS behavioural phenotype to be as robust and well conserved across mice strains to be also representative of a major characteristic of the disorder in humans. This lets the phenotype partly fulfil the requirements for face validity. Hence, within the years this assay has become a benchmark for predicting the therapeutic power of treatments under investigation (Min et al. 2009; Yan et al. 2005; Liu et al. 2011; Busquets-Garcia et al. 2013; Dölen et al. 2007; Osterweil et al. 2010; Osterweil et al. 2013). Several studies investigating different genetic and pharmacological treatments regularly found the efficacy of a treatment at reducing AGS incidence to indicate its effectiveness at rescuing also other *Fmr1* KO phenotypes. These include: antagonism of $mGluR_5$ receptor with MPEP, 50% genetic reduction of $mGluR_5$ expression, lithium treatment, pharmacological inhibition of ERK1/2 activity with inhibitor compound SL 327 or lovastatin, blockade of the endocannabinoid system receptor CB1R, positive allosteric

modulation of muscarinic receptor 4 (Min et al. 2009; Yan et al. 2005; Liu et al. 2011; Busquets-Garcia et al. 2013; Dölen et al. 2007; Muscas et al. 2019; Osterweil et al. 2010; Osterweil et al. 2013; Thomson et al. 2017). Therefore, this assay is currently regarded as an excellent *in vivo* measurement for treatment potential of compounds for later investigation in human trials.

However, it is important to note that treatments successful at rescuing AGS and other phenotypes in rodent models, in later human trials showed little promise. For example, trials with $mGluR_5$ antagonists and $GABA_A$ agonists (Erickson et al. 2017). This could be due to a number of reasons, including underpowered analyses of the effects of treatment in humans and crucial differences between the rodent and human systems still to be overcome in our research.

Model validation is used for establishing how much a model is reflective of the real system, and although the *Fmr1* KO AGS phenotype is a good model of FXS seizures, it does not truly reflect the 20% incidence of spontaneous seizures in FXS patients. Indeed, the audiogenic seizures present in the *Fmr1* KO mouse models manifest with sound-induction, being not truly reflective of human epilepsy. Moreover, the exact brain areas and mechanisms underlying epilepsy in FXS patients and AGS in mice are still to be identified. Hence, construct validity is yet to be met in this method of analysis.

On the other hand, for predictive validity to be assessed, clinical trials with the aforementioned treatments should be repeated with better measures of treatment outcome and less parent-focused assessment of the effects (Erickson et al. 2017).

1.4 Rat animal model in behavioural research

Now that both mice and rat species can be genetically manipulated to produce models of disease, the use of rat in research is having a comeback. Despite their similar appearance, mice and rats were evolutionarily separated between 16-40 million years ago (Springer et al. 2003), over three-four times over the time of human and non-human primates last common ancestor (Kumar & Hedges 1998). Thus, these two rodents present with numerous anatomical, structural and molecular differences

determining specific dissimilarities in drug reactivity and disease phenotyping (Logan et al. 1988; Till et al. 2015; Witte et al. 2010). Although biochemical and electrophysiological research data from both species is generally not strikingly different (Radermacher & Haouzi 2013). Moreover, their difference in body size can make them better or less suited for specific experiments. For example, the larger body of rats makes surgical procedures easier to carry out, particularly brain or spinal cord surgeries. Whereas the smaller brain size of mice is advantageous in optogenetic studies (Ellenbroek & Youn 2016).

When it comes to use in behavioural research, rat models can present some advantages. Mice have been shown to perform similarly to rats in an adaptive decision-making study (Jaramillo & Zador 2014), and to learn just as fast in a visual acuity test (Prusky et al. 2000). However, they are known to be less social and more prone to stress with human contact. In a social interaction test, whereas mice spent only 17% of their time interacting with each other, rats spent 79% of it (Kummer et al. 2014). Moreover, cage enrichment and handling have been shown to increase mice stress levels rather than decreasing them (Meijer et al. 2007).
Differently, handling of rats seems to reduce their stress and anxiety during performance in behavioural tests (Costa et al. 2012). Rats are also less territorial and less hierarchical than mice, being less aggressive towards other males (Lund 1975). Furthermore, a study of social transferred fear showed the ability of rats to process empathy in a way that allows naïve rats to acquire aversive learning from a shocked cage mate (Knapska et al. 2010). Hence, its less susceptibility to stress with human contact and higher sociability make the rat a popular animal in neuroscience studies and model for neuropsychiatric disorders.

Overall, having more than one animal model to investigate the pathology underlying a disorder can be very advantageous. One model could be more suitable for a diverse array of experiments than the other; and comparison of conserved or respective phenotypes could give insights on disease differences between models of different species, helping in the translation of notions and treatment to humans. If investigating a novel compound shown to successfully rescue core biochemical,

electrophysiological and behavioural phenotypes in mice, then further testing it in a second mammalian species such the rat could best assess its overall treatment potential for future clinical trials in humans.

1.4.1 The *Fmr1* KO rat model

The advent of the *Fmr1* KO rat has allowed for the beginning of cross-mammalian comparison of FXS pathology, giving confirmation of conserved core biochemical and electrophysiological phenotypes characteristic of the *Fmr1* KO mouse. These include elevated protein synthesis, altered synaptic plasticity and abnormal dendritic spine density (Rais et al. 2018; Till et al. 2015). More importantly, the advantageous characteristics of the rat animal for behavioural studies have prompted thorough testing of the *Fmr1* KO rat for phenotyping variance in tasks for social preference, anxiety, and complex learning and memory paradigms.

In 2014, *Fmr1* KO rats were shown to perform not differently from their WT littermates in a social preference task, nor have elevated levels of anxiety in the open filed and elevated zero maze (Hamilton et al. 2014). However, they were found to present some strain-independent and strain-specific deficits in learning and memory tasks. A study from 2015 showed *Fmr1 KO* rats to present a deficit in a complex hippocampal-dependent form of associative short-term memory, requiring the association of a specific object with a spatial location and context (Till et al. 2015). Another study showed *Fmr1* KO rats on the Sprague-Dawley background to present a long-term deficit in object placement recognition, and *Fmr1* KO rats in the Long-Evans background to also present a deficit in long-term novel object recognition (Asiminas 2017). For these listed advantages and already identified memory deficits, the *Fmr1* KO rat was used in this PhD thesis to run memory paradigms in which to assess the effects of a compound found to successfully treat the *Fmr1* KO mouse core phenotypes (data shown and described later).

1.5 Targeting the ERK1/2 pathway for FXS treatment

As already discussed, the mGluR theory of FXS postulates many protein-synthesis dependent effects downstream mGluRs activation to be exaggerated in the absence of FMRP (Bear et al. 2004). Consequently, it proposes the investigation of therapies targeting the activation of Gp1 mGluR5 and its downstream signalling pathways to correct core FXS deficits.

Several independent studies have followed this therapeutic approach, showing numerous beneficial effects of mGluR5 antagonism in mice and later, although to much less extent, in clinical trials with FXS patients (Erickson et al. 2017). Downstream mGluR5 signalling, the ERK1/2 pathway showed great potential as a more specific therapeutic target, and MEK1/2 or ERK1/2 pharmacological inhibition rescued core deficits in the *Fmr1* KO mouse (Osterweil et al. 2010; Wang et al. 2012; Chuang et a. 2005; Gkogkas et al. 2014). Although modulation of this pathway might cause worrying due to its major role in protein synthesis regulation throughout the body, early findings of this research led to the investigation of statins as potential drug treatment for FXS. Indeed, work by Osterweil et al. 2013, showed the statin lovastatin could rescue several phenotypes of the *Fmr1* KO mouse.

1.5.1 Statins for targeting Ras/ERK1/2 signalling

Statins are a group of cholesterol-lowering drugs that work by binding to and inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, reducing the production of mevalonate upstream cholesterol synthesis (Istvan 2003). Worldwide, they are the primary medication for therapy of hypercholesterolemia and coronary heart disease (Vaughan et al. 1996) (Vaughan et al., 1996**)**, with a good safety profile for long-term use at both young and adult ages (Kavey et al. 2015; Kapur & Musunuru 2008). By inhibiting HMG-CoA conversion to mevalonate, statins decrease not only the synthesis of the downstream cholesterol but also of isoprenoid intermediates (Ling & Tejada-Simon 2016). These include farnesyl and geranylgeranyl pyrophosphates, important substrates for the posttranslational

modification of proteins, including the small GTPases Ras, Rho and Rac (Liao & Laufs 2005; Ling & Tejada-Simon 2016; Nürenberg & Volmer 2012). Farnesylation of the Ras GTPase upstream ERK1/2 with a farnesyl moiety is a posttranslational step necessary for its association to the inner membrane and consequent binding to GTP (Schafer et al. 1989; Mendola & Backer 1990), and statins appear to variably affect this binding in different tissues and models.

Of the statins, lovastatin was the first to be developed and is now part of the first-generation class, able to efficiently reduce cholesterol levels with minimal side effects (Kapur & Musunuru 2008; Bradford et al. 1993). By reducing cholesterol, lovastatin has been shown to reduce the availability of the farnesyl pyrophosphate precursor required for Ras GTPase activation upstream the ERK1/2 pathway (Schafer et al. 1989; Mendola & Backer 1990). Hence, when investigated for treatment in a mouse model for neurofibromatosis type1 (NF1), a neurodevelopmental disorder caused by overactive Ras (Bollag et al. 1996), lovastatin was found to safely reduce Ras-ERK1/2 activation *in vivo* in the brain and rescue deficits in synaptic plasticity, spatial learning and attention (Li et al. 2005). These relevant findings on lovastatin inhibition of ERK1/2 signalling prompted its investigation in FXS.

1.5.2 Lovastatin for treatment of FXS and the simvastatin alternative

In FXS, lovastatin was similarly found to reduce Ras binding to GTP and consequently reduce ERK1/2 activation in the hippocampus of *Fmr1* KO mice, rescuing hippocampal epileptogenesis, exaggerated mGluR-LTD and elevated protein synthesis, as well as neocortical hyperexcitability and AGS susceptibility (Osterweil et al. 2013). In later behavioural studies with the *Fmr1* KO rat model, lovastatin treatment at juvenile ages was shown to prevent the arising of complex memory-associated cognitive phenotypes (Asiminas et al. 2019). Clinical trials for lovastatin treatment in both children and adults with FXS followed with promising results. Two open-label studies reported remarkable clinical improvements after treatment, with little side effects and well toleration of the drug (Çaku et al. 2014; Pellerin et al. 2016),

and a double-blind placebo-controlled study is currently taking place (Berry-Kravis et al. 2018). However, lovastatin is not licensed for medical use in several European countries, including the United Kingdom (UK). For this reason, the statin simvastatin has been suggested as a potential substitute in FXS therapy.

Simvastatin shares a very similar chemical structure to lovastatin, and is a member of second generation statins, which present significantly increased efficacy at reducing LDL-cholesterol compared to first class members (Kapur & Musunuru 2008). Indeed, a 10 mg daily dose of simvastatin has been reported to reduce plasma cholesterol by 25-30%, similarly to the effect of a 20 mg daily dose of lovastatin (Jones et al. 1998a; Schaefer et al. 2004). Several studies reported simvastatin to have higher levels of lipophilicity and be more blood brain barrier (BBB) penetrant than lovastatin and other statins. This property could make simvastatin a better choice than lovastatin in the treatment of conditions affecting the central nervous system (Tsuji et al. 1993; Sierra et al. 2011). Simvastatin can also be legally prescribed in significantly more countries worldwide than lovastatin, including the UK (Neuvonen et al. 2008).

Similarly to lovastatin, simvastatin was also shown to modulate Ras and/or ERK1/2 activity dependently on its HMG-CoA reductase inhibition *in vitro* in mouse osteoblasts and myoblasts (Chen et al. 2010; Yamashita et al. 2008). However, the effect of simvastatin on the Ras-ERK1/2 pathway in the brain is yet to be defined, and its treatment has not been studied in a *Fmr1* KO model. Acquisition of this information before the start of a FXS clinical trial with simvastatin is of primary importance, especially because trials with NF1 patients have shown that lovastatin treatment, but not simvastatin, is beneficial for specific cognitive phenotypes (Krab et al. 2008; Payne et al. 2016; van der Vaart et al. 2013; Bearden et al. 2016).

First aim of this PhD project was to investigate the impact of simvastatin on core deficits of our *Fmr1* KO mouse model.

1.6 Identifying the excessively translating mRNAs responsible for disrupted synaptic signalling in the *Fmr1* KO mouse

In the most recent years of research, the development of novel research techniques, bioinformatics and the expansion of online biological databases allowed for more in-depth analyses of FMRP target mRNAs, and proteins with altered expression in the *Fmr1* KO brain and FXS patients derived cells (Darnell et al. 2011; Brown et al. 2001; Klemmer et al. 2011; Liao et al. 2008; Tang et al. 2015). Recently, a novel study was conducted by Thomson and colleagues (2017) aimed at identifying the mistranslated mRNAs in the CA1 pyramidal neurons of the *Fmr1* KO hippocampus, the same neurons responsible for exaggerated mGluR-LTD due to elevated protein synthesis. This study used cell-type-specific translating ribosome affinity purification (TRAP) technique to isolate the translating ribosome-bound mRNAs in the *Fmr1* KO CA1 region and RNA-sequencing analysis to identify them. Overall, 121 genes were found significantly upregulated or downregulated in *Fmr1* KO mice compared to WT littermates. Surprisingly, upregulated mRNAs did not include FMRP targets, as they were found subtly downregulated in both input and CA1-TRAP samples. This suggested a homeostatic shift in translation developed to compensate for FMRP loss (Thomson et al. 2017).

Primary aim of the study was to identify the excessively translating mRNAs responsible for disrupted synaptic signalling. Interestingly, the most enriched mRNA transcripts in the CA1 TRAP samples were found to be from the G-protein-coupled acetylcholine receptor (muscarinic) (mAChR) family. Of these, the transcripts from *Chrm1* and *Chrm4* genes encoding for mAChR1 and mAChR4 (M_1 and M_4) were found significantly enriched in the CA1 translating mRNAs. However, only M_4 was also found significantly overexpressed in *Fmr1* KO hippocampal homogenates and synaptoneurosomes. Differently, its transcription levels were unchanged compared to WT, indicating that its increase in expression is driven by changes in translation and not transcription (Thomson et al. 2017). Because FXS is regarded as a disorder of protein translation, this finding sparked interest and prompted Thomson and

colleagues to test the effects of M_4 pharmacological manipulation on core *Fmr1* KO phenotypes.

1.6.1 mAChRs in the brain

mAChRs are part of the GCPRs family and are divided into five (M_1-M_5) subtypes. They mainly modulate autonomic responses in the peripheral nervous system, and, in the brain, they participate in motor control, synaptic excitability and plasticity, learning and memory (Kruse et al. 2014). M_2 and M_4 receptors mainly couple to $G_{i/o}$ subunit inhibiting adenylyl cyclase, and M_1, M_3 and M_5 to $G_{q/11}$ subunit promoting phosphatidylinositol hydrolysis (Hulme et al. 1990), however they couple to ion channels as well (Higashida et al. 1990; Hulme et al. 1990; Fukuda et al. 1988; Jones et al. 1988b).

In the brain, M_1, M_2 and M_4 are the most expressed, with M_1 and M_2 having higher levels than M_4 in the cortex, and with no differences between overall expression of the three in the neocortex, although their regional expression varies within layers (Levey et al. 1991). All five subtypes are variably present in the different hippocampal regions, , M_1 and M_4 being the most enriched in the CA1 area (Levey et al. 1995; Zang & Creese 1997).

mAChRs can regulate hippocampal and cortical activity both pre- and post-synaptically. Presynaptically, mAChRs can act as autoreceptors to regulate acetylcholine release in both rat cortex (M_1, M_2 and M_4) and hippocampus (M_1 and M_4 only) (Vannucchi & Pepeu 1995). They can block excitatory transmission by inhibiting aspartate and glutamate release (Raiteri et al. 1990). Postsynaptically, they can regulate excitatory signalling, for example by enhancing NMDA receptor currents in CA1 pyramidal cells through M_1 activity (Marino et al. 1998).

mAChRs have also been associated to striatal dopamine release, particularly M_4 (Zhang et al. 2002; Threlfell et al. 2010; Smolders et al. 1997), and presynaptic M_4 was indicated as the major regulator of corticostriatal glutamatergic input to striatum spiny neurons (Pancani et al. 2014). Moreover, M_4 can specifically promote excitatory

activity in the cortex by increasing glycine release and hence NMDAR activation (Russo et al. 1993).

1.6.2 M_4 in the hippocampus and learning and memory

In the hippocampus, mAChRs are particularly important for processes such learning and memory, and M_1 activity was until recently the most studied. As the acetylcholine binding sites of all mAChR subtypes are very conserved, it was thanks to the availability of genetically engineered rodents and the development of positive allosteric modulator (PAM) compounds that studies could be carried out differentiating between the specific activities of each receptor subtype.

Thus, hippocampal stimulation with a M_4-specific PAM showed to inhibit excitatory synaptic transmission in CA1 pyramidal cells by decreasing glutamate release, but it did not increase further the mAChR-driven CA1 pyramidal cell depression of inhibitory synaptic signalling (Shirey et al. 2008). M_4 therefore seems to be a primary regulator of glutamatergic transmission but not inhibitory signalling in the CA1 area of the hippocampus.

Moreover, *in vivo* administration of different M_4-specific PAMs was shown to reverse amphetamine-induced and NMDAR antagonism-induced hyperlocomotion in WT rats and mice (Bubser et al. 2014; Byun et al. 2014). Later, administration of the M_4 PAM VU0467154 enhanced memory and associative learning in contextual and cued fear conditioning assays in WT mice, whereas M_4 KO mice presented with deficits in the same tasks and could not be rescued by the treatment. The PAMs, administered via intraperitoneal (i.p.) injection, did not cause any adverse effects in autonomic function or somatosensory circuits for motor responses (Bubser et al. 2014). These findings suggest a prominent role of M_4 receptors in motor control and regulation of memory processes and associative learning, both hippocampal-dependent and -independent.

Although the currently available data is controversial, cholinergic transmission has been increasingly correlated to Alzheimer's disease (AD) loss of cognition and memory, as changes in levels of enzymes for acetylcholine synthesis and breakdown,

acetylcholine itself, and both nicotinic and muscarinic receptors were found in the post-mortem brains of AD patients compared to healthy individuals. In one of these studies, whereas M_1 and M_2 receptor levels were found unchanged in the hippocampus of AD patients, M_4 levels were significantly decreased in CA4 region and dentate gyrus, proposing a primary role for M_4 in cognitive functioning (Mulugeta et al. 2003). These findings showing M_4 regulation of excitatory transmission in the hippocampus and a prominent role for M_4 in working memory, cognition and learning suggest the result of Thomson et al. of increased M_4 expression in the *Fmr1* KO hippocampus could give important insights on the learning and memory deficits characteristics of FXS in both patients and animal models.

1.6.3 mAChRs and M_4 in FXS

Altered mAChR signalling has been recently correlated to FXS. For instance, mAChR activation with carbachol results in CA1 *de novo* protein synthesis (Feig & Lipton 1993), and M_1 activation induces a protein synthesis-dependent hippocampal LTD that is independent of mGluR and NMDA signalling (Volk et al. 2007). Similarly to mGluR-LTD, M_1-LTD is increased in *Fmr1* KO mice and no longer dependent on protein synthesis (Volk et al. 2007). This could be correlated to the increase in M_1 translation found by Thomson et al. (2017) in the *Fmr1* KO mouse CA1 region. Overactive M_1 in *Fmr1* KO rodents has been indicated by several studies, and M_1 inhibition was shown to reduce the number of marbles buried and AGS incidence in *Fmr1* KO mice, although increasing open field activity in WT controls and having no effect on learning and memory in a passive avoidance test (Veeraragavan et al. 2011). Interestingly, M_1 antagonism did not selectively rescue the elevated *Fmr1* KO hippocampal protein synthesis but decreased its levels in both WT and KO genotypes (Thomson et al. 2017). This prompted Thomson and colleagues to investigate the role of M_4 receptor instead.

When Thomson and colleagues (2017) found increased translation and expression levels of mAChR M_4 in the hippocampal CA1 region, they examined the effects of M_4 antagonism in core biochemical *Fmr1* KO phenotypes. By their surprise,

instead of ameliorating the deficits, M_4 antagonism caused a further increase in the elevated hippocampal protein synthesis of *Fmr1* KO mice, and increased protein levels in WT controls. On the contrary, treatment of hippocampal slices with the M_4 PAM VU0152100 rescued both the elevated hippocampal protein synthesis and the exaggerated mGluR-LTD in *Fmr1* KO mice without affecting the WT counterparts (Thomson et al. 2017). These surprising results showed a beneficial effect in enhancing the activity of an already overexpressed receptor in FXS, prompting the suggestion of M_4 being overtranslated in FXS as a homeostatic response to compensate for the overexpression of FMRP targets. These findings are consistent with the ones of a previous study showing that reducing M_4 expression in *Fmr1* KO mice has no positive effect on learning and memory, repetitive behaviours or AGS phenotype (Veeraragavan et al. 2012).

The second part of this PhD thesis focused on investigating the effects of M_4 PAM treatment *in vivo* on the AGS phenotype in *Fmr1* KO mice and on paradigms for deficits in learning and memory in *Fmr1* KO rats.

1.7 Aims of this thesis

Finding effective and appropriate therapeutic strategies for the core pathologies of FXS is one of the main focuses of the field. There is not definitive treatment for the syndrome, and therapies are based on the targeted treatment of specific symptomatology rather than underlying pathology. The finding of exaggerated protein synthesis-dependent responses downstream mGluR signalling in FXS led to the formulation of the mGluR theory of FXS (Bear et al. 2004), and the speculation that FXS core deficits are due to hypersensitive responses downstream $mGluR_5$ (Osterweil et al. 2010). Since then, research has been focusing on the investigation of therapies aimed at the targeting of $mGluR_5$ downstream pathways involved in protein synthesis, particularly ERK1/2 and mTOR. Of these therapies, the use of the statin lovastatin is showing strong potential, with clinical trials currently ongoing with positive preliminary results (Çaku et al. 2014; Pellerin et al. 2016; Berry-Kravis et al. 2018).

However, another potential therapeutic approach has recently emerged: positively modulating the muscarinic acetylcholine receptor 4 (M_4).

This thesis will focus on exploring two methods to reduce the elevated protein synthesis in the *Fmr1* KO mouse:
1) Statin treatment to target ERK1/2 activation with simvastatin, a more potent alternative to lovastatin.
2) Positively modulating M_4 with the positive allosteric modulator VU0152100, recently shown to rescue core biochemical and electrophysiological *Fmr1* KO mouse phenotypes.

Chapter layout:

Chapter 2: Methods

Chapter 3: Investigate the effects of simvastatin stimulation on core biochemical and behavioural *Fmr1* KO phenotypes to test if:

1) Simvastatin treatment rescues the elevated hippocampal protein synthesis in *Fmr1* KO mice.
2) Simvastatin treatment targets Ras-ERK1/2 or Rheb-mTOR pathways downstream mGluRs.
3) Simvastatin treatment rescues the increased sensitivity to AGS phenotype in *Fmr1* KO mice *in vivo*.

Chapter 4: Investigate the effects and mechanisms of M_4 PAM treatment in juvenile *Fmr1* KO mice testing if:

1) M_4 PAM treatment rescues the increased incidence and severity of AGS in *Fmr1* KO mice.
2) M_4 PAM treatment affects the activation of ERK1/2 or mTOR pathways via modulation of ERK1/2 or p70S6K components.

3) M_4 PAM treatment affects the activity of eukaryotic elongation factor 2 (eEF2).

Chapter 5: Run behavioural assays for long-term memory measurements in adult *Fmr1* KO mice and rats to test if:

1) Adult *Fmr1* KO mice present decreased freezing rate compared to WT in contextual fear-conditioning assay for associative learning and long-term memory.
2) Adult *Fmr1* KO rats present long-term memory impairment in object displacement behavioural task.
3) Adult *Fmr1* KO rats present long-term memory impairment in novel object recognition behavioural task.

Chapter 6: Investigate the effects of M_4 PAM treatment at adult ages, testing if:

1) M_4 PAM treatment rescues the elevated protein synthesis in adult *Fmr1* KO rat hippocampus.
2) M_4 PAM treatment rescues the elevated protein synthesis in adult *Fmr1* KO mouse hippocampus.
3) M_4 expression is elevated in adult *Fmr1* KO rats.

Chapter 7: Conclusions

Chapter 2

Methods

2.1 Animals

2.1.1 Mice background strain

Fmr1 KO mice from Jackson Labs 003025 were bred on either a C57BL/6J (Charles River) or a mixed C57BL/6J x FVB background (C57BL/6J backcrossed to FVB by two generations). These mutant mice were generated with the insertion of a neomycin cassette to replace the coding region of exon 5 of the *FMR1* gene, resulting in the loss of the product FMRP.

2.1.2 Rats background strain

Long Evans Hooded *Fmr1* KO rats were acquired from Sigma Advanced Genetic Engineering (SAGE) laboratories. Zinc-finger nuclease technology was used to insert an eGFP (eGFPnlsSV40pA) cassette in the coding region of exon 1 of the *FMR1* gene, causing full loss of the FMRP product. Full knock-out of the *FMR1* gene is checked using primers targeting the inserted eGFP cassette: while KO animals present a 421bp PCR product, WTs present none. On the other hand, genotyping with primers targeting the FMRP sequence to either side of the cassette yield to no product in KO animals and a 400bp PCR product in WTs (Figure 2.1, obtained from Asiminas 2017).

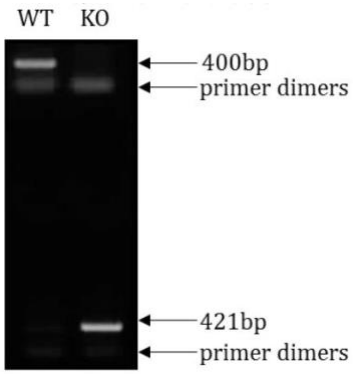

Figure 2.1. Genotyping of LEH Fmr1 KO and WT rats
Two sets of primers are used to genotype each sample in two separate reactions. The first set of primers (left in image) targets FMRP coding regions flanked by the eGFP

cassette, the second set ofp rimers targets the cassette (right in image). Figure from Asiminas 2017.

2.1.3 Housing and breeding

All experimental animals were males. While all experimental mice were naive to drug and behavioural testing prior to experimentation, some experimental rats used for biochemical analyses of brain tissue had been previously used for drug and behavioural testing, details specified later in Chapter 6. Mice and rats were housed in separate facilities, both were group caged, maintained in an environment with constant temperature of 21 ± 2 °C, with a 12h light-dark cycle and unlimited food and water provision.

2.2 Genotyping

2.2.1 DNA extraction mice

Ear tissue was collected at the time of weaning in the form of ear clip, for identification and genotyping. In Eppendorf tubes, the tissue was dissolved in 50mM NaOH at 100°C for 30 minutes. Once cooled, it was vortexed and 75µl of ddH_2O were added, plus 7.5µl of 1 M Tris-HCL. The tubes were centrifuged at top speed for 20 minutes and stored at 4°C.

2.2.2 Primers for mice colony

Single reaction:
Neo Forward Primer – AGGATCTCCTGTCATCTCACCTTGCTCCTG
Neo Reverse Primer – AAGAACTCGTCAAGAAGGCGATAGAAGGCG

2.2.3 Polymerase Chain Reaction for mice samples

Each reaction was prepared as follows: sample DNA, 1 µl; PCR Master Mix (Promega), 12.5µl; Neo Forward Primer, 0.125µl, Neo Reverse Primer, 0.125µl; ddH_2O 11.25µl. Thermocyclin conditions steps are listed below in Table 2.1. The obtained PCR products and a 100bp DNA ladder (Promega) were mixed with loading dye and run on a 1.5% agarose gels with Sybersafe (5µl per gel) at around 70mV for about 30mins, then imaged.

Table 2.1 Thermocycling conditions for polymerase chain reaction

Step1	95°C for 2mins
Step2	95°C for 30secs
Step3	58°C for 30secs
Step4	72°C for 30secs
Step5	Go to step2, 35 times
Step6	72°C for 5mins
Step7	4°C forever

2.2.4 Non-manual rat genotyping

Ear notches from WT and *Fmr1* KO rats were taken at time of weaning and shipped to the genotyping company TransnetYX for a charged genotyping service. Below are the sequences for forward and reverse primers used:
Forward primer – CGTCGTCCTTGAAGAAGATGGT
Reverse primer – CACATGAAGCAGCACGACTT

2.3 Metabolic labelling

2.3.1 Solutions

The artificial CSF (aCSF) was made fresh prior to experimentation using chemicals purchased from Sigma-Aldrich. The solution was saturated with constant flow of carbogen (95% O_2 and 5% CO_2).

Table 2.2 ACSF composition (in mM)

	mM
NaCl	124
KCl	3
NaH_2PO_4	1.25
$NaHCO_3$	26
Dextrose	10
$CaCl_2$	2
$MgCl_2$	1

2.3.2 Hippocampal tissue slicing

This assay was used to investigate the effects of several compounds on differing species and ages: juvenile mice (P25-32), adult mice (>p60) or adult rats (>p100). At all cases, male WT and *Fmr1-/y* animals - littermates for juvenile mice testing or age-matched for adult mice and rat testing - were anaesthetized with isoflurane and quickly decapitated in an interleaved fashion, with the experimenter blind to genotype all times. The brain was quickly extracted and the hippocampus (consistently taken from the same side hemisphere) dissected into ice cold freshly prepared aCSF. The dorsal side of the hippocampus was sliced into 500μm thick slices using a Tissue Slicer from

Stoelting. Each slice was separately stored in netwells from Life Technologies and left to recover in oxygenated aCSF at 32.5°C for about 4 hours.

2.3.3 Hippocampal tissue stimulation

At the end of the 4-hour recovery period the netwells with slices were transferred to new chambers with 32.5°C warm aCSF and 25 μM Actinomycin D (Tocris) plus either vehicle (0.05% DMSO in ddH_2O) or drug (see Table 2.2) for 30 minutes. After blocking transcription with Actinomycin D, to quantify the levels of de-novo synthesized proteins and investigate the effects of tested compounds, the slices were moved to a third set of chambers with fresh oxygenated warm aCSF containing 10 μCi/ml of ^{35}S-Met/Cys protein labelling mix (Perkin Elmer), and again either vehicle or drug as previously for a further 30 minutes. Slice were then frozen and stored at -80°C until processing together with control aCSF samples needed for data analysis.

Table 2.3 Dose (in μM) and compounds for biochemical analysis

	μM
Lovastatin sodium salt (Calbiochem Merck Millipore)	50
Simvastatin sodium salt (Cayman Chemical)	0.1-5
VU0152100 (Abcam)	5

2.3.4 Processing

Previously stimulated slices were thawed and homogenized in ice-cold buffer (10 mM HEPES pH 7.4, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100) with protease and phosphatase inhibitors from Sigma-Aldrich (cOmplete™, mini, EDTA-free Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 2, Phosphatase Inhibitor Cocktail 3). The resulting homogenate of each sample was separated in half. The first half was boiled in 4X Laemmli sample buffer for later use in immunoblotting. The remaining

half was used to precipitate proteins by incubating in trichloroacetic acid (TCA: 12.5% final) for 10 minutes on ice and being centrifuged at 16,000 rpm for another 10 minutes. While the supernatant was discarded, the pellet was washed once in ice-cold ddH_2O and re-suspended in 1 N NaOH, with the addition of 0.33 N HCl to adjust pH back to neutral. In a 96-well plate each sample was added in triplicates to wells previously filled with a liquid scintillation cocktail (Ultima Gold, from PerkinElmer) and thoroughly mixed, the same was done with the control aCSF samples taken at the end of stimulation in^{35}S-Met/Cys. The plate was read with a scintillation counter obtaining triplicate counts per minute (CPM) values for incorporation of ^{35}S isotope into newly synthesized proteins.

2.3.5 BSA protein assay for measuring protein levels in hippocampal slices

Protein concentration was calculated using the BioRad DC protein assay kit (BioRad). BSA standards were made from a 10mg/ml stock (New England Bio Labs) diluted on the day to 2mg/ml with ddH_2O and from there into serial dilutions from 2 to 0.125mg/ml. 5µl from each BSA standard and samples were loaded in triplicates into a 96 well Nunc plate (Scientific Laboratory Supplies Ltd). To each well were added 25µl of the premixed reagent A' (2ml of reagent A plus 40µl of reagent S from the kit per every full plate) and 200µl of reagent B. The plate was then left incubate at room temperature for 15 minutes and later read on a FluoStar Optima plate reader (BMG Labtech) with a set absorbance of 740nm. The triplicate values obtained were averaged and the mg/ml protein values for each sample calculated using the BSA standard curve.

2.3.6 Data analysis

The scintillation CPM triplicates for ^{35}S incorporation were divided by the mg/ml protein values obtaining CPM per µg of protein values. Each of these values was normalized to the ^{35}S-Met/Cys ACSF control samples taken during the incubation step and to the volume of samples added to each well with scintillation cocktail: 35µl per

sample. Finally, each triplicate value was averaged obtaining CPM/µg/µl for each differently treated hippocampal slice. Values of all slices belonging to each pair (WT with mutant paired sibling or age-matched or group caged animal) were each normalized to the average of that same pair. Statistics was performed using these normalized values, which were subsequently expressed as percentage ± SEM to the average of WT control values for graphical purposes. Each animal represented a single n.

2.4 Western Blotting

2.4.1 Gel preparation

10% gels were made of two different phases: a separating phase to host the run protein and a stacking phase to help initial running and proper alignment of proteins. The separating phase at the bottom was made as 10% acrylamide gel: ddH2O, 72ml; 40% Acrylamide (BioRad), 37.5ml; Resolving Buffer, 37.5ml (BioRad); 10% SDS, 1.5ml; 10% Ammonium Persulfate (APS), 1.5ml; Temed, 75µl (BioRad). The stacking gel, on top of the separating gel, was made as 4%: ddH2O, 47.25ml; 40% Acrylamide, 7.5ml; Stacking Buffer, 18.75ml; 10% SDS, 0.75ml; 10% APS, 0.75ml; Temed, 75µl; few crystals of 10% Bromophenol Blue. The separation gel preparation was poured into a gel caster and the top surface covered with water saturated isopropanol (50:50) and left polymerise for about 40 minutes. Then the isopropanol was accurately washed several times with ddH_2O and the stacking gel preparation was poured on top, 15-well coms were added to the top, and it was left to set again for another 20 minutes. Once ready, gels were either used immediately for running or stored a 4°C for a maximum of 48-hours into a closed plastic container and wrapped in paper wet with running buffer.

2.4.2 Running and Blotting

The metabolically labelled hippocampal slices previously homogenized and boiled in 4X Laemmli sample buffer were loaded in the prepared SDS-polyacrylomide gels for gel electrophoresis. Gels were run at a constant speed of 100V in 1X Tris-Glycine Running Buffer (BioRad) diluted in ddH_2O from a 10X stock containing: 25mM Tris, 192 mM Glycine. Proteins run on the gel were then transferred into 0.45µm think nitrocellulose membranes (BioRad), either ON at 4°C at a constant speed of 35mA or at RT for 2 hours at a constant speed to 250mA, in 1X Tris-Glycine-SDS Blotting Buffer (BioRad) diluted in ddH_2O and 20% methanol from a 10X stock containing: 25mM Tris, 192 mM glycine, 0.1% SDS.

2.4.3 Total protein memcode stain

To visualize the consistency of protein loading and later quantify total protein levels per lane of membrane, we used the Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes (Thermo Fisher Scientific). Following protocol: membranes were incubated 1 to 2 minutes in the protein-binding blue stain, gently shaking at room temperature, then washed repeatedly with Destain solution form the kit first and ddH_2O after, to clean membrane from all excess stain. The stained membranes were transferred into transparent film and scanned on greyscale and 300dpi. Then stain was completely removed with the Erase solution and washed 2x5 minutes with ddH_2O.

2.4.4 Immunostaining

Membranes were cut at determined molecular weights in order for more than one protein per membrane to be probed for: at 35, 50 or 75kDa. Later were blocked in 5% BSA [made up in 1X Tris Buffered Saline (BioRad) with 0.1% Tween-20 (BioRad) (TBS-T)] at RT for one hour and incubated ON at 4°C with primary antibody (also

made up in 5% BSA in TBS-T) (see table 2.3). Following incubation strips of membranes were washed 3x10 minutes in TBS-T and incubated at RT for 30 minutes with horseradish-peroxidase- (HRP-) conjugated secondary antibody, anti-rabbit or mouse IgG (Cell Signaling Technology), made up in 3% BSA in TBS-T. After incubation membranes were washed again for 3x10 minutes and incubated for 5 minutes with Clarity ECL substrates (mixed 1:1) (BioRad) to then be exposed to development on X-ray film. If probing for phosphorylated proteins, after development on film the membranes were stripped for 5 minutes using 1X Re-Blot Plus Strong Antibody Stripping Solution (Millipore) diluted in ddH_2O from 10X stock, then blocked again for 1 hour with 5% BSA in TBS-T and probed for the corresponding total protein following the same protocol as described above. All gels were loaded and membranes probed by experimenter blind to genotype and treatment.

Table 2.4 Primary antibodies used in Western Blotting

Antigen	Host	Dilution	Expected MW (kDa)	Company
p44/42 MAPK (ERK1/2)	Rabbit	1:2000	44+42	CST
eEF2	Rabbit	1:2000	95	CST
M_4	Rabbit	1:1000	53	Abcam
M_4	Rabbit	1:1000	53	Elabscience
p70 S6 Kinase	Rabbit	1:1000	70	CST
Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	Mouse	1:2000	44+42	CST
Phospho-eEF2 (Thr56)	Rabbit	1:1000	95	CST
P-p70S6Kinase (Thr389)	Rabbit	1:1000	70	CST

2.4.5 Image analysis

Developed X-ray films were scanned with a canon LiDE110 scanner with 300dpi resolution and later opened with ImageJ software and converted into 8-bit, Tiff files. Densitometry of the individual bands of each blot was performed using ImageStudioLite (Li-Cor) software, while total membrane protein was quantified in

each lane using 300dpi greyscale scanned images of MemCode staining with FIJI software. The images used for the quantification were never processed or manipulated in any way. Only the representative bands shown within the figures in the following chapters were appropriately processed with an equally applied change in brightness and contrast to adjust the sharpness of the image. Quantified densitometry data for expression of selected phosphorylated proteins was normalized to expression of corresponding total protein and, to correct for blot-to-blot variance, each signal was normalized to the average signal of all lanes on the same blot. If only the total levels of a specific protein were of interest, densitometry data was normalized to total MemCode signal for the corresponding lane, then each signal was normalized to the average signal of all lanes on the same blot. Statistics was performed on these normalized values. For graphic purposed we subsequently expressed them as percentage ± SEM to the average of WT control values. All films were analysed by experimenter blind to genotype and treatment. Each animal represented a single *n*.

2.5 Acquisition of autoradiograph representative images

To acquire representative images of metabolic labelling of de-novo synthesized proteins with ^{35}S-Met/Cys mix, slice homogenates were resolved on SDS-PAGE gels and transferred to nitrocellulose membrane as described in 2.3.2 and 2.3.3. The full blot was exposed to a phosphorimaging screen (GE Healthcare) protected from light for about 15 to 30 days. Phosphorimages were acquired using a Typhoon scanner (GE Healthcare) and compared in a representative graph to total protein MemCode staining of the same membrane.

2.6 Audiogenic seizure assay in juvenile mice

2.6.1 Treatment injection

WT and *Fmr1*$_{-/y}$ male mice from both C57BL/6J or mixed C57BL/6J x FVB background between P18-30 were weighed and injected intraperitoneally (i.p.) with vehicle (DMSO in PBS with 10% Tween-80) or drug (see table 2.4) in a volume of 10µl per g. Then left to rest for 1 hour in a quiet room (< 60 dB ambient sound), in a cage with *ad libitum* food and water.

Table 2.5 Dose of compounds and vehicle tested in AGS assay

Drug	Dose	Vehicle
Simvastatin prodrug (CAS 79902-63-9)	3 mg/kg	3% DMSO + 10% Tween-80 in PBS
Simvastatin active form (CAS 101314-97-0)	50 mg/kg	20% DMSO + 10% Tween-80 in PBS
Lovastatin active form (CAS 75225-50-2)	100 mg/kg	50% DMSO + 10% Tween-80 in PBS
M_4 PAM VU0152100 (CAS 409351-28-6)	56 mg/kg	10% DMSO + 10% Tween-80 in PBS

For the compound VU0152100 we chose a dose of 56 mg/kg as it was the highest concentration we could administer due to its limited solubility.

2.6.2 Testing

At the end of the 1-hour wait, mice were placed into a transparent plastic test chamber. Following 1 minute of habituation, seizures were induced with a stimulus of >120dB (repeated sampling of alarm, Radioshack model 49-1010) played for 2 minutes with a pair of KRK Rokit RP5 G3 speakers (Studio Monitor). Every testing session conducted

included mice from both genotype and treatment groups, with the experimenter blind to both.

2.6.3 Scoring

Occurrence and severity of audiogenic seizures (AGS) were scored live during the testing, with identification of the following stages of severity: wild running (WR; prominent, uncontrolled running and flailing), clonic seizure (loss of balance and intense convulsions), or tonic seizure (cessation of movement and extension and stiffening of limbs and tail). Videos of the test acquired with QuickTime were kept to allow for an eventual second scoring. When reaching tonic stage of severity, animals were immediately humanely euthanized.

2.7 Contextual fear conditioning in adult mice

2.7.1 Handling

WT and *Fmr1* KO male mice of same or similar age were caged in groups of four with balanced genotypes at soon after weaning. After 100 postnatal days, mice were habituated to the journey through the lift from the animal facility down to the experimental rooms and back. When in the experimental room, mice were handled according to previously assigned experimental order for about 2 x 10 minutes for two days prior to training.

2.7.2 Training

After the two days of handling, the mice were put in a small chamber with a grid floor, transparent walls and scent of disinfectant wipes. They were let explore for 3 minutes and then were given a set number (1 or 3) of mild foot shocks through the grid floor (0.8-1.2mA), with an interval of 70 seconds between each shock. The training was recorded at all times with a camera placed to the siling of the experimental chamber.

2.7.3 Testing for memory retrieval

To test for long-term memory association, 24-hours later the mice were split into two different cohorts and brought back to either the experimental chamber from the previous day (familiar context) or to a novel context. The novel context had a plastic grey floor, walls with black and white stripes and ethanol scent. The mice were placed in the assigned context for the same amount of time spent in the familiar context the previous day; however, this time without receiving a foot shock. Later, the groups were switched over and all mice that went back to the familiar context first where then exposed to the novel and vice versa. Again, all testing phases were recorded for later examination. Freezing was scored manually as counted seconds of total immobility, and percentage of freezing was calculated. The data was analysed different times. First, we binned the freezing scores according to context and genotype, ignoring the order of context exposure. Then, to account for potential artefacts from context order, we examined the data taking into consideration the order of context exposure. The results obtained with manual scoring were compared with automatic scoring performed by the software FreezeFrame and no differences were found. All scoring showed in the result sections are obtained with manual scoring from the experimenter. The experimenter was blind to genotype during the entire handling, experimenting and scoring phases.

2.7.4 Testing for memory extinction

To test for extinction, 48hours post-training and 24hours post-testing for memory retrieval, the same mice were exposed a second time to both familiar and novel contexts, and their freezing scored. Again, no foot shock was administered. Data was analysed as detailed above.

2.8 Non-associative memory paradigms in adult rats

2.8.1 Handling

WT and *Fmr1* KO male rats of same or similar age were caged in groups of four with balanced genotypes at weaning. After six-seven postnatal weeks, the rats were handled between four to five days a week for at least two weeks, and habituated to the journey through the lift from the animal facility down to the experimental rooms and back. On the last few days of handling, they were exposed to various objects of different sizes and shapes (never identical to the experimental ones) to eliminate their potential experience of fear of the objects during the test.

2.8.2 Habituation for Object Displacement task

After handling, the animals were habituated to the experimental arena. Having assigned them a numerical order, one by one they were placed into a squared arena for 10 minutes for three consecutive days prior to testing. The four walls of the arena were transparent, each with cues of different shape, colour and texture attached externally. This in order for the animals to associate the objects later inserted to their location in the environment. At the end of each habituation session, before going back to their home cage, each rat was also habituated for 2 minutes to a holding plastic bucket used in the Probe Phase of testing. The bedding on the floor of the arena was cleaned from

excrements and thoroughly mixed in between each session to prevent smell from concentrating more in one area versus another.

2.8.3 Testing for Object Displacement task

On the fourth day, called sample phase, the rats were brought back to the arena, left identical from the previous days in position and cues attached but with two identical novel objects placed on the inside (attached to the arena floor). The object pair was either placed on the north side of the arena, one to the left and one to the right, or on the south side, one to the left and one to the right. Either way the animal was introduced to the arena always facing the same corner, the only corner that was calculated to never present an object during the days of testing. In this phase, each animal was left explore the arena for 5 minutes for three consecutive times. Between each of the three explorations, to not go back to the home cage until when finished with experimentation for the day, the animal was placed inside the holding bucket for a few minutes, while the objects were cleaned and the bedding of the arena mixed.

24 hours later, in the probe phase, rats were introduced again to the arena following the same numerical order, at the same time as the previous day. This time, one of the two identical objects had been moved to the opposite corner. Each rat was placed in the arena from the same corner as the previous day and left explore for 3 minutes only.

There were two different pairs of objects used in the test. The object pair used, its initial location and the new location of the moved object were all counterbalanced for in each experimental group tested. The experimenter was blind to genotype during the entire handling, experimenting and scoring phases. Every exploration session from habituation to sample and probe phase was recorded for analysis and scoring.

2.8.4 Habituation for Novel Object Recognition task

After handling, the animals were habituated to the experimental squared arena for the three days prior to testing, 10 minutes per day. The four walls of the arena were white and without any cues attached, a coloured pin wheel was kept visible externally on the top of the north wall. The bedding on the floor of the arena was cleaned from excrements and thoroughly mixed in between each session to prevent smell from concentrating more on one area versus another.

2.8.5 Testing for Novel Object Recognition task

Sample phase: on the day following the end of the habituation phase, two identical objects (novel to all animals) were placed either to the north or south wall of the arena. Following a set numerical order, the rats were left explore for 5 minutes only.

Probe phase: 24-hours later rats were placed again into the arena and left explore once for 3 minutes. For this phase, one of the two objects explored the day before was substituted by a novel one (in the same location).

In both days of testing (for sample and probe phases), each rat was placed into the arena facing the middle of the wall opposite to the objects, not a corner. There were four different pairs of objects used in the test, either as familiar pair at the start of the test or one of them as novel substitute. The object type used as familiar or novel, their location north or south, and the location of novelty to the left or right were all counterbalanced for in each experimental group. The experimenter was blind to genotype during the entire handling, experimenting and scoring phases. Every exploration session from habituation to sample and probe phase was recorded for analysis and scoring.

2.8.6 Treatment for Novel Object Recognition task

To test the effects of compound VU0152100 on memory consolidation, rats were weighted at the end of the last day of habituation. On the day of the sample phase, immediately after the 5 minutes of exploration (pair of identical objects) and before returning to their home cage, each rat was given a single i.p. injection of 56mg/kg M_4 PAM VU0152100 or vehicle (10% DMSO in 10% Tween-80 PBS).

2.8.7 Scoring

The video files acquired from the sample phase of both memory tasks were scored to check for a minimum of 15 seconds of exploration per object and a non-significantly different total exploration time between experimental groups. The video files acquired from the probe phase were used to score each animal performance in terms of long-term memory and novelty recognition. Rats that remember the previous experiences are expected to spend more time exploring the object in the novel location (Object Displacement task (OD)) or the novel object itself (Novel Object Recognition task (NOR)). Rats with memory and learning deficits should not be able to discriminate between the novel and familiar object location or object itself. Engaging with the object comprises sniffing, licking and touching with direct attention to the object itself, for e.g.: a rat climbing on top of the object to then looking at the ceiling or sniffing around is not regarded as engaging with the object. The averaged final values of each experimental group were expressed as mean ± SEM. The experimenter was blind to genotype during all scoring phases.

2.9 Statistics

Statistical testing was performed using GraphPad Prism software.

2.9.1 For biochemistry experiments

Outliers greater than 2 SD from the mean were removed and significance determined by repeated measures two-way ANOVA ($p < 0.05$) and post hoc Sidak multiple comparisons test ($p < 0.05$). Results of all statistical analyses are reported in detail in the figure legends.

2.9.2 For behavioural experiments

In AGS experiments, significance for seizure incidence differences between treatment and vehicle groups was determined by Fisher's Exact Test ($p < 0.05$), whereas Mann-Whitney U test was used to compare seizure severity.

In contextual fear conditioning, we used repeated measures two-way ANOVA ($p < 0.05$) for context and genotype differences, and post hoc Sidak multiple comparisons test ($p < 0.05$).

Power analysis was performed with package "PWR2", with specified significance level of 0.05. The package calculates the power effect by using the number of samples, the expected difference between genotypes (calculated from data: 4.252201 for novel and 9.457475 for familiar), the number of conditions (WT and KO: n in the formula), and the standard deviation. It uses the formula: $\sqrt[2]{(1/\mathrm{n} * \mathrm{difference\hat{}2} * 2)/\mathrm{standard\ deviation}}$. The effect size obtained was: 0.1995983 for novel and 0.3536383for familiar.

In long-term memory assays, significance for discrimination above chances was determined using one sample t-test with theoretical mean at 0 ($p < 0.05$). Differences in total exploration time for either both object or left and right locations were determined with unpaired t test or one-way ANOVA. In NOR assay, paired t-test was used for determining significance between exploration of familiar versus novel object of each experimental group ($p < 0.05$). Detailed results of all analyses are shown in the figure legends.

Chapter 3

Simvastatin treatment on core biochemical and behavioural *Fmr1* KO phenotypes

3.1 Introduction

The hippocampus of *Fmr1* KO mice is characterized by elevated levels of basal protein synthesis and exaggerated mGluR-dependent LTD (Huber et al. 2002; Qin et al. 2005). These changes were found to occur downstream mGluR5 signalling through the ERK1/2 pathway, and work in the *Fmr1* KO mouse has shown that reduction of ERK1/2 activation is beneficial for the disorder (Osterweil et al. 2010; Gkogkas et al. 2014). Indeed, a study by Osterweil et al. in 2013 has shown that the statin lovastatin can be used to this end and is able to rescue core *Fmr1* KO phenotypes.

The statins are a group of drugs widely used as therapy for hypercholesterolemia in both children and adults (Kapur & Musunuru 2008). They are HMG-CoA reductase inhibitors, which work by inhibiting the mevalonate pathway upstream cholesterol production, and are thought to consequently reduce the activation of the Ras GTPase upstream ERK1/2 (Nürenberg & Volmer 2012). Of the statins, lovastatin was shown to safely reduce Ras-ERK1/2 activation *in vivo* in the mouse brain (Li et al. 2005). Hence, it was tested as new therapeutic strategy for FXS in the *Fmr1* KO mouse model, and found to rescue several hippocampal-associated phenotypes, including the elevated protein synthesis, as well as the core behavioural phenotype of AGS susceptibility (Osterweil et al. 2013). Clinical trials with FXS children and adults followed the finding of 2013 reporting significant beneficial effects of lovastatin in two open-labelled studies (Caku et al. 2014; Pellerin et al. 2016), the results of a double-blind placebo-controlled study are currently awaited (Berry-Kravis et al. 2018).

As lovastatin is not licensed for medical use in the UK, the first part of this PhD project illustrated in this chapter was to evaluate the potential of an alternative statin for the treatment of FXS, simvastatin. Simvastatin and lovastatin share a similar chemical structure, however, simvastatin presents with higher brain permeability and is able to reduce plasma cholesterol to the same levels of lovastatin with half its dose (Jones et al. 1998a; Schaefer et al. 2004; Sierra et al. 2011; Tsuji et al. 1993). They present almost identical side effects and no significant differences were found between them

in the treatment of primary hypercholesterolemia in a large screening study (Frohlich et al. 1993). Overall, simvastatin is simply described as a more potent version of lovastatin, without any substantial differences being acknowledged. Hence, as it is licensed for medical use in the UK (Neuvonen et al. 2008), its use as an alternative therapeutic to lovastatin for FXS has been suggested.

In this chapter, I describe the effects of *in vitro* simvastatin treatment on the elevated protein synthesis levels found in the *Fmr1* KO hippocampus. Then, I investigate whether the found effects are mediated by simvastatin modulation of the Ras-ERK1/2 pathway or the alternative Rheb-mTOR cascade downstream mGluR5. In conclusion, I thoroughly evaluate the clinical potential of this statin by testing its effects on AGS susceptibility, behavioural gold standard assay for prediction of drug treatment efficacy in FXS patients. All this work has been published in Muscas et al. 2019.

3.2 Results

3.2.1 Simvastatin increases hippocampal protein synthesis levels dose-dependently

Fmr1 KO mice present increased protein synthesis levels in the dorsal hippocampus. This phenotype can be measured *in vitro* by incubating acutely cut slices into a bath of aCSF with a mix of ^{35}S-methionine/cysteine amino acids (Figure 3.1A). The incorporated amino acids labelled with the radioactive isotope give a readout of the rate of newly synthesized proteins, and the assay can be easily manipulated pharmacologically to examine protein synthesis under the effect of selected compounds.

Using this assay, and knowing 10μM of lovastatin to decrease the elevated protein synthesis phenotype in *Fmr1* KO mice, and 50 μM to completely rescue it (Osterweil et al. 2013), Dr. Susana R. Louros tested first a 5μM dose of simvastatin. Differently

from what expected, we found a 5μM dose of simvastatin to significantly exacerbate the protein synthesis phenotype in *Fmr1* KO slices and significantly increase protein synthesis in the WT as well (Figure 3.1B) (WT veh = 100.00% ± 2.70%, WT 5μM = 153.49% ± 6.32%, KO veh = 111.02% ± 4.27%, KO 5μM = 170.61% ± 9.43%).

As unpublished data from Osterweil et al. showed that higher doses (>50μM) of lovastatin would also result in an increase in protein synthesis, we decided to test lower doses of simvastatin (0.1-0.5μM). Again, even at these very low doses, simvastatin increased protein synthesis levels in both genotypes (Figure 3.1C) (WT veh = 100% ± 2.21%, WT 0.1μM = 106.99% ± 3.51%, WT 0.3μM = 117.79% ± 4.08%, WT 0.5μM = 124.13% ± 4.23%, KO veh = 115.61% ± 3.48%, KO 0.1μM = 116.52% ± 2.21%, KO 0.3μM = 129.15% ± 3.99%, KO 0.5μM = 137.01% ± 3.08%).

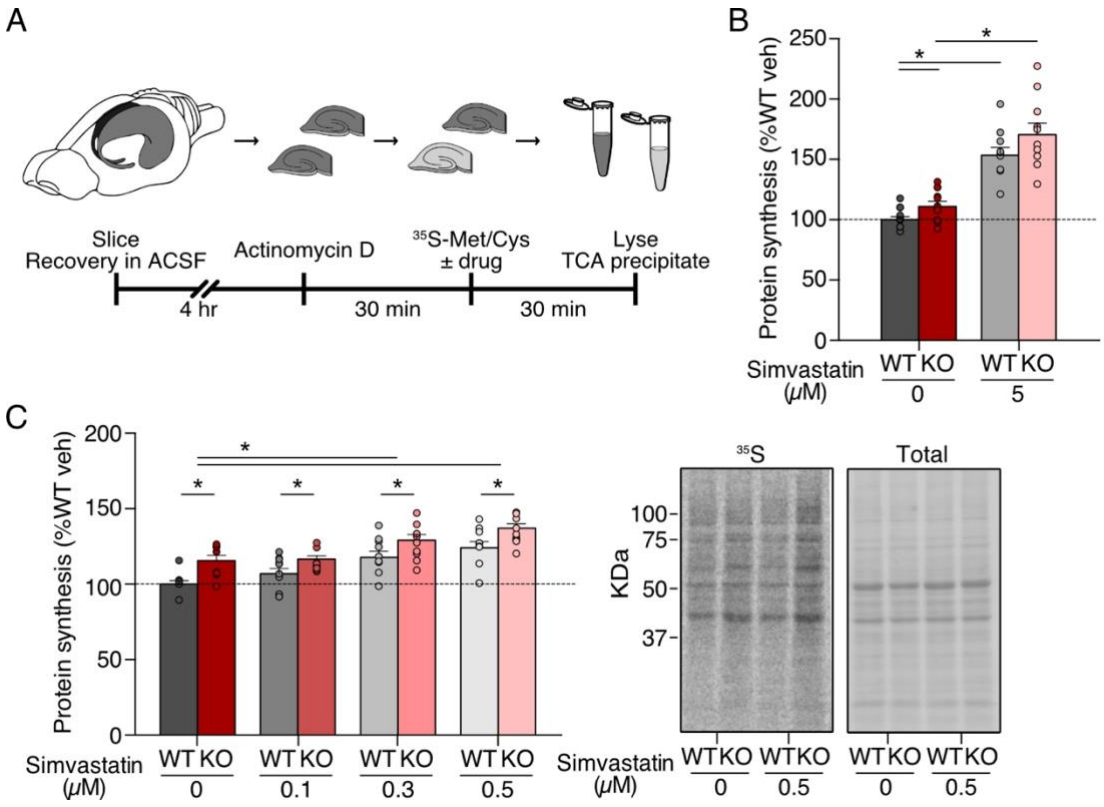

Figure 3.1. Simvastatin exacerbates the elevated protein synthesis levels in the *Fmr1* KO hippocampus

(A) Time course for metabolic labelling experiments. **(B)** Treatment with simvastatin (5μM) in WT and *Fmr1* KO slices, **conducted by Dr. Louros** (ANOVA treatment *p < 0.0001, genotype *p = 0.0294; Paired *t*-test WT veh vs KO veh *p = 0.0366; Sidak WT veh vs simva 5μM *p = 0.0001, KO veh vs simva 5μM *p < 0.0001; n = 10). **(C)** Dose response treatment with simvastatin (0.1, 0.3 and 0.5μM) in WT and *Fmr1* KO slices (ANOVA treatment *p <0.0001, genotype *p = 0.0068, Sidak WT veh vs 0.3 μM *p = 0.0002, WT veh vs 0.5 μM *p < 0.0001, KO veh vs 0.3 μM *p = 0.0035, KO veh vs 0.5 μM *p <0.0001, WT veh vs KO veh *p = 0.0005, WT 0.1μM vs KO 0.1μM *p = 0.0406, WT 0.3 μM vs KO 0.3μM *p = 0.0115, WT 0.5 μM vs KO 0.5 μM *p = 0.0038; n = 9). On the right of dose response graph are representative samples of ^{35}S-labelled proteins visualized via phosphor imaging and the respective total protein from staining of the same membrane. Error bars = SEM. N = littermate pairs. Panel A is adapted from Muscas et al., 2019.

3.2.2 Lovastatin rescues elevated hippocampal protein synthesis in *Fmr1* KO mice

After the unexpected results with simvastatin treatment of hippocampal slices, we wanted to confirm we could replicate the previous findings from Osterweil et al. 2013, with lovastatin treatment rescuing the protein synthesis phenotype in *Fmr1* KO mice. Consistently with those findings, we found that 50μM lovastatin significantly rescued the elevated protein synthesis in *Fmr1* KO mice without affecting the WT protein levels, **Dr. Louros contributed to this dataset** (Figure 3.2) (WT veh = 100 ± 1.48%, WT 50μM = 100.06 ± 4.87%, KO veh = 117.97 ± 4.27%, KO 50μM = 106.04 ± 4.93%). This proved the unexpected results obtained with simvastatin to be unrelated to our different experimenter and mice colony in respect to the Osterweil et al. previous study.

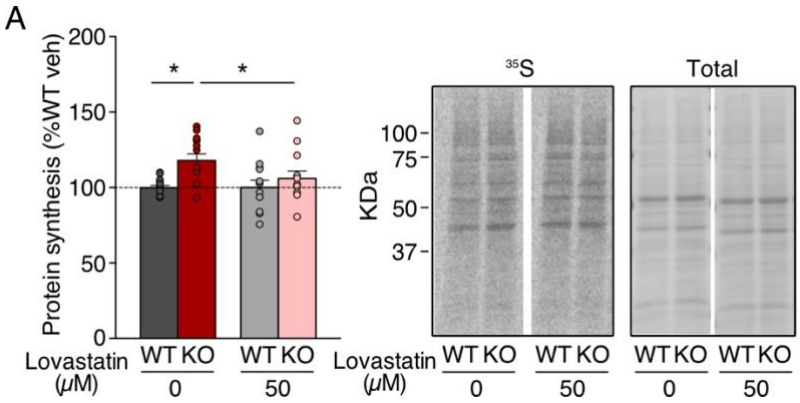

Figure 3.2 Lovastatin normalizes the elevated hippocampal protein synthesis in *Fmr1* KO slices to WT levels.
(A) Graph shows treatment with lovastatin (50 μM) in WT and *Fmr1* KO slices, (ANOVA treatment p = 0.1010, genotype *p = 0.0106; Sidak WT veh vs KO veh *p= 0.0032, WT lova vs KO lova p = 0.3516, KO veh vs KO lova *p = 0.0368; n = 12). **Dr. Louros contributed to this dataset**. On the right are representative samples of ^{35}S-labelled proteins visualized via phosphor imaging and the respective total protein from staining of the same membrane. Error bars = SEM. N = littermate pairs.

3.2.3 Lovastatin inhibits Ras-ERK1/2 activation whereas simvastatin has no impact on either Ras-ERK1/2 or Rheb-mTORC1 pathways

Since we found that 50 μM lovastatin rescues the elevated protein synthesis in the *Fmr1* KO hippocampus, and both 0.5 and 5 μM simvastatin oppositely increase protein synthesis in both genotypes, we decided to investigate if these two statins were differently affecting the signalling cascades involved in regulation of translation downstream mGluRs activity. The two main signalling pathways involved in synaptic protein synthesis regulation downstream the metabotropic receptors are the ERK1/2 and mTORC1 pathways, which lie downstream the GTPases Ras and Rheb respectively, both regulated by farnesylation and hence potentially influenced by upstream cholesterol reduction (Basso et al. 2005; Schafer et al. 1989) (Figure 3.3A). We therefore stimulated dorsal hippocampal slices with 50 μM lovastatin, 0.1-0.5 μM simvastatin and respective vehicles and performed quantitative immunoblotting for phosphorylated (p-) ERK1/2 and the mTORC1 downstream target p70S6 kinase.

Confirming the metabolic labelling results, we found lovastatin to significantly reduce ERK1/2 activation compared to vehicle (Figure 3.3B) (WT vehicle = 100 ± 4.32%, WT lova = 99.28 ± 4.42%, KO vehicle = 91.83 ± 4.74%, KO lova = 76.28 ± 3.76%), and simvastatin to have no effect on the kinase at any dose investigated (Figure 3.3C) (WT vehicle = 100 ± 4.51%, WT 0.1 μM = 102.87 ± 3.42%, WT 0.3 μM = 108.45 ± 4.10%, WT 0.5 μM = 101.01 ± 2.09%, KO vehicle = 105.63 ± 4.97%, KO 0.1 μM = 98.94 ± 4.46%, KO 0.3 μM = 94.71 ± 4.53%, KO 0.5 μM = 106.93 ± 3.65%). This last finding suggests that simvastatin, at doses where is found to dramatically increase protein synthesis in both *Fmr1* KO and WT hippocampi, has no positive or negative impact of the Ras-ERK1/2 pathway.

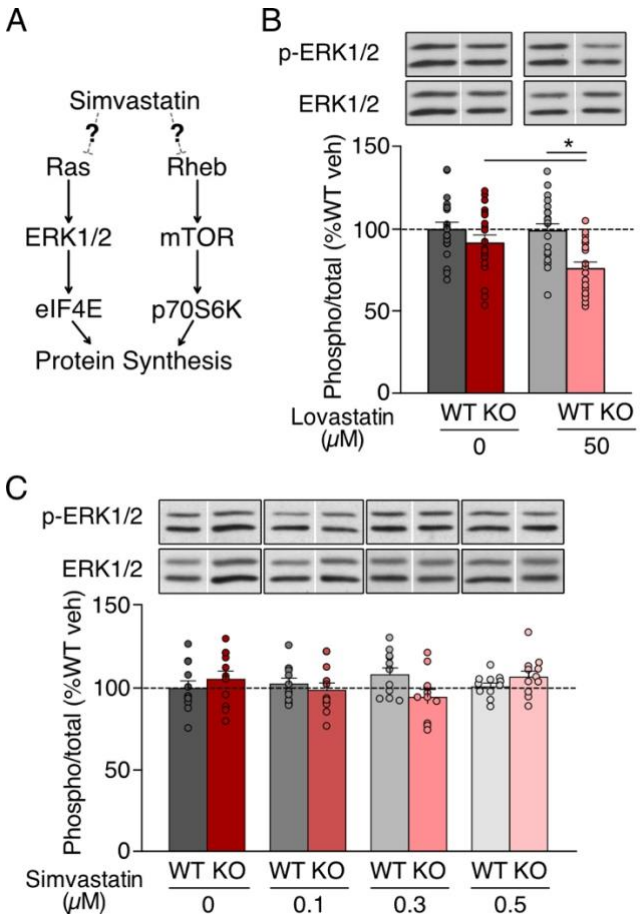

Figure 3.3 Lovastatin, but not simvastatin, reduces ERK1/2 phosphorylation. **(A)** Schematic of potential pathways affected by simvastatin through Ras or Rheb inhibition. **(B)** Lovastatin treatment (50 μM) of dorsal hippocampal slices significantly decreases ERK1/2 phosphorylation in *Fmr1* KO slices (ANOVA treatment p = 0.0795, genotype *p = 0.0146; Sidak KO veh vs KO lova *p = 0.0048; n = 19). **(C)** Simvastatin treatment (0.1-0.5μM) of dorsal hippocampal slices does not reduce or increase ERK1/2 phosphorylation in *Fmr1* KO or WT slices (ANOVA treatment p = 0.8761, genotype p = 0.7010; n = 11). Representative bands shown above each graph were cut from the original blots used for quantification. Error bars = SEM. N = littermate pairs. Panel A is adapted from Muscas et al. 2019.

After verifying that simvastatin has no effect on ERK1/2 phosphorylation, we investigated whether simvastatin could affect the activity of the homologous GTPase Rheb upstream the mTORC1 pathway, which had been previously shown by other studies to be inhibited under conditions of reduced cholesterol (Basso et al. 2005). To prove so, we immunoblotted for the mTORC1 effector protein p70S6K. We found no

effect of simvastatin (0.1-0.5μM) on p70S6K phosphorylation levels (Figure 3.4) (WT vehicle = 100 ± 11.14%, WT 0.1 μM = 112.94 ± 10.25%, WT 0.3 μM = 110.66 ± 9.47%, WT 0.5 μM = 98.89 ± 4.72%, KO vehicle = 92.87 ± 4.49%, KO 0.1 μM = 85.37 ± 11.82%, KO 0.3 μM = 101.71 ± 10.37%, KO 0.5 μM = 92.53 ± 10.64%).

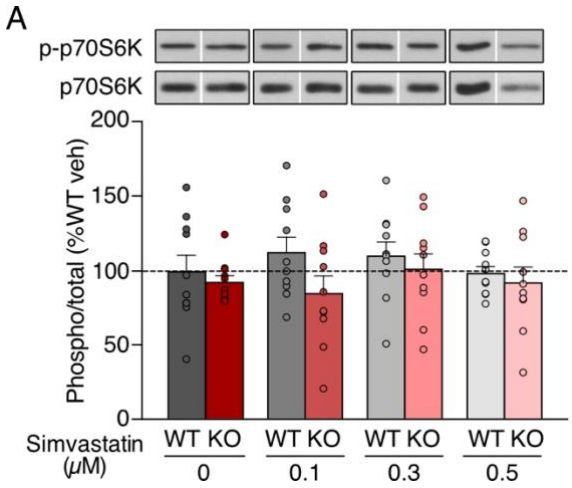

Figure 3.4 Simvastatin has no effect on p70S6K phosphorylation
Simvastatin treatment (0.1-0.5 μM) does not affect the phosphorylation levels of p70S6K in WT or *Fmr1* KO slices (ANOVA treatment p = 0.6206, genotype p = 0.2860, n = 10). Representative bands shown above each graph were cut from the original blots used for quantification. Error bars = SEM. N = littermate pairs.

3.2.4 Simvastatin administration for *in vivo* treatment of AGS phenotype in *Fmr1* KO mice with C57Bl6/J background strain.

Seen the unpromising results obtained with simvastatin treatment of *Fmr1* KO phenotypes *in vitro*, we wanted to lastly test the statin drug in the core behavioural *Fmr1* KO phenotype of increased AGS susceptibility *in vivo*. The AGS phenotype has been documented in both C57Bl6/J and FVB mouse background strains (Musumeci et al. 2000; Yan et al. 2004), hence we first tested simvastatin treatment effect on the same C57Bl6/J line used for the biochemical experiments.

Previous studies from the literature showed single oral administration of 1 mg/kg simvastatin to reduce kainite-induced seizures in one mouse study (Xie et al. 2011)

and reach the ventral midbrain protecting against loss of nigrostriatal neuronal processes in a Parkinson's disease mouse model (Ghosh et al. 2009). Moreover, using the US Food and Drug administration guidelines we calculated the 20 mg human dose of simvastatin for cholesterol treatment to correspond to just less a 3 mg/kg dose in mouse (Nair & Jacob 2016). Therefore, we started testing a single dose of 3 mg/kg administered i.p. 1 hour prior testing. As for human administration, we chose to use the lactone prodrug form of simvastatin, which is enzymatically hydrolysed into active form (hydroxy-acid) in the liver (Corsini et al. 1995).

Unfortunately, several problems were encountered during the investigation. Our *Fmr1* KO mice colony bred in C57Bl/6J background, which had previously consistently shown an AGS incidence versus WT of about 70% (Osterweil et al. 2013; Thomson et al. 2017), stopped showing such robust phenotype. The reason for this is unknown to us, could be due to unidentified disturbances occurring in the animal house during pregnancy or after, due to changes in the genetic makeup of the colony, or due to technical problems with the AGS setup.

We thoroughly investigated the phenotype testing a large range of ages and changing the equipment several times, although without much success. Eventually, we rebuilt the assay chamber to be bigger enough to host two new speakers (KRK Rokit RP5 G3 Active Studio Monitor), and we managed to get again a strong output of the alarm sound (>120dB) into the experimental chamber. However, we could get a consistent AGS incidence of only about 30% in *Fmr1* KO C57Bl/6J mice versus a 0-5% incidence in WT. We still went on performing the experiment with a 3 mg/kg dose of simvastatin 1 hour prior testing, and found it did not decrease either AGS incidence or severity in *Fmr1* KO C57Bl/6J mice versus vehicle treatment (Figure 3.5) (Incidence = WT veh 4%, WT simvastatin 0%, KO veh 30%, KO simvastatin 22%; Severity = KO veh: wild running 5/27, clonic 3/27, tonic 0/27; KO simvastatin: wild running 2/27, clonic 4/27, tonic 0/27).

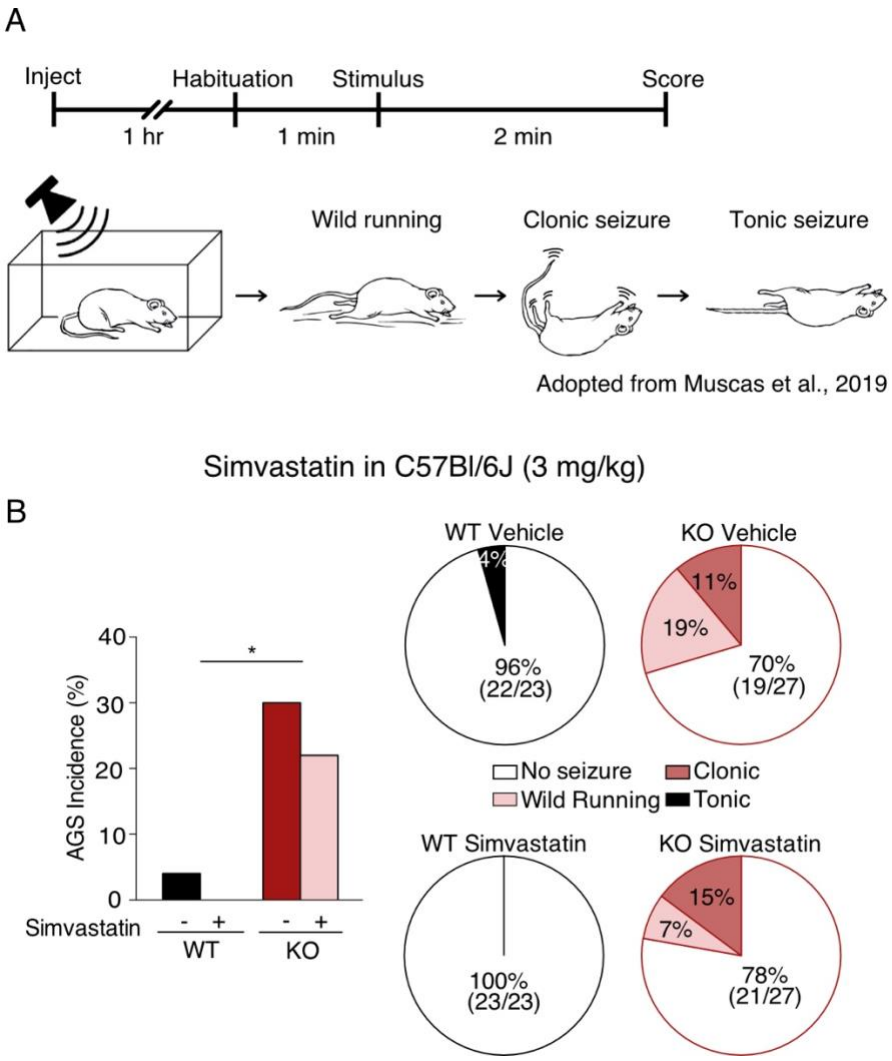

Figure 3.5 Simvastatin fails to reduce increased AGS incidence in *Fmr1* KO mice with C57Bl/6J background.

(A) Schematic of the scoring system experimental timeline for AGS testing. **(B)** Injection of 3 mg/kg simvastatin in *Fmr1* KO C57BL/6J background mice does not reduce incidence of AGS versus vehicle (Fisher's exact test WT vs KO veh *p = 0.0279, WT vs KO simva *p = 0.0250, KO veh vs simva p = 0.7570). AGS severity, shown in pie charts, is also not reduced.

3.2.5 Simvastatin and lovastatin administration for *in vivo* treatment of AGS phenotype in *Fmr1* KO mice with C57Bl6/J x FVB hybrid background strain.

As previously said, the AGS phenotype has been documented in both C57Bl6/J and FVB mouse background strains (Musumeci et al. 2000; Yan et al. 2005). However, the AGS incidence and severity scored in this round of 3 mg/kg simvastatin treatment in C57Bl/6J *Fmr1* KO mice were unexpectedly low compared to previous studies conducted in our same lab and with the same colony of mice. Therefore, we wondered whether the inability to see an effect of simvastatin on seizures was perhaps due to the reduced magnitude of the phenotype to start with. Even though the AGS phenotype is spread across background strains, the FVB strain is known to be seizure-prone and FVB *Fmr1* KO mice present a higher AGS incidence that persists through adulthood (Yan et al. 2004; Yan et al. 2005).

Hence, we crossed our C57Bl/6J mice with FVBs to obtain a C57Bl/6J X FVB hybrid strain. First we did only one generation crossing (F1) and assessed their incidence and severity of AGS. F1 hybrids are closer to inbred strains as they are uniform in their genetic and phenotypic makeup, presenting heterozygosity in all genes for which their mother and father carry different variants. However, when tested for AGS susceptibility we found F1 C57Bl/6J X FVB hybrids had 0 incidence of AGS, suggesting the strain resistant character of the C57Bl/6J strain to be still uniformly present. Then, we tried backcrossing our F1 hybrid mice to FVB mice (obtaining N2 of C57BL/6J backcrossed to FVB by two generations). This time, the C57Bl/6J X FVB backcrossed hybrid mice showed a robust and consistent incidence of AGS. Thus, we repeated the same AGS experiment with 3mg/kg simvastatin. We obtained a 75% AGS incidence in our hybrid Fmr1 KOs compared to <10% in WT mice, but again, simvastatin treatment had no positive impact on AGS incidence compared to vehicle (Figure 3.6A) (WT veh 8%, WT simva 9%, KO veh 75%, KO simva 75%).

When sent for publication in Molecular Autism Journal, one reviewer asked for the pie charts representing AGS severity (Figure 3.6B) to be translated into a histogram graph and run statistical analysis to it (Figure 3.6C). Therefore, all following figures showing AGS experiments present the AGS severity data in both pie charts and histogram graphs as representative examples of the obtained data and appropriate

statistics is shown within. Simvastatin treatment had no positive impact on AGS severity in *Fmr1* KO mice (Figure 3.6D) (WT veh: wild running 0/12, clonic 0/12, tonic 1/12; WT simva: wild running 0/11, clonic 0/11, tonic 1/11; KO veh: wild running 1/12, clonic 5/12, tonic 3/12; KO simva: wild running 3/12, clonic 2/12, tonic 4/12) (Figure 3.6B-C) nor on latency to seizures (in seconds) (KO veh = 26.33 ± 3.80 sec, KO simva = 42.11 ± 12.32 sec).

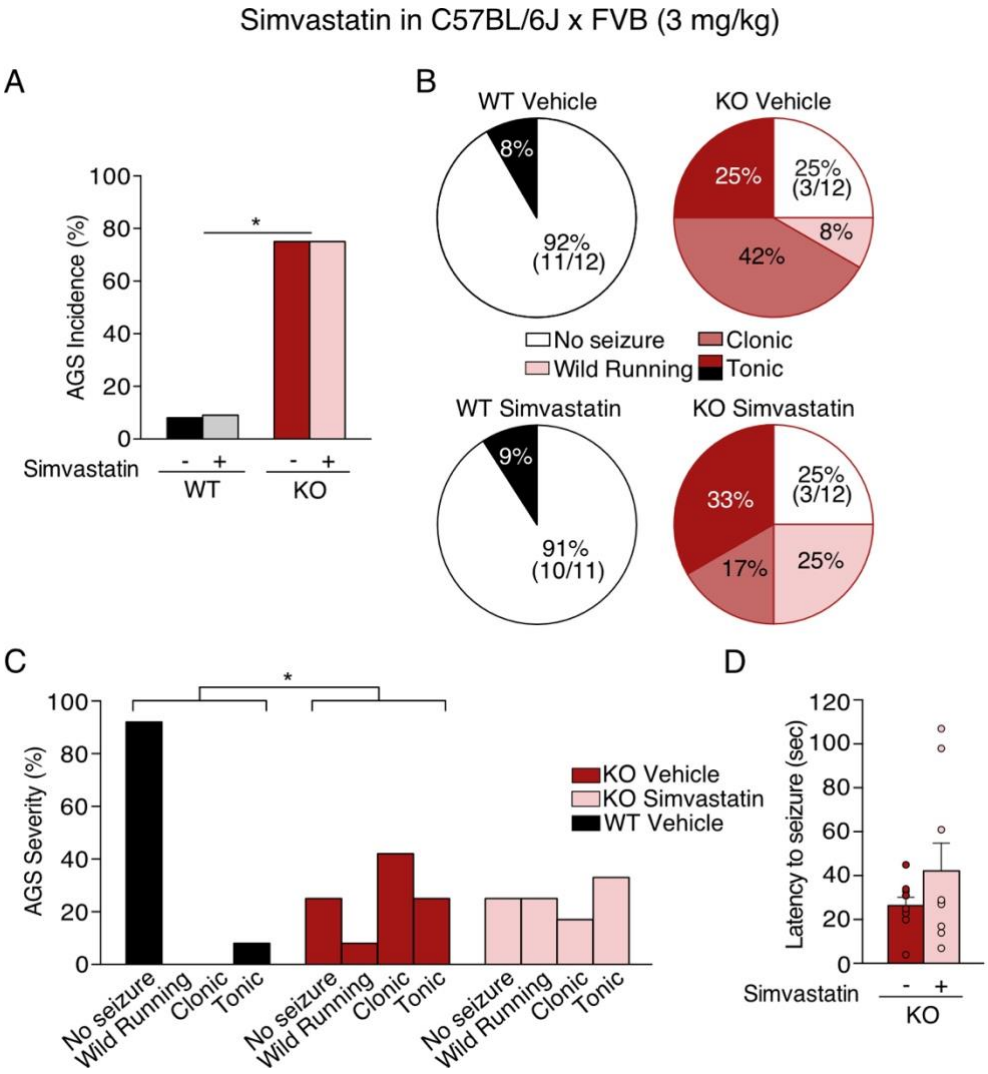

Figure 3.6 Simvastatin fails to reduce increased AGS incidence in *Fmr1* KO mice with C57Bl/6J x FVB background.

(**A**) Injection of 3 mg/kg simvastatin does not reduce incidence of AGS in *Fmr1* KO mice bred on a C57Bl/6J x FVB background (Fisher's exact test WT vs KO veh *p = 0.0028, WT vs KO simva *p = 0.0028, KO veh vs simva p > 0.999). (**B-C**) Pie charts and histogram graph both represent AGS severity, showing that is also not reduced (Mann-Whitney WT vs KO veh *p = 0.0028, KO veh vs KO simva p = 0.9510). (**D)**

3mg/kg simvastatin does not increase latency to first seizure (seconds) in *Fmr1* KO mice (unpaired t-test p = 0.239). Error bars = SEM.

When neither testing 3 mg/kg simvastatin in *Fmr1* KO mice with C57Bl/6J x FVB hybrid background proved the statin to be beneficial for the disease, we wondered whether the dose tested was perhaps too low to show an effect in this specific model. Although a 1 mg/kg dose has been reported in the literature as beneficial for KA-induced seizures and in a mouse model for Parkinson's disease (Xie et al. 2011; Ghosh et al. 2009), doses up to 50 mg/kg have also been studied (Ramirez et al. 2011; Li et al. 2006).

As a dose of 100 mg/kg lovastatin was previously shown to significantly reduce AGS incidence and severity in *Fmr1* KO mice with FVB background (Osterweil et al. 2013), and 10 mg simvastatin is known to reduce cholesterol to the same percentage as 20 mg lovastatin, a dose of 50 mg/kg in mice would be more comparable as equipotent dose to the previously shown beneficial 100 mg/kg of lovastatin. Therefore, we chose to investigate a 50 mg/kg dose of simvastatin (Figure 3.7) and to repeat, in parallel, the previously published experiment with 100 mg/kg injection of lovastatin (Figure 3.8). Both the prodrug lactone form of lovastatin and the active hydroxy acid were proved to significantly reduce AGS incidence and severity in our previous study (Osterweil et al. 2013), hence, we chose to inject the active hydroxy acid form of both drugs, to ensure the lack of effect of 3 mg/kg simvastatin was not due to potential confounds in the liver metabolism of the prodrug.

We found that the lovastatin equipotent dose of 50 mg/kg hydroxy acid simvastatin does not rescue the increased incidence of AGS in *Fmr1* KO mice (Figure 3.7A) (WT veh 8%, WT simva 8%, KO veh 64%, KO simva 55%). This higher dose of simvastatin also has no effect on AGS severity (Figure 3.7B-C) (WT veh: wild running 0/13, clonic 0/13, tonic 1/13; WT simva: wild running 0/13, clonic 0/13, tonic 1/13; KO veh: wild running 0/14, clonic 1/14, tonic 8/14; KO simva: wild running 0/11, clonic 3/11, tonic 3/11), and it has no influence on latency to first seizure (Figure 3.7D) (KO veh = 27 ± 2.95 sec, KO simva = 25.67 ± 3.61 sec).

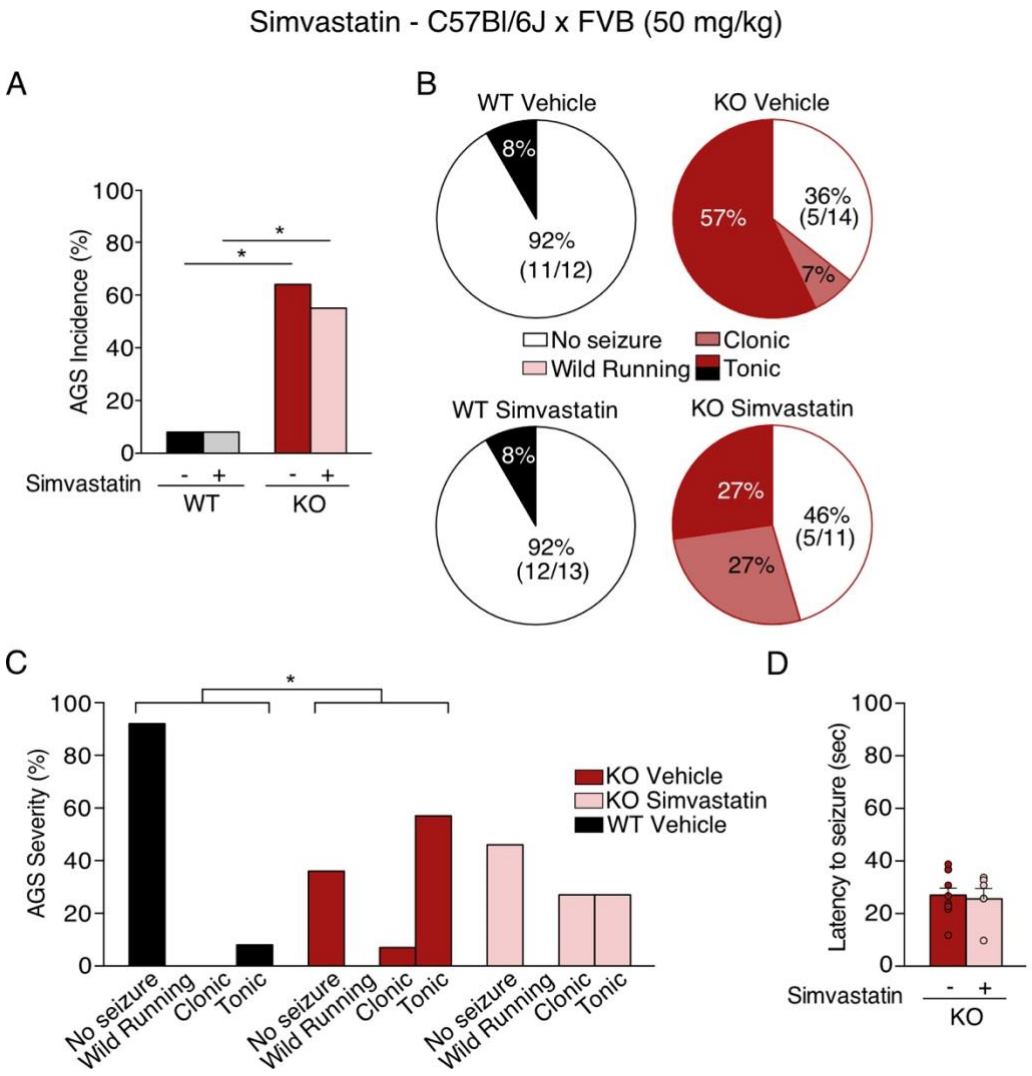

Figure 3.7 50 mg/kg hydroxy acid simvastatin fails to reduce increased AGS incidence in *Fmr1* KO mice with C57Bl/6J x FVB background.

(A) 50 mg/kg hydroxy acid simvastatin does not reduce AGS incidence in *Fmr1* KO C57BL/6J x FVB mice (Fisher's exact test WT vs KO veh *p = 0.0053, WT vs KO simva *p = 0.0233, KO veh vs simva p = 0.6968). (**B-C**) Pie charts and histogram graph both represent AGS severity, showing that 50mg/kg simvastatin does not reduce AGS severity in *Fmr1* KO mice (Mann-Whitney WT vs KO veh *p = 0.0036, KO veh vs KO simva p = 0.2254). **(D)** Latency to first seizure is not significantly different between vehicle and 50 mg/kg simvastatin treated *Fmr1* KO mice (unpaired t-test p = 0.779). Error bars = SEM.

Differently, and consistent with the literature, 100 mg/kg of hydroxy acid lovastatin significantly rescued the increased AGS incidence in *Fmr1* KO mice (Figure 3.8A) (WT veh 13%, WT lova 12%, KO veh 69%, KO lova 21%), and the increased AGS severity (Figure 3.8B-C) (WT veh: wild running 0/15, clonic 0/15, tonic 2/15; WT lova: wild running 2/17, clonic 0/17, tonic 0/17; KO veh: wild running 0/16, clonic 5/16, tonic 6/16; KO lova: wild running 0/14, clonic 1/14, tonic 2/14). Lovastatin treatment also significantly increased the latency to first seizures versus KO vehicle animals (Figure 3.8D) (KO veh = 28 ± 3 sec, KO lova = 45.33 ± 4.84 sec).

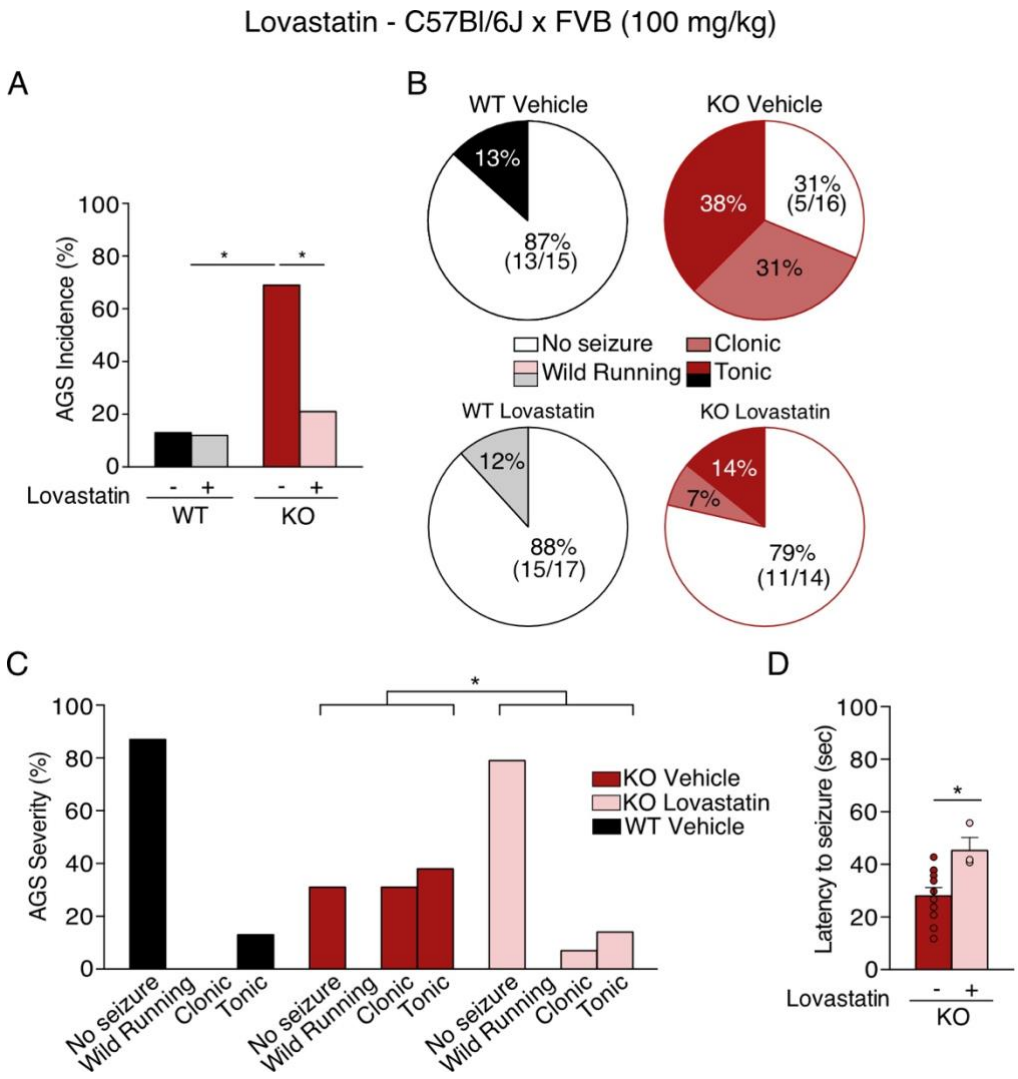

Figure 3.8 100 mg/kg hydroxy acid lovastatin rescues the increased AGS incidence in *Fmr1* KO mice with C57Bl/6J x FVB background.

(**A**) Lovastatin treatment (100 mg/kg hydroxy acid) significantly decreases the AGS incidence in *Fmr1* KO C57Bl/6J x FVB mice (Fisher's exact test WT vs KO veh *p = 0.0032, WT vs KO lova p = 0.6358, KO veh vs lova *p = 0.0136). (**B-C**) Pie charts and histogram graph both represent AGS severity, showing that 100 mg/kg lovastatin decreases AGS severity in *Fmr1* KO mice compared to vehicle (Mann-Whitney WT vs KO veh *p = 0.0064, KO veh vs KO lova *p = 0.0204). **(D)** Lovastatin also significantly increases the latency to first seizure of *Fmr1* KO treated mice compared to vehicle KO group (unpaired t-test KO veh vs lova *p = 0.0176). Error bars = SEM.

3.3 Discussion

In this chapter, we wanted to assess the prediction that simvastatin could be used as an alternative to the promising lovastatin in the treatment of FXS. To do so, we compared the two statins side-by-side on two core biochemical and behavioural phenotypes of the *Fmr1* KO mouse model for FXS. We found that, differently from lovastatin, simvastatin does not target Ras-ERK1/2 activity in the *Fmr1* KO mouse brain, and worsens the increased protein synthesis phenotype in the *Fmr1* KO hippocampus. Moreover, simvastatin is ineffective at rescuing seizures at various doses tested.

3.3.1 Statin modulation of Ras-ERK1/2 and protein synthesis

By reducing mevalonate production statins decrease the synthesis of isoprenoid intermediates, necessary for membrane-binding and activation of GTPase proteins, including Ras (Nürenberg & Volmer 2012). Although all statins share a common binding structure to HMG-CoA reductase, they differ in the rest of their structure. These structural differences could explain their differing blood brain barrier permeability and diverse potencies at reducing cholesterol (Shepardson et al. 2011; Istvan 2003), as well as potential dissimilarities in their level of depletion of isoprenoid intermediates.

In vitro, both lovastatin and simvastatin were found to affect Ras-ERK1/2 activity in numerous cell lines, normal and tumorous, from both mouse and human (Chen et al. 2010; Cerezo-Guisado et al. 2007; Ghittoni et al. 2005; Laezza et al. 2008; Khanzada et al. 2006; Dai et al. 2007; Mendola & Backer 1990; Yamashita et al. 2008; Kang et al. 2009; Hohl & Lewis 1995). *In vivo*, 1 mg/kg of orally administered simvastatin was found to significantly decrease Ras activation in the ventral midbrain of a mouse model with elevated Ras activity. But, it showed no effect in healthy controls with normal levels of Ras activation (Ghosh et al. 2009). Two studies showed long-term treatment of 40 mg/kg and 0.2 mg/kg simvastatin respectively to inhibit Ras-ERK1/2 activation in cardiac muscle tissue preventing cardiac hypertrophy in rats (Indolfi et al. 2002;

Takayama et al. 2011). However, we were not able to find any studies in the literature showing simvastatin to inhibit Ras-ERK1/2 activation in the brain and/or specifically in the hippocampus of animal models with physiologically active Ras. To our knowledge, we are the first to investigate the effect of simvastatin on hippocampal tissue of *Fmr1* KO mice and compare it side-by-side with lovastatin.

Our western blot analysis of stimulated hippocampal slices showed no effect of simvastatin on ERK1/2 activation, whereas lovastatin significantly reduced ERK1/2 phosphorylation in the *Fmr1* KO, similarly to what previously published (Osterweil et al. 2013). We believe the ability of lovastatin to inhibiting Ras-ERK1/2 signalling to be the reason for its ameliorating effects on protein synthesis levels and other protein synthesis-related phenotypes of *Fmr1* KO mice. This is consistent with the missing mechanistic interaction between simvastatin and the Ras-ERK1/2 pathway and its inability to rescue the *Fmr1* KO elevated protein synthesis phenotype; and supports the idea that the impact of a pharmacological treatment on ERK1/2 signalling is an important variable to be considered for successful FXS therapeutics.

We show that 0.5 to 5 μM simvastatin treatment significantly exaggerates the already elevated protein synthesis levels in the hippocampus of the *Fmr1* KO mouse, and significantly increases the normal protein synthesis levels in WT mice, posing an argument for a mechanism of activity by which simvastatin acts on protein synthesis/degradation pathways that are not dysregulated in FXS. Here, through its effect on isoprenoid synthesis, simvastatin is likely to be preferentially targeting a different protein, such as a GTPase other than Ras. For instance, even though we found no effects of simvastatin treatment on the phosphorylation levels of the mTORC1 downstream target p70S6K (regulated via the homologous Ras GTPase Rheb), a study with prolonged (2 hour) simvastatin treatment of hippocampal slices has demonstrated activation of the PI3 kinase-Alt pathway, which could be involved in the simvastatin-driven increase in protein synthesis (Mans et al. 2010).

It is possible that only much higher concentrations of simvastatin than the ones tested in this study would inhibit Ras farnesylation enough to then reduce the activity of physiologically active Ras in the mouse brain. However, seen the dose-dependent

increase in protein synthesis with increasing simvastatin doses, from 0.1 to 5 μM, we argue that treating *Fmr1* KO slices with even higher doses would not ameliorate the protein synthesis phenotype and likely trigger off-target effects.

3.3.2 *In vivo* statin administration for AGS treatment

To confidently exclude simvastatin as a potential alternative to lovastatin in the treatment of FXS or any of the so called RASopathies, we finally examined its effects on the *Fmr1* KO AGS phenotype *in vivo*. Again, comparing equipotent doses of simvastatin to lovastatin, we found no positive effect of simvastatin treatment versus a rescue with lovastatin administration.

We chose intraperitoneal injection as administration route for several reasons. It allows for precise administration of desired dose, it is a quick method for delivery with minimal and fast restraint of the animal. It does not require habituation and the animals do not present any stress-associated behaviour post-injection. Moreover, although being a parenteral administration route, i.p. injections present pharmacokinetic mechanisms closer to oral routes of delivery, as the drug is mainly absorbed by the mesenteric vessels and passed to the liver for metabolism (Turner et al. 2011). Rodent studies from the literature show that simvastatin peaks in the blood at 30 min-1 hour post single oral administration of 10-20 mg/kg (van de Steeg et al. 2013; Xu et al. 2014), and in the mouse brain after 1 hour from acute administration (Johnson-Anuna et al. 2005). This assured us that a single i.p. injection of 3 to 50 mg/kg simvastatin would reach its peak in blood and body tissues by the time of AGS testing 1 hour post administration. Therefore, we are confident that the lack of effect of simvastatin treatment to the AGS phenotype is not due to dosage or metabolism related inaccuracies. This is further supported by the finding that an equipotent dose of lovastatin, 100 mg/kg, delivered through the same administration route, does rescue the phenotype.

3.3.3 Final considerations

Being prescribed worldwide for safe long-term therapy at both adult and young ages, the use of statins for the treatment of neurodevelopmental disorders such as FXS is very appealing. However, it is important to note that whereas the effect of each statin on cholesterol is well known, their variable impact on isoprenoid synthesis, hence potentially on Ras-ERK1/2 signalling, has not been thoroughly examined in all body tissues and systems. Indeed, we showed in this chapter that the effects of simvastatin and lovastatin on ERK1/2 phosphorylation in the mouse hippocampus are significantly different, with simvastatin further increasing the elevated protein synthesis levels in the *Fmr1* KO mouse, and failing to rescue its AGS phenotype. These results strongly imply a major difference in the mechanisms of activity of lovastatin and simvastatin in the brain. Although additional studies comparing a wider range of simvastatin doses and administration routes should be performed, our study suggests that simvastatin should not be assumed to be an effective replacement for lovastatin with respect to correction of FX pathology or other disorders of excess Ras. Indeed, whereas lovastatin improved some cognitive deficits in a pilot clinical trial for the NF1 RASopathy, simvastatin had no positive outcome in a second clinical trial conducted in children (van der Vaart et al. 2013; Payne et al. 2016; Bearden et al. 2016).

In conclusion, our study proves that compounds with very similar chemical structure and almost identical clinical effects on specifically investigated targets do not necessarily share all major molecular targets or act through the same molecular pathways, particularly if this is assumed for different cell types or tissues. Before recommending a compound for clinical trials in humans for any medical condition or disorder it is therefore imperative to first investigate its effects and molecular targets with the available animal models.

Chapter 4

M_4 positive allosteric modulation in *Fmr1* KO mice

4.1 Introduction

The hippocampus is a primary structure involved in the defining of FXS pathology. In *Fmr1* KO rodent models, elevated protein synthesis in the hippocampal CA1 region is responsible for the core phenotype of exaggerated mGluR-LTD (Nosyreva & Huber 2006). Hence, a recent study conducted within our research group aimed at identifying the mistranslated mRNAs in the CA1 area of the *Fmr1* KO hippocampus, to unveil the proteins underlying FXS hippocampal pathology and disrupted synaptic signalling (Thomson et al. 2017). We found that the most enriched mRNA transcripts were from the G-protein-coupled acetylcholine receptor (muscarinic) (mAChR) family. Among these, the transcript for the muscarinic receptor 4 (M_4) was found significantly enriched in the CA1 translating mRNAs and significantly overexpressed in KO hippocampal homogenates and synaptoneurosomes (Thomson et al. 2017).

Following the notion of FXS pathology being characterized by elevated protein synthesis due to absence of FMRP, we first investigated the effects of M_4 antagonism on the *Fmr1* KO hippocampal protein synthesis. Surprisingly, M_4 antagonism exacerbated the already elevated protein synthesis in the *Fmr1* KO, and caused an increase in normal protein levels in the WT. Hence, as there are no available agonists for M_4-selective binding, we tried using a positive allosteric modulator (PAM) instead. The PAM VU0152100 is a powerful, brain penetrant and highly selective M_4-specific positive allosteric modulator. It binds to an allosteric site of the M_4 receptor and increases acetylcholine affinity for the orthosteric site, enhancing M_4 responses to acetylcholine binding (Brady et al. 2008). Stimulating hippocampal slices with this M_4-specific PAM rescued both the elevated protein synthesis and exaggerated mGluR-LTD phenotypes in the *Fmr1* KO mice, and had no effect in the respective WT controls (Thomson et al. 2017).

Moving on from these results, we wondered if treatment with this M_4 PAM would also rescue a core behavioural FXS phenotype: the increased susceptibility to AGS. First part of this chapter will illustrate these results, now published in Thomson et al. 2017.

Then, we decided to investigate what signalling cascades downstream of M_4 positive allosteric modulation were underlying its ameliorating effects. According to the literature, M_4 signalling recruits and influences the activity of numerous pathways known to regulate protein synthesis, including ERK1/2, mTOR and cyclic AMP (cAMP) signalling (Wiseman et al. 2013; Rosenblum et al. 2000; Chan et al. 2009). Thus, research illustrated in the second part of this chapter focused on investigating whether hippocampal stimulation with the M_4 PAM results in the activation or inhibition of any of these pathways.

4.2 Results

4.2.1 M_4 PAM treatment rescues the increased incidence and severity of AGS in *Fmr1* KO mice.

The finding that treatment of *Fmr1* KO hippocampal slices with the M_4 PAM could rescue both the elevated protein synthesis and the exaggerated mGluR-LTD, prompted us to further investigate this treatment on the *Fmr1* KO AGS phenotype. A previous study showed a single i.p. administered dose (30-56 mg/kg) of the M_4 PAM VU0152100 to rescue amphetamine-induced hyperlocomotion in rats in a dose-dependent manner (Byun et al. 2014). We hence decided to similarly treat our juvenile KO and WT mice with a dose of 30 mg/kg M_4 PAM or respective vehicle. Differently from what described in Chapter 3, at the time of this study our AGS apparatus was working satisfactorily and our C56Bl/6J *Fmr1* KO mice were consistently showing about 70% AGS incidence compared to WT. Thus, we proceeded with the experiment using this very same colony.

Consistent with the literature (Dölen et al. 2007; Osterweil et al. 2010; Osterweil et al. 2013; Thomson et al. 2017), we found our C56Bl/6J *Fmr1* KO mice with vehicle treatment to have a 62% AGS incidence compared to 6% in WT vehicle mice. Instead, the 30 mg/kg PAM treated KOs showed a slightly decreased AGS incidence of 48%, however not statistically significant from the KO vehicle group (Figure 4.1A) (WT veh 6%, WT PAM 6%, KO veh 62%, KO PAM 48%).

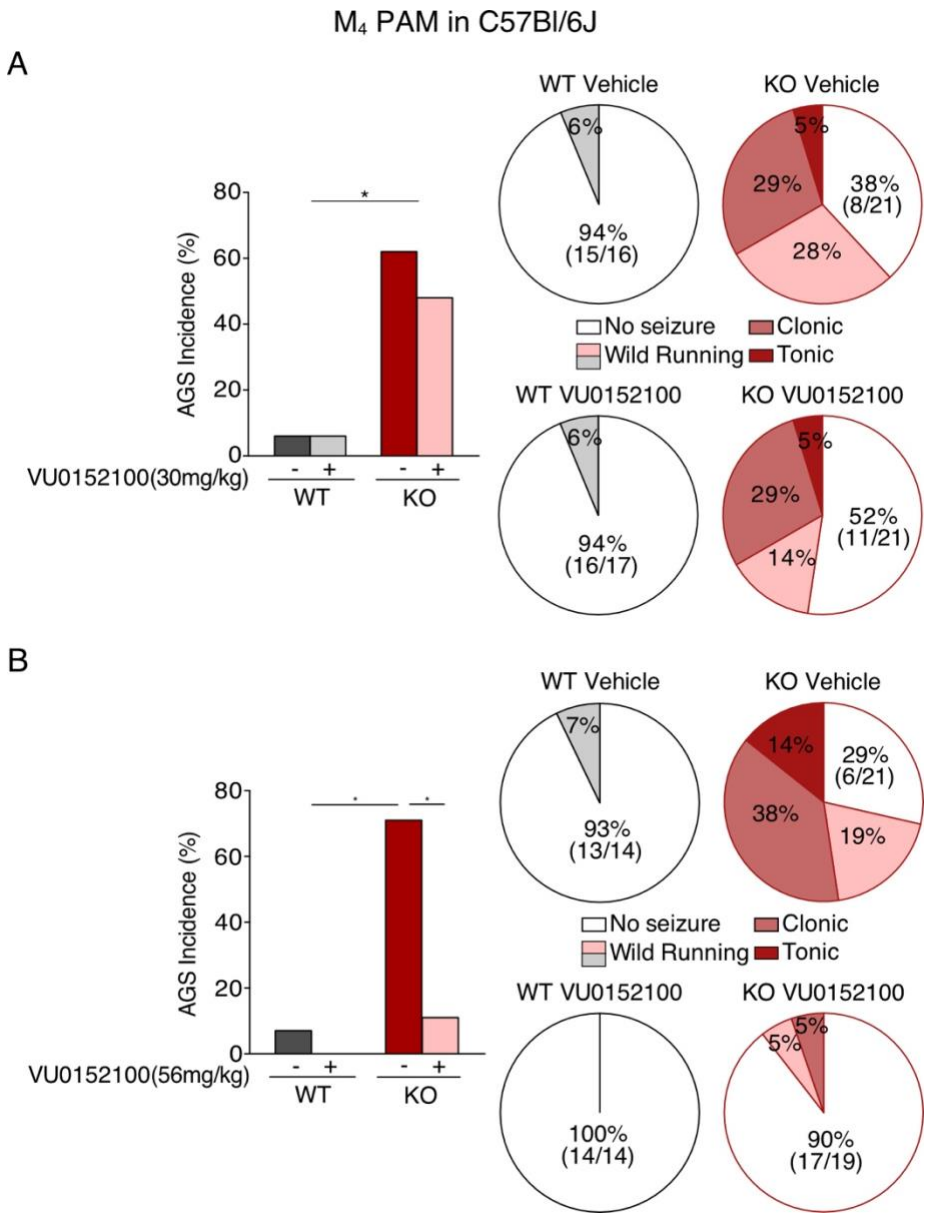

Figure 4.1 56 mg/kg M4 PAM treatment rescues the increased AGS incidence in *Fmr1* KO mice with C57Bl/6J background.

(A) Injection of lower M4 PAM dose (30 mg/kg) in *Fmr1* KO C57BL/6J background mice does not reduce incidence of AGS versus vehicle (Fisher's exact test WT vs KO veh *p = 0.0006, WT vs KO PAM *p = 0.0099, KO veh vs PAM p = 0.5359). AGS severity is also not reduced (KO veh: wild running 7/21, clonic 5/21, tonic 1/21; KO PAM: wild running 4/21, clonic 5/21, tonic 1/21). **(B)** Injection of higher M4 PAM dose (56 mg/kg) in *Fmr1* KO C57BL/6J background mice significantly rescues the elevated AGS incidence in the *Fmr1* KOs versus vehicle (Fisher's exact test WT vs KO veh *p = 0.0003, WT vs KO PAM p = 0.4962, KO veh vs PAM *p = 0.0001). AGS severity is also rescued (KO veh: wild running 4/21, clonic 8/21, tonic 3/21; KO PAM: wild running 1/19, clonic 1/19, tonic 0/19). This result is published in Thomson et al. 2017.

We therefore tried a second round of experiment increasing the M_4 PAM treating dose to 56 mg/kg. With this higher dose, both incidence and severity of AGS in *Fmr1* KO mice were successfully rescued, with no effect on WT treated animals (Figure 4.1B) (WT veh 7%, WT PAM 0%, KO veh 71%, KO PAM 10%) (Thomson et al. 2017).

4.2.2 Potential downstream mechanisms of M_4 activity in *Fmr1* KO and WT mice

The metabolic labelling assay adopted by our research group to measure hippocampal levels of newly synthesized protein is conducted with drug or vehicle treatment and concomitant inhibition of transcription with actinomycin D to isolate translation changes. Because of this, we focused on studying the effects that M_4 PAM treatment could exert on protein synthesis without passing through transcription first. Using processed hippocampal tissue treated with actinomycin D plus M_4 PAM or vehicle (provided by Dr. Susana Louros), we used western blotting to probe for phosphorylated (p-) levels of the protein synthesis regulators ERK1/2, p70S6K and eEF2. A schematic in Figure 4.2A shows how M_4 activation could affect either or all these downstream protein regulatory effectors.

M_4 receptors mainly couple to G proteins associated with the α $G_{i/o}$ subunit, and are known for inhibiting the enzyme adenylyl cyclase (AC) (Migeon et al. 1995). By inhibiting AC, $G_{i/o}$-associated G proteins reduce ATP conversion to cyclic AMP (cAMP), which normally works as second messenger in several signalling pathways important for cellular growth, differentiation, transcription and even protein synthesis (Yan et al. 2016). At the root of cAMP regulation of protein synthesis-associated signalling is the cAMP-dependent protein kinase A (PKA). PKA has numerous substrates, many of which are associated to regulation of gene transcription, while others affect translation directly. As for that, PKA activation causes the turnover of the eukaryotic elongation factor-2 kinase (EF2K), which when active phosphorylates and inhibits the eukaryotic elongation factor-2 (eEF2), inhibiting elongation of nascent

polypeptides and hence their translation (Wiseman et al. 2013). Therefore, PKA activation promotes protein synthesis via eEF2 disinhibition (Figure 4.2A).

As M_4 signalling through the $G_{i/o}$ α subunit inhibits cAMP, M_4 PAM treatment should prevent the PKA-driven increase in protein synthesis. However, blocking the PKA-driven turnover of EF2K does not necessarily imply a consequent decrease in protein synthesis.

To clarify the nature of the connection between M_4 and its downstream eEF2 target in both WT and *Fmr1* KO mice, and the effects of M_4 positive modulation, we probed for phosphorylated eEF2 at the phospho site Thr56, targeted for inhibition by EF2K. We found M_4 activation to cause, in the WT hippocampus, a significant increase in eEF2 phosphorylation by EF2K (Figure 4.2B). In accordance with the literature, this could be due to M_4 positive modulation to decrease cAMP levels, hence PKA activation, which would prevent EF2K turnover. This results in increased levels of phosphorylated, hence inhibited, eEF2. However, Thomson et al. 2017 did not find treatment with the M_4 PAM to change hippocampal protein synthesis in the WT. This suggests this found change in eEF2 phosphorylation to not be sufficient to make a difference in overall hippocampal protein levels.

What is surprising is that, in the *Fmr1* KO hippocampus, we found no effect of M_4 PAM treatment on eEF2 phosphorylation, indicating that the ameliorating effects of M_4 positive modulation in the *Fmr1* KO mouse are not related to eEF2 activity (Figure 4.2B) (WT vehicle = 100.00 ± 4.52%, WT 5 μM = 114.95 ± 6.22%, KO vehicle = 101.75 ± 4.64%, KO 5 μM = 96.62 ± 2.83%).

Besides inhibiting cAMP production, G proteins associated to the $G_{i/o}$ α subunit affect ion channels and numerous other molecular pathways in different cells or tissues of the body. Their Gβ and Gγ subunits also present important regulatory roles (Schmidt et al. 1992; Bondar & Lazar 2014). However, the specific targets of the $G_{i/o}$ Gβγ subunits have not been all identified, and seem to vary between cell types and tissue. Gβγ subunits activated by $G_{i/o}$ proteins have for example been correlated to Raf-ERK1/2 pathway activation in fibroblasts cell cultures (Winitz et al. 1993). Activation of muscarinic receptors *in vivo* in the rat has also been correlated to activation of the

ERK1/2 pathway, with M_4 specific stimulation in fibroblast-like COS7 cell cultures having the same effect (Rosenblum et al. 2000) (Figure 4.2A). Although previous studies have shown inhibition rather than activation of ERK1/2 to be beneficial in FXS (Osterweil et al. 2010; Osterweil et al. 2013), because M_4 positive modulation rescues the elevated protein synthesis of *Fmr1* KO mice, it was important for us to investigate its effects on this crucial kinase for FXS pathology and treatment.

Interestingly, we found M_4 PAM treatment to have no significant effect on ERK1/2 activity in either *Fmr1* KO or WT mice (Figure 4.2C) (WT vehicle = 100.00 ± 4.43%, WT 5 μM = 94.68 ± 3.87%, KO vehicle = 91.55 ± 3.84%, KO 5 μM = 92.27 ± 5.29%). It is not through modulation of the ERK1/2 pathway or the PKA-downstream eEF2 elongation factor that M_4 works on the *Fmr1* KO mouse model.

Other studies proved a relationship between muscarinic receptor subtypes and p70S6K activation. One showed M_4 to promote p70S6K activation via Rheb/mTOR pathway in the PC12 cell line, model for neuronal cells (Chan et al. 2009) (Figure 4.2A). Hence, we finally tested if the M_4 PAM was affecting the activity of the protein synthesis regulator p70S6K. We found a significant treatment effect with ANOVA analysis between vehicle and M_4 PAM groups, however, post hoc analyses revealed no significant differences between each KO and WT group (Figure 4.2D) (WT vehicle = 100.00 ± 5.66%, WT 5 μM = 109.99 ± 7.88%, KO vehicle = 86.80 ± 2.55%, KO 5 μM = 102.02 ± 5.57%). While it is possible that an increased sample size would reveal differences, the current analysis does not support the role of Rheb/mTOR in the effect of M_4 PAM on *Fmr1* KO protein synthesis.

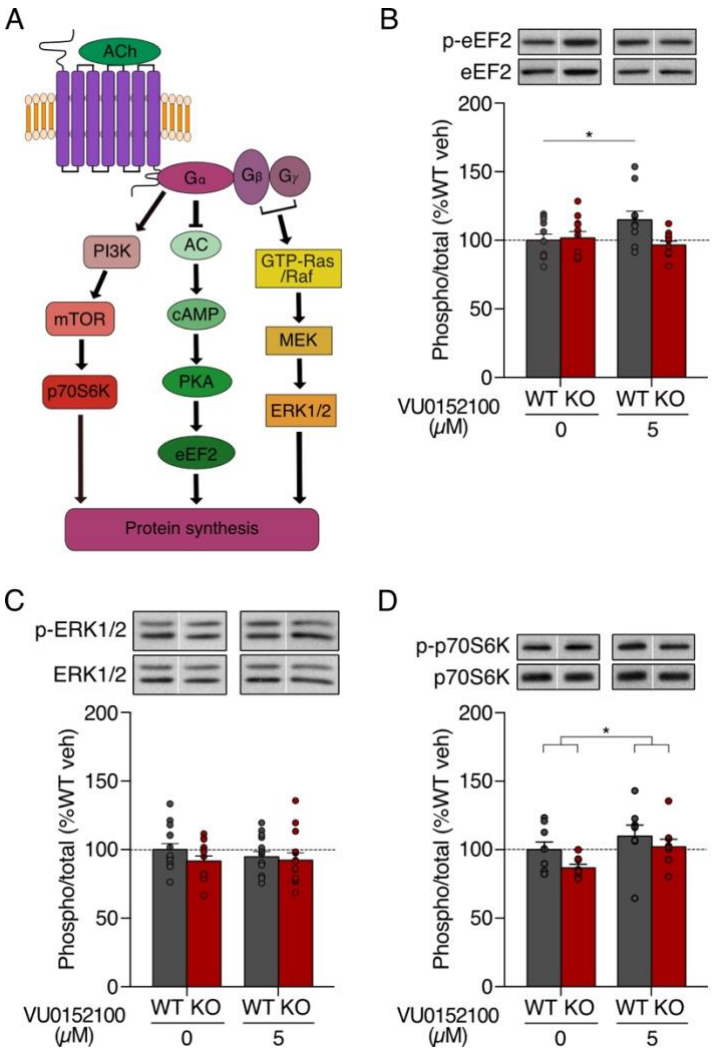

Figure 4.2 M_4 amelioration of the *Fmr1* KO phenotypes does not seem to occur through modulation of either ERK1/2, mTOR or PKA-eEF2 pathways.
(A) Simplified schematics of possible protein synthesis regulatory pathways in play downstream M_4 signalling. (**B**) M_4 PAM treatment (5μM) does not affect the phosphorylation levels of eEF2 in *Fmr1* KO slices, but it significantly increases them in WT slices (ANOVA treatment p = 0.4458, genotype p = 0.1910, treatment x genotype *p = 0.0123; Sidak WT veh vs WT PAM *p = 0.0187; n = 10). (**C**) M_4 PAM treatment (5μM) has no effect on ERK1/2 phosphorylation in either WT and *Fmr1* KO slices (ANOVA treatment p = 0.6412, genotype p = 0.3046, n = 13). (**D**) Phosphorylation of p70S6K is also not significantly changed with M_4 PAM treatment (5μM) with post-hoc Sidak's analysis, although there is a significance in ANOVA treatment (ANOVA treatment *p = 0.0134, genotype p = 0.0876, n = 8). Representative bands were cropped from original blots. Error bars = SEM. N = littermate pairs.

4.3 Discussion and future directions

Previous work by Thomson and colleagues found treatment with the M_4 PAM VU0152100 to rescue core biochemical and electrophysiological phenotypes of the *Fmr1* KO mouse. Following on those results, we show at the beginning of this chapter that a single M_4 PAM treatment can also fully rescue both incidence and severity of AGS in juvenile *Fmr1* KO mice (Thomson et al. 2017).

Intrigued by these very promising findings, we investigated what signalling mechanisms underlie M_4 activity in the *Fmr1* KO and WT hippocampus. We found that the M_4 PAM does not significantly modulate the ERK1/2 pathway under these conditions, and the impact of M_4 PAM treatment on eEF2 phosphorylation in WT hippocampal slices does not explain its selective effect on hippocampal protein synthesis in the *Fmr1* KO. The mTOR-p70S6K pathway also does not seem to be significantly involved in the ameliorating mechanism, although further experiments should be performed to finalise its dataset.

Interestingly, our data suggests the cAMP-PKA-eEF2 pathway may be disconnected from M_4 signalling in *Fmr1* KO mice, perhaps indicating a homeostatic connection between M_4 and a new downstream target capable of counteracting for FMRP loss. Furthermore, it is interesting to note that cAMP production upon AC stimulation is found to be reduced in platelets and neuronal cells of both *Fmr1* KO mice and FXS patients, and in the head of a drosophila model for FXS (Kelley et al. 2007; Berry-Kravis & Huttenlocher 1992; Berry-Kravis et al. 1995). Moreover, increasing cAMP production was shown to rescue several phenotypes in the *Fmr1* KO mouse and FXS drosophila (Choi et al. 2015; Choi et al. 2016). However, we know that inhibiting M_4 activity to increase the production of cAMP exacerbates the *Fmr1* KO protein synthesis. Thomson et al. (2017) also looked at the effects of M_4 PAM stimulation on cAMP levels, and found no significant effect in either WT or *Fmr1* KO hippocampal slices. This strongly suggests that, although increasing cAMP levels might ameliorate other phenotypes, correction of the *Fmr1* KO hippocampal protein synthesis by the M_4 PAM is not related to cAMP levels.

M_4 receptors have yet to be found to be targets of FMRP regulation, and their elevated expression in the hippocampus of *Fmr1* KO mice is hypothesized to be due to a homeostatic response to FMRP loss (Thomson et al. 2017). So, what could M_4 be signalling to downstream?

The elongation factor 1A2 (eEF1A2) could be one option. eEF1A2 is crucial for protein synthesis as it is necessary for the binding of aminoacyl-tRNA to the ribosome binding site, and is known to directly interact with M_4 through its third intracellular loop. *In vitro*, eEF1A2 was found to co-localize with M_4 in both neuronal soma and processes, independently of agonist stimulation. As M_4 was shown to act as a guanine exchange factor (GEF) for eEF1A2, exchanging its bound GDP for GTP and driving its activation, M_4 stimulation was hypothesized to drive a conformational change for activating this GEF activity (McClatchy et al. 2002).

To better tackle the question of what is lying downstream M_4 signalling in FXS, our next step in the investigation would be to adopt a cell culture system for hippocampal neurons. Using a cell culture system has several benefits when investigating potentially small biochemical changes in the brain. It would give us much bigger samples for more thorough analyses of proteins or mRNAs levels; it would allow us to perform transcriptional or translational manipulations with the use of shRNA and plasmid vectors. In a cell culture system with *Fmr1* KO hippocampal neurons, we would knock down M_4 expression by using a lentiviral vector transfer plasmid for anti-M_4 shRNA expression (developed by Okada et al. 2014). Within that system, we could measure protein synthesis using the alkyne analog of puromycin, o-propargyl-puromycin, which is incorporated into newly translated proteins and can be fluorescently visualized with an easily performed assay for copper-catalized Click chemistry. By measuring protein synthesis levels under silencing of M_4 expression with an anti-M_4 shRNA, we would be looking to confirm or disprove the finding that M_4 antagonism exacerbates protein synthesis in both WT and *Fmr1* KO hippocampus. On the other hand, a cell culture system with *Fmr1* KO hippocampal neurons with endogenous M_4 expression would allow us to measure protein synthesis levels with M_4 PAM stimulation.

If M_4 PAM treatment would prove beneficial also in cultured *Fmr*1 KO hippocampal neurons, then we would move forward to investigate the downstream M_4 targets. Small changes in protein translation or phosphorylation should be more easily detectable in a culture system due to the higher levels of overall proteins available to lyse or stain for fluorescence. Knowingly good antibodies for potential M_4 targets could be used for pull-downs for investigating M_4 direct interactions in *Fmr1* KO neurons, such as for eEF1A2. With concomitant M_4 PAM treatment or M_4 antagonism to compare the opposing scenarios.

Moreover, going back to hippocampal slices, we would complete the dataset for phosphorylation levels of p70S6K upon M_4 PAM stimulation.

These are only starting points for future research. By carrying out some of the listed investigations it would possible to plan further ahead to discover how M_4 acts downstream in the *Fmr1* KO mouse.

Chapter 5

Behavioural assays with adult *Fmr1* KO mice and rats

5.1 Introduction

FX patients present abnormally increased hippocampus (Kates et al. 1997), decreased amygdala and posterior cerebellar vermis, and show abnormalities in cortical and subcortical volumes and connectivity (Gothelf et al. 2008). Because intellectual impairments are leading characteristics of FXS, the *Fmr1* KO models are being extensively investigated for deficits and impairments in behavioural tasks involving these aforementioned brain structures (Cornish et al. 1999; Paradee et al. 1999). In assessing disease relevant mechanisms, it is indeed helpful to have measurable and quantifiable behavioural deficits in the FXS animal models. Thus, to deeply investigate the potential benefits of M_4 pharmacological manipulation for FXS treatment, we were interested in testing for associative learning and non-associative memory-related behaviours at adult ages. Because core phenotypes of the *Fmr1* KO are its increased hippocampal protein synthesis and exaggerated mGluR-dependent LTD, and M_4 PAM treatment proved beneficial for both, we looked for memory paradigms preferentially requiring the activity of hippocampal structures.

In this chapter of the thesis we will investigate the performance of *Fmr1* KO and WT adult mice and rats in long-term memory tasks with and without primary involvement of the hippocampus. At the end, we select one of these assays to investigate the therapeutic potential of the M_4 PAM.

5.2 Contextual fear conditioning

Fear conditioning paradigms work on associative learning, and according to their category they can involve different brain structures and to variable extents. For example, cued-associated fear conditioning primarily involves the amygdala, whereas contextual fear conditioning depends on both amygdala and hippocampus (Phillips & LeDoux 1992; Kim & Fanselow 1992; Fanselow & Kim 1994). Importantly, both memory consolidation and extinction of contextual fear conditioning were found to be dependent on hippocampal protein synthesis (Motanis & Maroun 2012; Fischer et al.

2004), as the hippocampus has been correlated to long-term memory for contextual information by numerous studies (Maren & Fanselow 1997; Young et al. 1994; Kim et al. 1993).

For this reason, we decided to pursue an investigation for contextual fear conditioning phenotypes in our colony of *Fmr1* KO mice, assessing whether KO mice present decreased fear responses to context compared to WT animals. We chose to conduct the assay with young adult mice as opposite to juvenile for several reasons. Firstly, we wanted to investigate the potential beneficial effects of the M_4 PAM at adult ages; secondly, because of the complexity of the task; thirdly, as we hoped for a more consistent performance of the animals; and finally, for potentially less stress of the animals with human contact and easier handing.

In the literature, studies on contextual fear conditioning with *Fmr1* KO mice show controversial results, and some adopt a protocol with both context- and cue-associated adverse stimulus. Paradee et al. (1999) tested C57Bl/6J *Fmr1* KO mice for contextual fear conditioning by returning them to the training cage 24 hours after the shock (2 seconds long, at 0.35 mA and paired with a 30-second-long tone) and scoring their freezing rate. While WT mice showed an overall freezing of 50%, KO mice showed only 31%. When inserted into a novel context, both genotypes showed significantly less freezing, with 9% score. Similarly, another study with a 24-hour delay and a single 2 seconds-long foot shock at 0.7 mA found that male C57Bl/6J *Fmr1* KO mice showed 30% freezing in the familiar context while WT males showed about 70% (Ding et al. 2014). Other research groups showed the contrary, one found no significant differences in the freezing rate of *Fmr1* KO mice in both C57Bl/6J and FVB-129 hybrid background strains compared to their WT littermates. However, the overall freezing percentage of both genotypes were at or below 30% (Dobkin et al. 2000). Another group, which investigated 2 mild foot shocks, showed a 60% freezing rate with both *Fmr1* KO and WT mice with C57Bl/6J background strain (Spencer et al. 2006).

5.2.1 Results for contextual fear conditioning

Because studies have shown the hippocampus to be primarily involved in not cue-associated contextual memory (Phillips & LeDoux 1992; Kim & Fanselow 1992), we decided to compare the level of freezing of animals in a familiar, adverse stimulus-associated context with a novel one. This to measure the ability of mice to formulate associative memories via their discrimination of contexts. For this reason, we had two chambers. One with a grid floor to administer the foot shock on the day of training, with black and transparent walls and neutral scent from disinfectant wipes; the other with black and white striped walls, grey plastic floor and ethanol scent.

In the first experimental round, we started with a cohort of 8 adult mice (>120 postnatal days) and exposed them a single foot shock of 1.2mA after 3 minutes of exploration of the training chamber. To test each animal in both familiar and novel contexts and account for potential artefacts caused by the order of context exposure, we counterbalanced both WT and KO groups for their order of context exposure. Hence, 24 hours post-training, half the mice (group1) went back to the original context, now familiar, and the other half (group2) to the novel context. Then the two groups were switched over: animals from group1 were exposed to the novel context and animals from group2 to the familiar (Figure 5.1A). After scoring of the freezing, we binned the data according to context and genotype independent from the order of context exposure. Unfortunately, we found the percentage of freezing to be very low in both genotype groups. However, we did see a significant difference in percentage of freezing of WT animals between the familiar and novel contexts (Figure 5.1B) (WT familiar = 21.91% ± 5.35%, WT novel = 11.24% ± 2.01%, KO familiar = 15.29% ± 3.94%, KO novel = 14.11% ± 3.70%).

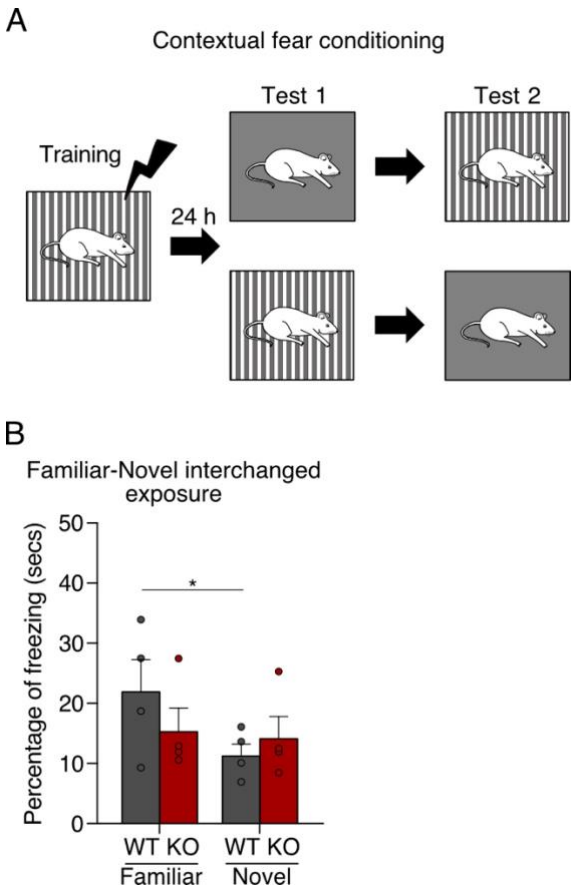

Figure 5.1 Post-training freezing rate of WT and *Fmr1* KO mice with single foot shock of 1.2mA

(**A**) Schematics of protocol for fear conditioning assay. (**B**) Freezing rate in seconds, manually scored, of *Fmr1* KO and WT mice exposed to both familiar and novel contexts (in counterbalanced order) 24 hours after shock (ANOVA context *p = 0.0480, genotype p = 0.7216; Sidak WT familiar vs WT novel *p = 0.0390; n = 4). Error bars = SEM. N = littermate/age-matched pairs.

Because of the small percentage of freezing rate of WT animals, we decided to repeat the experiment with a second cohort of mice and increase the number of foot shocks to three, 70 seconds apart one to the other. Again, data was pulled together independently from the order of context exposure.

Even with three separate shocks, the rate of freezing was still lower than expected, with about 30% freezing compared to a 50% or higher in the literature (Paradee et al. 1999; Ding et al. 2014) (Figure 5.2A) (WT familiar = 32.70% ± 4.60%, WT novel = 16.86% ± 7.07%, KO familiar = 23.24% ± 8.26%, KO novel = 12.61% ±

3.63%). Regardless of that, and despite the low number of total animals per experimental group, ANOVA statistical testing showed context significance, with both WT and KO mice showing a trend for context discrimination, although KO mice to less extent. Moreover, we found the KO animals to be maybe showing a small trend for decreased freezing rates in both contexts compared to WTs.

To confirm the absence of artefacts in the calculated freezing percentage due to exposure to a novel context, we further examined the data and split the mice according to their order of context exposure. We wondered whether the first context exposure could be having a significant influence on the freezing time in the second context exposure. To assess this, we split the data to compare all mice that were exposed to the familiar context first (Figure 5.2B) to all mice exposed to the novel context first (Figure 5.2C). We found the two graphs to show a similar trend, with mice freezing more in the familiar context and less in the novel (Figure 5.2B: WT familiar = 29.18% ± 10.12%, WT novel = 4.99% ± 3.52%, KO familiar = 21.85% ± 9.24%, KO novel = 9.97% ± 7.62%; Figure 5.2C: WT familiar = 36.22% ± 0.15%, WT novel = 28.74% ± 2.35%, KO familiar = 24.63% ± 17.89%, KO novel = 15.25% ± 2.64%). This result is consistent with the one of the original graphs with combined data of interleaved context exposure (Figure 5.2A).

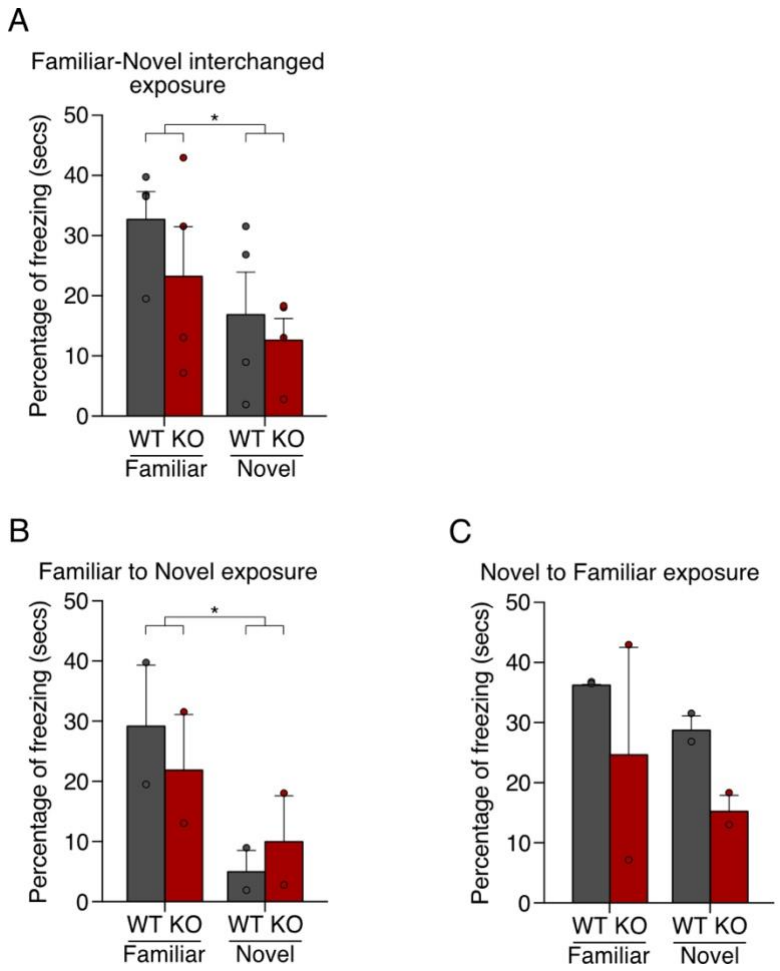

Figure 5.2 Post-training freezing rate of WT and *Fmr1* KO mice with 3 foot shocks of 1.2mA

(**A**) Graph showing freezing rate in seconds, manually scored, of *Fmr1* KO and WT mice exposed to both familiar and novel contexts (in interleaved, counterbalanced order) 24 hours after shock (ANOVA context *p = 0.0201, genotype p = 0.4047; Sidak WT familiar vs WT novel p = 0.0739; n = 4). (**B**) Percentage of freezing of *Fmr1* KO and WT mice exposed first to the familiar context, then to the novel (ANOVA context *p = 0.0337, genotype p = 0.9237; Sidak WT familiar vs WT novel p = 0.0731; n = 2). (**C**) Percentage of freezing of *Fmr1* KO and WT mice exposed first to the novel context, then to the familiar (ANOVA context p = 0.3880, genotype p = 0.3491; n = 2). Error bars = SEM. N = littermate/age-matched pairs.

Lastly, we wondered if the *Fmr1* KO mice would present an extinction phenotype (Dölen et al. 2007). To test for extinction, the day following the post-24-hour testing, we repeated the exact same paradigm: mice were exposed to both contexts following

the order of the previous day and their freezing rate was scored. No shock was given at any time point. We found the data to be quite variable, also due to the low number of animals per group, but it showed similar trend as the day before. Both WT and KO mice froze more in the familiar context versus the novel, and both presented with decreased freezing (by about 10%) compared to 24 hours earlier. We found no indication for an increased extinction phenotype in the *Fmr1* KO group (Figure 5.3) (WT familiar = 17.30% ± 2.57%, WT novel = 10.70% ± 5.21%, KO familiar = 11.07% ± 4.87%, KO novel = 4.40% ± 1.56%). A higher number of experimental animals per group would be required to reach statistically conclusive data.

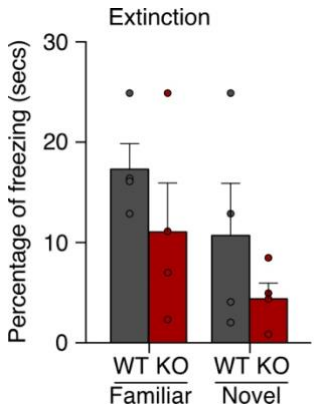

Figure 5.3 Testing for extinction: freezing rate 48 hours post-training of WT and *Fmr1* KO mice with three foot shocks of 1.2mA.
Graph showing freezing rate in seconds, manually scored, of *Fmr1* KO and WT mice exposed to both familiar and novel contexts (in variable, counterbalanced order) 48 hours after shock (ANOVA context p = 0.1562, genotype p = 0.1346; n = 4).

Overall, the average percentage of freezing seen with this assay was, in our opinion, quite low in both experimental groups, even with three foot shocks at 1.2 mA. In the literature, reported freezing levels of both *Fmr1* KO and WT mice are variable, with research groups obtaining freezing percentages from 30%, similarly to us, to 70%, with the administration of only one or two foot shocks (Table 5.1). In Table 5.1, we compared the protocol and results of these previous studies on fear conditioning experiments with *Fmr1* KO mice (Paradee et al. 1999; Ding et al. 2014; Dobkin et al. 2000; Spencer et al. 2006). Even accounting for the experimental differences of each

study, such as the age of the animals, the intensity and the number of the foot shocks administered, we were unable to identify one specific factor to influence the experimental outcome. Whereas some studies find a significantly reduced freezing rate of KO mice compared to WT, other do not.

Table 5.1 Details of literature fear conditioning studies

Paper:	**Species:**	**Age:**	**Background:**	**n. of foot shocks:**	**Freezing% familiar context**	**Freezing% novel context**
Paradee et al. 1999	Mouse, male	70-100 days old	C57Bl/6J	1 shock, 2secs, 0.35mA tone-paired	no cue: WT: 50% KO: 31%	Pre-cue: WT: 9% KO: 9% Cue: WT: 56% KO: 38%
Ding et al. 2014	Mouse, male and female	60-90 days old	C57Bl/6J	1 shock, 2secs, 0.7mA	WT: 70% KO: 30%	/ /
Dobkin et al. 2000	Mouse, male	120 days old	C57Bl/6J	1 shock, 2secs, 0.75mA, tone-paired	no cue: WT: 32% KO: 31%	Pre-cue: WT: 0% KO: 2% Cue: WT: 38% KO: 42%
Dobkin et al. 2000	Mouse, male	120 days old	FVB-129 hybrid	1 shock, 2secs, 0.75mA, tone-paired	no cue: WT: 30% KO: 22%	Pre-cue: WT: 17% KO: 11% Cue: WT: 40% KO: 51%
Spencer et al. 2006	Mouse, male	60-90 days old	C57Bl/6J	2 shocks, 2sec, 0.7mA, tone-paired	no cue: WT: 60% KO: 60%	Pre-cue freezing minus cue freezing: WT: 50% KO: 60%

Although we realised the *n* of experimental animals was low to draw definite conclusions, we thought 30% freezing was too low to yield robust results from a treatment study. Moreover, the low percentage of freezing found in WT animals could be preventing a phenotypic variance in the KOs to be visible, or else be significantly increasing the number of animals necessary for the phenotype to be detectable at later stages. To finalise a decision in regard to this dataset, Dr. Sang Seo from the Osterweil lab performed a power analysis to calculate the expected n of experimental animals necessary to obtain a sufficient statistical power to detect a genotypic variance (details of this analysis in page 66). Using the preliminary dataset discussed above, the power analysis showed a total n > 50 of animals per experimental group to be necessary to obtain a statistical power of 0.8 with p value threshold of 0.05. This is, n > 50 to reach 80% chance that a present change could be detected with 0.05 p value. With this information, and not wanting to increase either the power or number of electric foot shocks administered for both animal welfare and experimental reasons (the animals could develop generalized freezing), we decided to stop these experiments and look for a different phenotype in a second animal species, the rat.

5.3 Long-term memory paradigms for object displacement and novel object recognition in adult rats

As discussed in the introduction of the thesis, mice and rat species can now be genetically manipulated to produce models of disease, and research should take advantage from the availability of both species to investigate whether core disease deficits are conserved between them, and if successful treatment in one species is translated to the other. Moreover, *Fmr1* KO rats have been shown to present deficits in hippocampal-dependent forms of complex associative memory (Till et al. 2015). For these reasons, we investigated the performance of adult *Fmr1* full KO rats in long-term memory tasks associated with hippocampal function (see page 49 for details on full knock-out mutation).

For his PhD project thesis, the colleague Dr. Antonis Asiminas (from the research group of Dr. Emma Wood) conducted extensive and thorough investigations on short and long-term learning memory in WT and *Fmr1* KO adult rats. He tested for spontaneous recognition in non-associative memory tasks for novel object recognition (NOR) and spatial memory in object displacement (Object Displacement, OD), finding a long-term memory deficit in the performance of *Fmr1* KO adult rats on Long-Evans-Hooded (LEH) background strain (Asiminas 2017). We therefore decided to test for these same deficits in adult *Fmr1* KO rats on the same background strain.

The hippocampus has been widely associated with spatial memory, particularly the dorsal area for spatial learning in water maze (Moser et al. 1995; Moser et al. 1993), and with the formation and storage of context and object memory. With primary role of the CA1 hippocampal area, the hippocampus has also been implicated in both the acquisition (Assini et al. 2009) and retrieval (Gilbert & Kesner 2004; Haettig et al. 2011) of object-location memory, making the OD assay a new mean of measuring hippocampal deficits in FXS. As for NOR, data is more controversial, and brain regions other than the hippocampus appear to be more involved. Long-term novel object recognition was found blocked if protein synthesis was inhibited by anisomycin infusions in either perirhinal or insular cortices, but not in the hippocampus (Balderas et al. 2008). Numerous other studies report conflicting results, with hippocampal lesions through different means sometimes leading to long-term memory deficits in novel-familiar discrimination and sometimes not (Mumby 2001). Hippocampal lesions in rats did not impair short-term NOR but did affect long-term NOR (Clark et al. 2000). Similarly, infusion of the anaesthetic lidocaine in the CA1 region of dorsal hippocampus prior to sample phase impaired long-term and not short-term NOR in mice (Hammond et al. 2004). Other studies argue that only major (>75%) damages to the hippocampal structure can result in a long-term deficit (Broadbent et al. 2004).

Overall, it appears that the hippocampus might be involved in the process at specific time points and assay-related scenarios (such as object type, sample-test phase interval, extent of tissue lesion) but without necessary requirement for normal performance in novelty-preference paradigm (Dere et al. 2007). The studies with negative results addressing the involvement of hippocampus in NOR outnumber the

ones with positive data (Warburton & Brown 2015). Differently, the perirhinal cortex has been extensively shown to have a primary role in NOR memory (Warburton & Brown 2015; Barker et al. 2007; Balderas et al. 2008).

5.3.1 Results for long-term object displacement (OD) memory task in adult *Fmr1* KO LEH rats

First, we tested for long-term memory of OD with spontaneous exploration. Both OD and NOR paradigms are based on the assumption that normal rats will show a preference for novelty versus familiarity. The arena used for the OD assay had transparent walls decorated with several three-dimensional coloured cues attached to their surface externally or pending from the top, out of physical reach (Figure 5.4C). The walls were so rich of distinctive features for the animals to associate the spatial location of the two identical objects later inserted with the cues around them. On the training day, called sample phase, the animals explored the arena with the two identical objects for 3 minutes x 3 times, and 24 hours later they explored the same arena with one of the two objects in a different position in respect to the previous day (Figure 5.4A-B). The interaction of each animal with each object is scored and an index of discrimination (DI) calculated for positive interaction with the object in the novel location and negative interaction with the object in the old location. Because the animals are let explore the arena for an overall of 3 minutes, the data was binned in cumulative minutes to investigate the DI for old-new location at 1, 2 and 3 minutes of exploration. While WTs showed significance preference for novelty at 60 seconds of exploration, the KOs did at 120 and 180 seconds (Figure 5.5A) (WT DI 1min = 0.22 ± 0.10, KO DI 1min = 0.23 ± 0.11, WT DI 2min = 0.10 ± 0.11, KO DI 2min = 0.21 ± 0.09, WT DI 3min = 0.11 ± 0.09, KO DI 3min = 0.26 ± 0.06).

Hence, contrarily from what previously found by Dr. Asiminas, WT and *Fmr1* KO rats were found equally able to significantly discriminate between the old and new object location at various time points of the test phase. However, these results can be misleading. It should be noted that a good DI generally expected from WT adult rats should sit at around 0.3-0.4/1.0 (Till et al. 2015; Asiminas 2017). Instead, these WT

rats showed discrimination index values of 0.1-0.2/1.0. Therefore, the inability of us to see a deficit in the performance of the KO rats versus WTs could be due to the below-average performance of the WT animals. Finally, the length of object interaction (seconds) was examined and found consistent between groups during both sample and probing phases (Figure 5.5B-C).

Because we used more than a single pair of objects to make sure the results obtained were consistent with different objects (Figure 5.4B), we can confidently say the inconsistency of the phenotype between laboratories is given either by the experimental room, the experimenter or the arena used, or all. These are important and common difficulties in the replicability of behavioural phenotypes in complex cognitive tasks with elaborated and often original equipment.

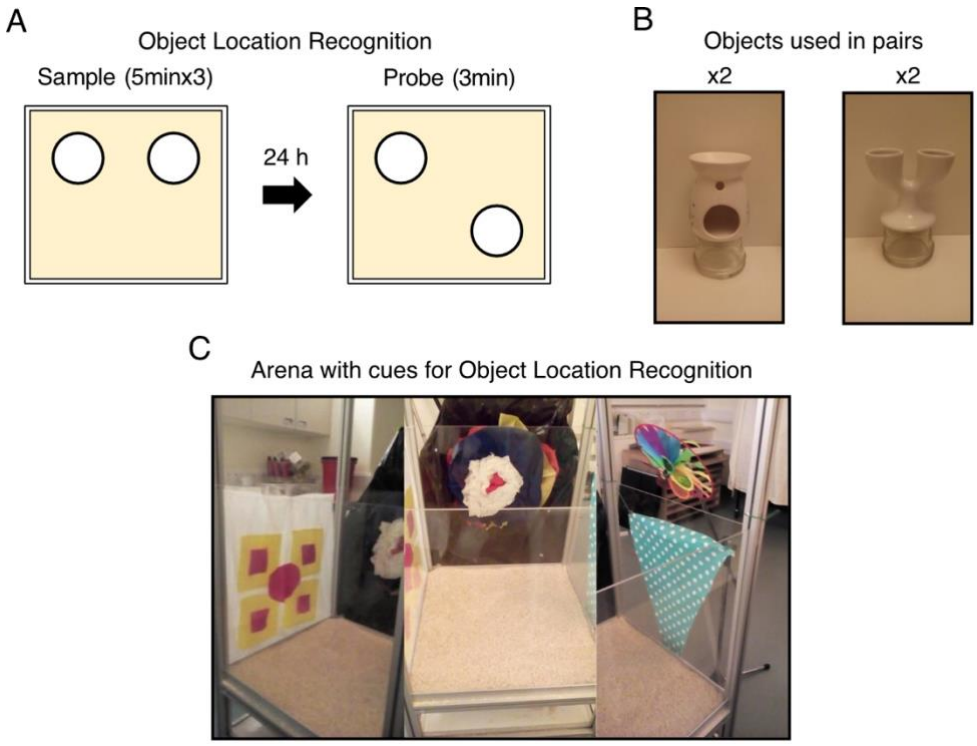

Figure 5.4 Equipment and assay schematic for object displacement task.
(**A**) Schematic of long-term OD memory task. (**B**) Photograph of the two different objects either used in pairs for the assay, to make sure the results obtained to be consistent with different object pairs. (**C**) Photograph of the arena with all cues.

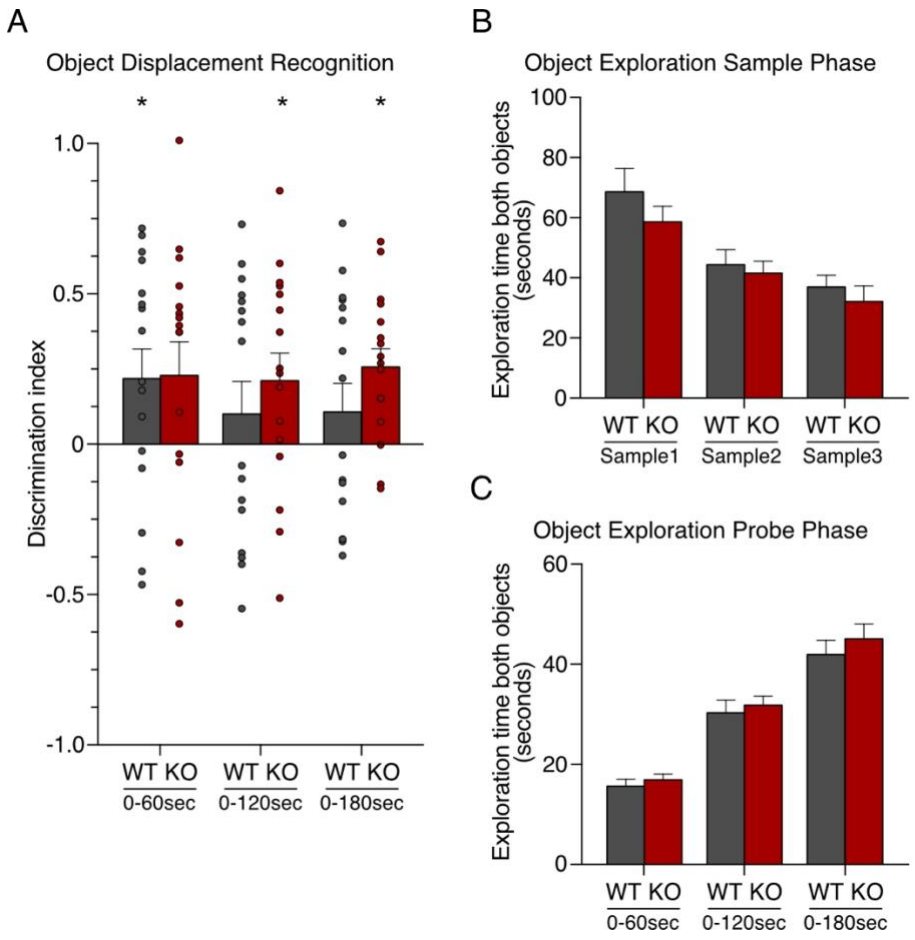

Figure 5.5 *Fmr1* KO rats do not present long-term memory deficits in object displacement task.

(**A**) Discrimination index of object in old location versus object in novel location. One sample t-test with theoretical mean of 0.0 (WT 1min *p = 0.0429, KO 1min p = 0.0570, WT 2min p = 0.3652, KO 2min *p = 0.0356, WT 3min p = 0.2743, KO 3min *p = 0.0008; n = 16). (**B**) Total exploration time of both objects in the three sample phases (WT sample1 = 68.60 ± 7.75, KO sample1= 58.59 ± 5.21, WT sample2 = 44.37 ± 5.02, KO sample2 = 41.51 ± 4.00, WT sample3= 36.93 ± 3.91, KO sample3= 32.10 ± 5.18; unpaired t-tests to compare each sample session between genotypes: WT vs KO sample1 p = 0.2924, WT vs KO sample2 p = 0.6594. WT vs KO sample3 p = 0.4629. (**C**) Total exploration time of both objects in the probe phase, in seconds (WT 1min = 15.64 ± 1.39, KO 1min = 16.91 ± 1.16, WT 2min = 30.29 ± 2.53, KO 2min = 31.80 ± 1.80, WT 3min = 41.93 ± 2.84, KO 3min = 45.03 ± 2.99; unpaired t test: WT vs KO 1min p = 0.4899, WT vs KO 2min p = 0.6310, WT vs KO 3min p = 0.4584).

5.3.2 Results for long-term novel object recognition (NOR) memory task in adult *Fmr1* KO LEH rats

We then tested the same cohort of rats plus a second new one for long-term memory in NOR. The arena used for this assay had no spatial cues. On the day of the sample phase, the animals explored the arena with two identical objects for 5 minutes. 24 hours later they were brought back to the same arena, where one of the two objects had been replaced with a novel one (Figure 5.6).

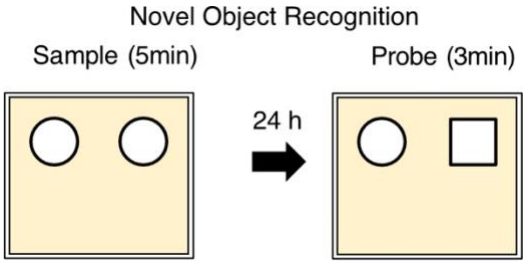

Figure 5.6 Schematic of long-term NOR memory task.

Because this task requires the presence of two different objects at the same time in the arena, and assesses memory for novelty versus familiarity via the one and the other, we wanted to ascertain the absence of confounds in the objects themselves. That includes one object being substantially bigger or smaller than the other, or visibly more interesting, such as richer in texture and structural elements. Hence, numerous pairs of non-identical, different objects were trialled to test for recognition of novelty. The objects were selected to be not too different nor too similar one to the other, and most importantly to be attractive to the animals to similar extents (Figure 5.7). The rats were counterbalanced for genotype and pair of objects.

After testing several different object pairs (Figure 5.7A-D shows some examples), we identified which objects would highly influence the outcome of the experiments. Both WT and KO rats showed consistent preference for one object over another at more than one instance, thus not carrying out the assay without unwanted influences (Figure 5.7D) (Pair D: WT DI 1min = 0.18 ± 0.10, KO DI 1min = 0.26 ± 0.14, WT DI 2min = 0.07 ± 0.12, KO DI 2min = 0.21 ± 0.12, WT DI 3min = 0.14 ±

0.10, KO DI 3min = 0.17 ± 0.12). Furthermore, we excluded the first pair tested because WT rats had difficulty to distinguish between the two test objects, too similar one to the other (Figures 5.7A) (Pair A: WT DI 1min = 0.27 ± 0.13, KO DI 1min = 0.19 ± 0.09, WT DI 2min = 0.19 ± 0.15, KO DI 2min = 0.13 ± 0.10, WT DI 3min = 0.07 ± 0.12, KO DI 3min = 0.12 ± 0.10). Only two pairs of different objects over a total of seven were selected based on optimal performance of WT rats and absence of preference between objects (Figure 5.7B-C) (Pair B: WT DI 1min = 0.50 ± 0.10, KO DI 1min = 0.24 ± 0.14, WT DI 2min = 0.34 ± 0.10, KO DI 2min = 0.25 ± 0.10, WT DI 3min = 0.16 ± 0.10, KO DI 3min = 0.17 ± 0.11. Pair C: WT DI 1min = 0.38 ± 0.08, KO DI 1min = 0.13 ± 0.10, WT DI 2min = 0.26 ± 0.05, KO DI 2min = 0.05 ± 0.08, WT DI 3min = 0.24 ± 0.05, KO DI 3min = 0.05 ± 0.09).

By pulling data from these two selected pairs together, we show both WT and KO groups to significantly discriminate above chances between novel and familiar objects, but we also find the KO rats to reach an overall lower DI, having a deficit in their discrimination for novelty compared to WT mates (Figure 5.8A) (WT DI 1min = 0.44 ± 0.07, KO DI 1min = 0.19 ± 0.09, WT DI 2min = 0.30 ± 0.05, KO DI 2min = 0.15 ± 0.07, WT DI 3min = 0.20 ± 0.06, KO DI 3min = 0.11 ± 0.07). Again, the total exploration time for both objects in both sample and probe phases was similar between genotypes (Figure 5.8B-C).

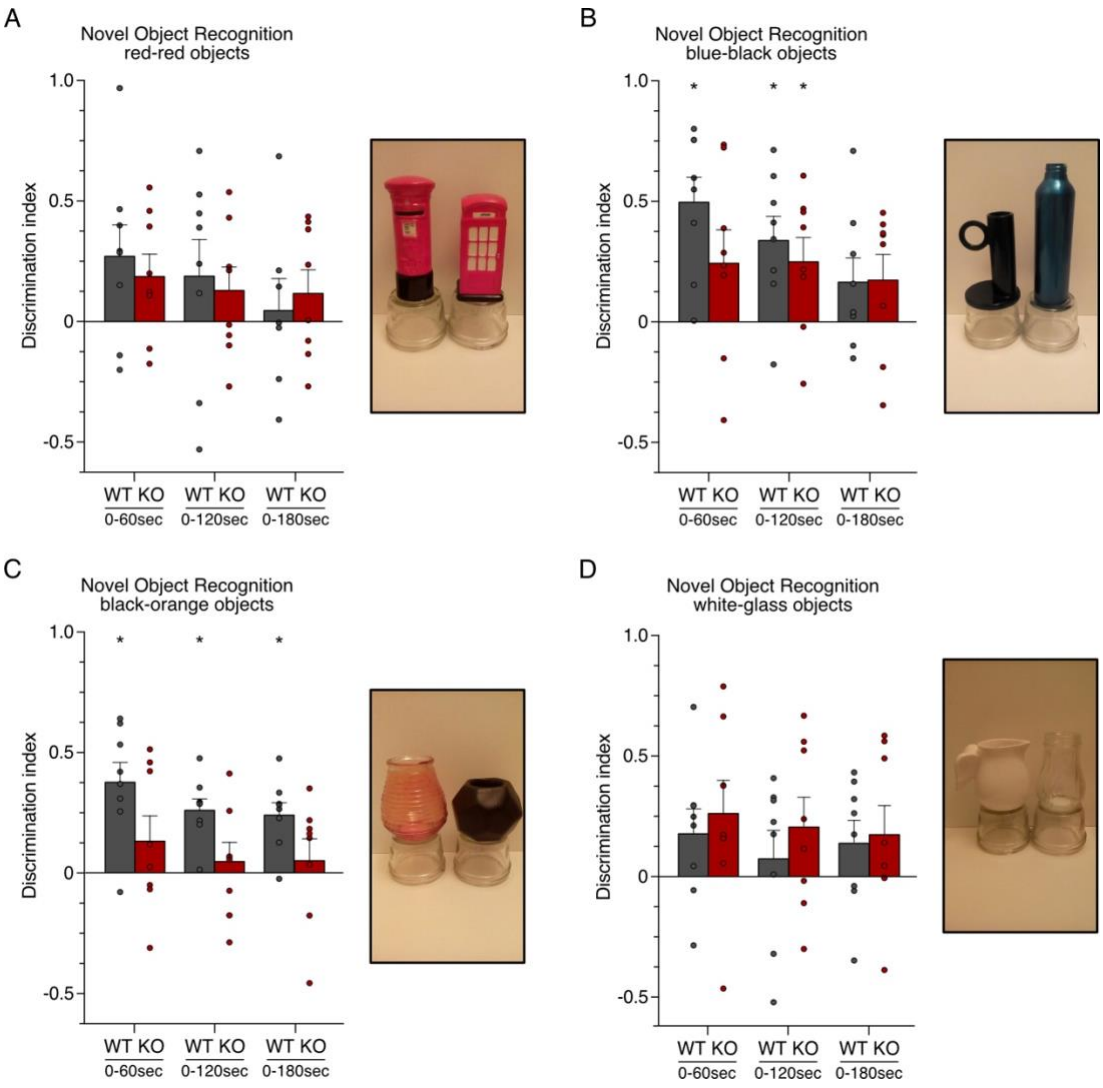

Figure 5.7 Testing for different object pairs in long-term NOR task
(**A**) Discrimination index object pair A. One sample t-test with theoretical mean of 0.0, none significant (n = 8). (**B**) DI object pair B. One sample t-test (WT 1min *p = 0.0020, KO 1min p = 0.1227, WT 2min *p = 0.0115, KO 2min *p = 0.0432; n = 8). (**C**) DI object pair C. One sample t-test (WT 1min *p = 0.0026, KO 1min p = 0.2517, WT 2min *p = 0.0009, KO 2min p = 0.5760, WT 3min *p = 0.0025, KO 3min p = 0.6006; n = 8). (**D**) DI object pair D. One sample t-test (WT 1min p = 0.1281, KO 1min p = 0.0975, WT 2min p = 0.5515, KO 2min p = 0.1399, WT 3min p = 0.1895, KO 3min p = 0.1926; n = 8).

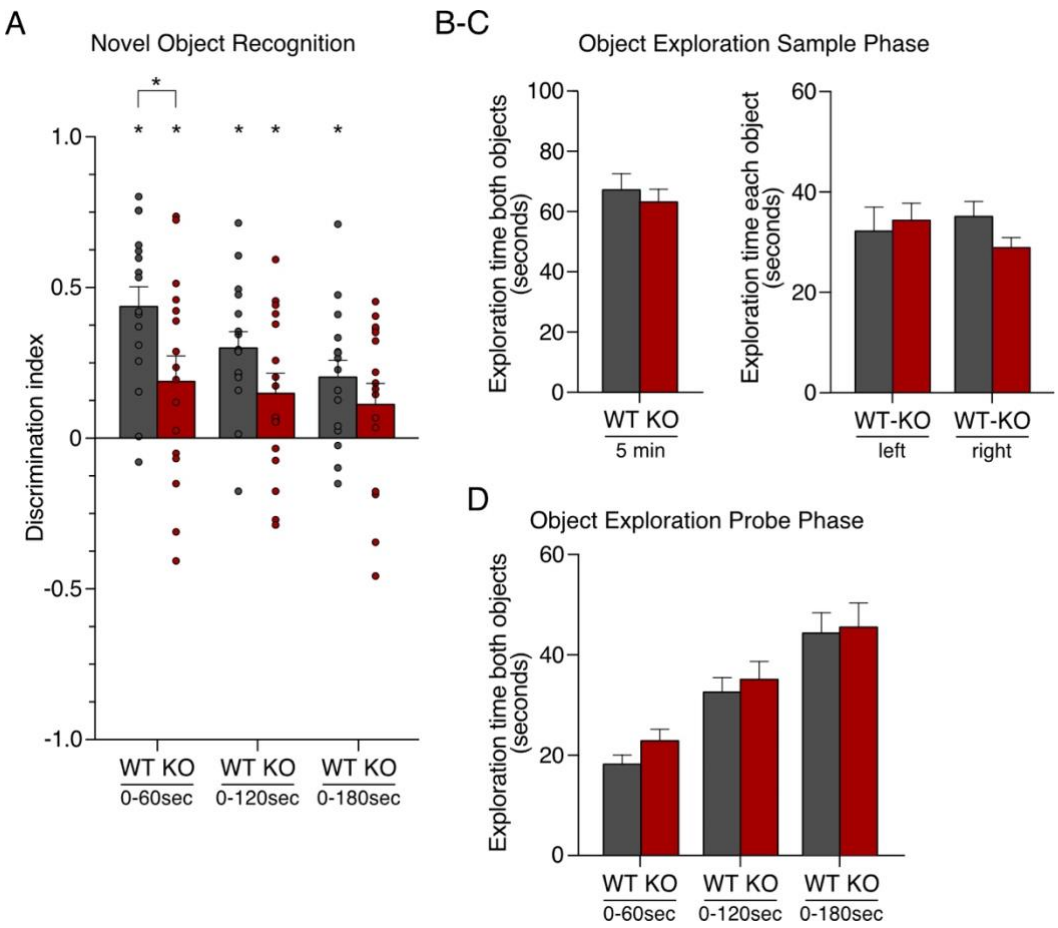

Figure 5.8 *Fmr1* KO rats present a long-term memory deficit for novel object recognition with selected, well-balanced object pairs.

(**A**) Discrimination index of novel object versus familiar. One sample t-test with theoretical mean of 0.0 (WT 1min *p = <0.0001, KO 1min *p = 0.0442, WT 2min *p = <0.0001, KO 2min *p = 0.0445, WT 3min *p = 0.0026, KO 3min p = 0.1303; unpaired t test: WT vs KO 1min *p = 0.0281, WT vs KO 2min p = 0.0917, WT vs KO 3min p = 0.3204; n = 16). (**B**) Total exploration time of both objects in the five-minute sample phase (WT = 67.26 ± 5.40, KO = 63.25 ± 4.22; unpaired t-test to compare sample phase exploration time between genotypes: p = 0.5630). (**C**) Total exploration time for left and right objects respectively in the 5minute sample phase (WT left = 32.17 ± 4.79, WT right = 35.09 ± 3.01, KO left = 34.35 ± 3.41, KO right = 28.91 ± 1.98; One-way ANOVA, p = 0.5876). (**D**) Total exploration time of both objects in the 3minute probe phase, in seconds (WT 1min = 18.19 ± 1.83, KO 1min = 22.84 ± 2.29, WT 2min = 32.55 ± 2.87, KO 2min = 35.08 ± 3.56, WT 3min = 44.44 ± 4.08, KO 3min = 45.64 ± 4.81; unpaired t test: WT vs KO 1min p = 0.1233, WT vs KO 2min p = 0.5835, WT vs KO 3min p = 0.8503).

5.4 Discussion

In this chapter, we tested adult mice and rats for phenotyping variance in one assay for associative-learning and two for non-associative memory respectively. Having already a gold-standard measure for therapeutic efficacy of treatments in our AGS assay in juvenile *Fmr1* KO mice, we looked for some memory-related phenotypes at a different time of development: adulthood.

First, we tested >100 post-natal days old *Fmr1* KO and WT mice in the associative-learning paradigm of contextual fear conditioning. In this assay, mice are given a mild foot-shock in a specific, defined context and tested 24 hours later for fear recall via freezing behaviour in both the same context and a novel one. Even administering the animals three separate moderately high foot-shocks, the WT mice freezing score never surpassed a 30% average score, which we thought was not strong enough to look for deficits in the KO siblings. Indeed, assuming for the presence of a genotype difference in freezing levels, a power analysis of the preliminary data showed we would have needed at least 50 animals per experimental group to see a genotype difference significantly. For this reason, we moved to a different species, the rat.

As previously mentioned, the rat is a popular animal in behavioural neuroscience studies, as it is less susceptible to stress with human handling and is a highly social animal. Hence, it is an excellent model for neuropsychiatric and neurodevelopmental disorders such as ASD. Finding a behavioural phenotype different from AGS in the adult *Fmr1* KO rat would enable to test the promising M_4 PAM in a novel species, at a different age, and on a phenotype affecting brain circuits not previously tested. We used *Fmr1* KO and WT rats with LEH background, at adult ages of >100 post-natal days. We looked into two long-term memory paradigms with spontaneous exploration: the object displacement task and the novel object recognition task.

With the OD memory assay, we found the WT rats unable to strongly and consistently discriminate between the old and new location of the moved object. They displayed a significant DI for novel location only within the first minute of exploration of the three-minute probe phase, with overall low quality of performance compared to

literature data (Till et al. 2015; Asiminas 2017). We therefore concluded not possible to investigate potential deficits in the performance of KO rats in this assay.

We hence tried the NOR task. After selecting two well-balanced pairs of different objects, we found consistent results. As in the literature (Till et la. 2015), WT rats performed better at NOR than OD, with a significantly above chances average DI of 0.4-0.5/1.0 across the three minutes of probe phase. However, we noticed their performance to be optimal within the first minute of exploration, which will be our primary focus in the follow-up experiments later discussed. The *Fmr1* KO rats were also able to significantly perform above chances, but with lower DI compared to WTs, therefore presenting a deficit in novelty preference.

This deficit could be correlated to known phenotypes of the FXS visual cortex. In the neurons of the visual cortex, FXS patients and *Fmr1* KO mice have been shown to present immature dendritic spines, with also higher densities compared to healthy controls. This results in abnormal brain structure and signalling, and its associated with the attentional deficits and impairments in working memory of FXS patients (Kogan et al. 2004), and the hyperexcitability of the visual cortex in *Fmr1* KO mice (Osterweil et al. 2013). Although there are no studies to show a direct association between the increased excitability of the visual cortex and the NOR deficit, it would be interesting to see if treatment of the former is necessary for amelioration of the latter.

In conclusion of these findings, we selected the NOR assay for the testing of M_4 PAM VU0152100 compound. The data is discussed in the following chapter.

Chapter 6

Investigating the effects of M_4 PAM treatment in *Fmr1* KO rat and mice at adult ages

6.1 Introduction

As discussed in Chapter 4, *in vitro* treatment of hippocampal slices with the M_4-specific PAM (VU0152100) normalizes both the elevated protein synthesis and the exaggerated mGluR-LTD phenotypes of juvenile *Fmr1* KO mice, and *in vivo* administration of the same compound rescues their AGS incidence and severity (Thomson et al. 2017). Following up these promising results, we were interested in investigating the potential of this treatment at later ages and in learning and memory tasks. Hence, in Chapter 5 we investigated long-term memory phenotypes in both adult *Fmr1* KO mice and rat models. We found that *Fmr1* KO rats present a deficit in novelty preference, and hence decided to test the effects of M_4 PAM treatment in this phenotype.

Acetylcholine is known to be required for memory formation in hippocampus and cortex, but the specific role of each muscarinic receptor subtype in memory acquisition, consolidation and retrieval stages is controversial (Leaderbrand et al. 2016). The processes of learning have been associated with increased levels of acetylcholine in the cortex and increased activity of cholinergic neurons. In a test for operant behaviour with food reward, extracellular cortical and hippocampal acetylcholine levels were found to increase during the learning acquisition of reward-driven responses, and not during recall (Orsetti et al. 1996). Similarly, acetylcholine levels were found to increase in the insular cortex at the presentation of novel taste stimuli, and decrease concomitantly with increased presentation of the same stimulus (Miranda et al. 2000). This evidence of increased release of acetylcholine immediately after learning in the cortex and hippocampus supports the hypothesis that acetylcholine is involved in long-term memory formation and consolidation. Indeed, there is evidence for a specific role of muscarinic receptors in memory consolidation. Post-sampling injection of either a general muscarinic acetylcholine receptor agonist or a cholinesterase inhibitor via i.p. injection supported memory consolidation in a task for long-term passive avoidance in mice (Baratti et al. 1979). Post-training i.p. injections of a presynaptic muscarinic receptor antagonist, hypothesized to work by inhibiting muscarinic autoreceptors, was also found to improve memory retention in a long-term

inhibitory avoidance test in mice (Kopf et al. 1998). Rats injected with the muscarinic inhibitor scopolamine immediately after training in a task for odour-reward associative learning were found profoundly impaired in their performance 24 hours later (Carballo-Márquez et al. 2007).

Overall, several studies demonstrate the involvement of amydgala, striatum, dorsal hippocampus and cortex in memory consolidation via muscarinic signalling in different memory paradigms (Power et al. 2003). Some of these prove the specific involvement of M_1, but very little is known regarding the role of M_4 in these processes.

In sight of these findings, we decided to investigate the role of M_4 in memory consolidation. In the first part of this chapter we describe the effects of M_4 PAM treatment in the deficit for novelty preference of adult *Fmr1* KO rats. Then we move on investigating whether M_4 PAM stimulation of adult mouse hippocampal slices can rescue the elevated hippocampal protein synthesis, which we know from unpublished work to persist into adulthood and be conserved in the rat model (Asiminas et al. 2019). Furthermore, we investigate whether M_4 expression levels are increased in adult *Fmr1* KO rats as found in juvenile *Fmr1* KO mice by Thomson et al. 2017.

6.2 Results

6.2.1 M_4 PAM treatment rescues the age-dependent long-term memory impairment of *Fmr1* KO rats in novel object recognition task

The NOR assay requires exposure of the animals to two different objects at the same time, one is novel and the other is familiar. For the assay to be reliable and give an accurate measure of the discrimination for novelty of the rats, it is important for the objects to present a similar level of inherent interest for the animals. Hence, after investigating several object pairs, we selected the two pairs of objects that yielded the best discriminatory performance of WT controls, and we found that, in comparison, their KO age-matched cage mates presented a deficit in discrimination for novelty (see Chapter 5).

A few weeks post-training and testing, we re-tested this original cohort of 32 rats on NOR, this time with treatment of either 56 mg/kg M_4 PAM or vehicle. The animals were divided into smaller cohorts as previously done, and the objects pairs used in the precedent rounds switched over, so that whatever selected pair of objects they had already seen, they would not see a second time. The first cohort of 16 rats that did a NOR trial with the selected pair-B (see Figure 5.7), did this final round with treatment with the object pair-C, and vice versa. To test the effects of the M_4 PAM on memory consolidation and not acquisition, the animals were injected with either PAM or vehicle immediately after the 5-minute sample phase. 24 hours later they were tested for recognition of novelty. This batch of animals had an age range of 120-140 postnatal days.

Because in the previous testing of NOR the WT rats showed consistent optimal performance at the 60-seconds time point of the probe phase, here we show only the results obtained at the end of the first minute of exploration. However, it should be noted that all three minutes of probe phase were analysed, and no conclusive differences were found between them.

Unexpectedly, instead of showing a deficit in their performance, the KO + vehicle rats showed a full impairment, being completely unable to discriminate between the novel and familiar object. The WT rats of both vehicle and treated groups showed very promising levels of discrimination for novelty, although not reaching an above chances significance due to the low number of animals per group, n = 8. The KO + M_4 PAM rats were fully rescued from the impairment, and were the only experimental group to significantly discriminate above chances (Figure 6.1A) (WT veh = 0.27 ± 0.16, KO veh = 0.07 ± 0.09, WT PAM = 0.27 ± 0.13, KO PAM = 0.30 ± 0.10). For more explicative graphing of the data, we show here not only the DI of the animals, but also the seconds of exploration per single object, novel versus familiar (Figure 6.1B) (WT veh, novel = 9.72 ± 1.82, WT veh, familiar = 5.22 ± 1.09, WT PAM, novel = 10.85 ± 1.18, WT PAM, familiar = 6.91 ± 1.74, KO veh, novel = 10.54 ± 1.00, KO veh, familiar = 9.58 ± 1.59, KO PAM, novel = 10.99 ± 1.45, KO PAM, familiar = 5.94 ± 0.98). To check that the treatment was not affecting the exploratory

activity of any animal group, we also measured the total seconds of exploration in the post-treatment probe phase, finding no significant difference between groups (Figure 6.1C) (WT veh = 14.97 ± 1.38, WT PAM = 17.76 ± 2.02, KO veh = 20.12 ± 1.77, KO PAM = 16.92 ± 1.85).

To increase the n of animals per group, we assembled a new cohort of 32 rats (in groups of four animals per cage and genotype balanced) and repeated the experiment. To make sure the animals received a similar pre-test experience, in groups of 16 the animals went through either two or three rounds of NOR with objects pairs not selected for the final NOR + treatment test. After few weeks of break from that experience, as done with the previous cohort, this second batch of 32 rats went through a NOR test with M_4 PAM or vehicle treatment post-sample phase. This test was conducted with the same two objects pairs used for the previous cohort.

Surprisingly, we found that by adding the data acquired with this second cohort to the original n = 8, the impairment in novel discrimination previously seen in *Fmr1* KO rats was no longer present. All four experimental groups were found able to significantly discriminate above chances between novel and familiar objects (Figure 6.2A-B) (DI: WT veh = 0.28 ± 0.09, KO veh = 0.23 ± 0.07, WT PAM = 0.28 ± 0.07, KO PAM = 0.29 ± 0.07; seconds of exploration: WT veh, novel = 10.12 ± 0.96, WT veh, familiar = 5.68 ± 0.73, WT PAM, novel = 10.77 ± 0.80, WT PAM, familiar = 6.46 ± 0.98, KO veh, novel = 11.85 ± 0.93, KO veh, familiar = 7.97 ± 1.07, KO PAM, novel = 10.89 ± 1.09, KO PAM, familiar = 6.37 ± 0.83). No differences in their total seconds of exploration were found (Figure 6.2C) (WT veh = 15.82 ± 0.85, WT PAM = 17.22 ± 1.29, KO veh = 19.82 ± 1.44, KO PAM = 17.25 ± 1.68).

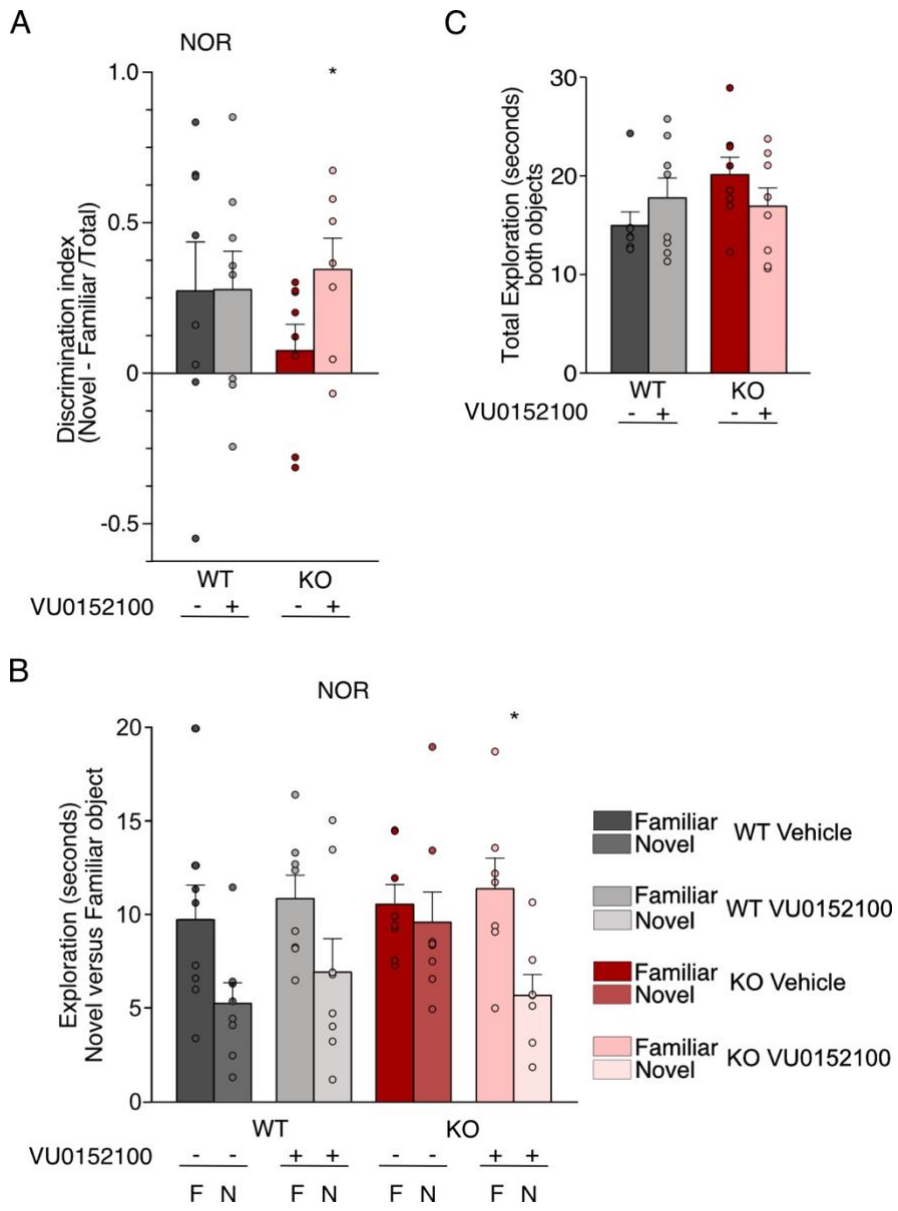

Figure 6.1 First batch: *Fmr1* KO rats present a long-term memory impairment for novel object recognition and are fully rescued with M_4 PAM treatment (>120 postnatal days).

(**A**) Discrimination index of novel object versus familiar of four experimental groups. One sample t-test with theoretical mean of 0.0 (WT veh p = 0.1392, WT PAM p = 0.0672, KO veh p = 0.4264, KO PAM *p = 0.0162; unpaired t test: WT vs KO PAM p = 0.0659, n = 8). (**B**) Total seconds of exploration of novel vs familiar object per experimental group in the probe/test phase (Paired t test, KO PAM familiar vs novel *p = 0.0171). (**C**) Total exploration time for both objects in the first minute of probe phase (One-way ANOVA, p = 0.2490, n = 8).

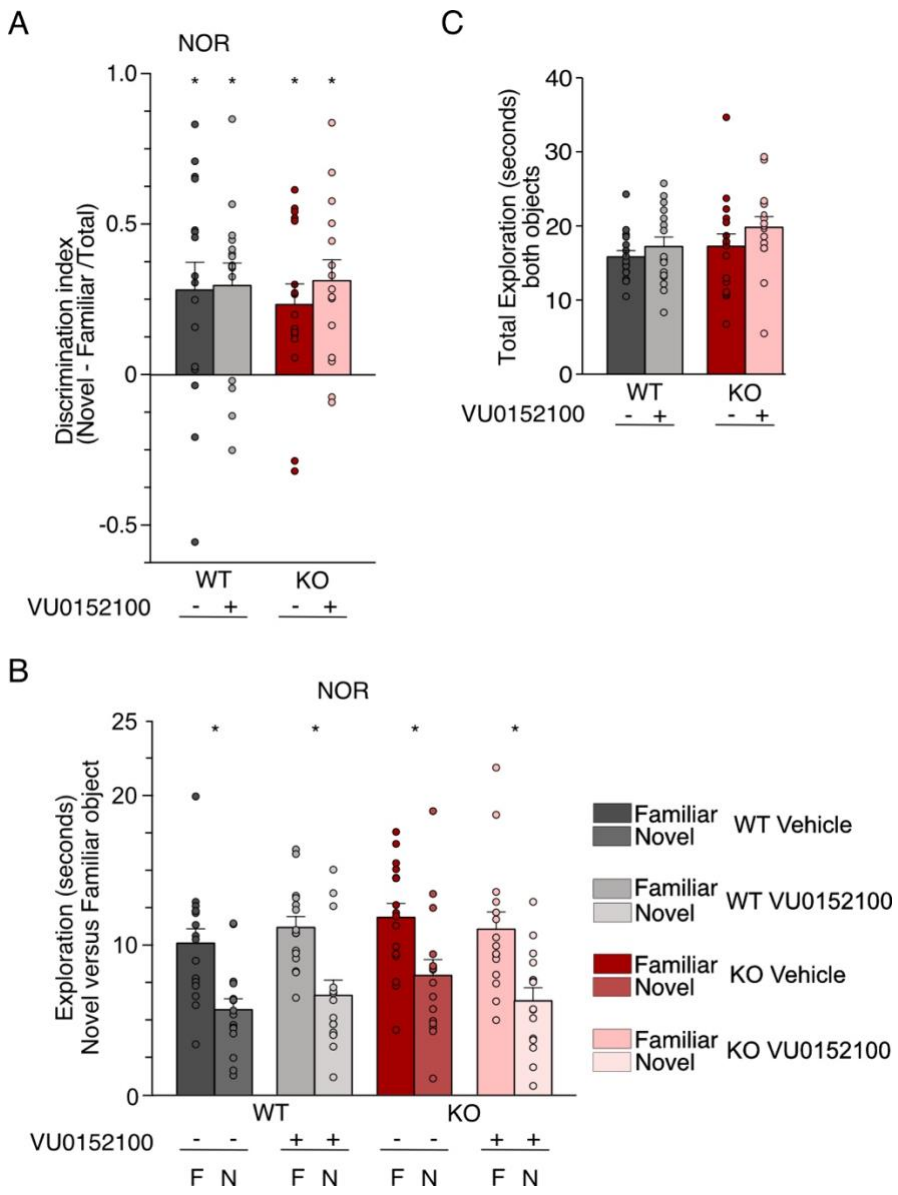

Figure 6.2 First + second batch: *Fmr1* KO rats no longer present a long-term memory impairment for novel object recognition.

(**A**) Discrimination index of novel object versus familiar of four experimental groups. One sample t-test with theoretical mean of 0.0 (WT veh *p = 0.0082, WT PAM *p = 0.0014, KO veh *p = 0.0042, KO PAM *p = 0.0005; unpaired t test: WT vs KO veh p = 0.6772, KO veh vs KO PAM p = 0.4211, n = 16). (**B**) Total seconds of exploration of novel vs familiar object per experimental group in the probe phase (Paired t test, WT veh familiar vs novel *p = 0.0087, WT PAM familiar vs novel *p = 0.0037, KO veh familiar vs novel *p = 0.0139, KO PAM familiar vs novel *p = 0.0003) (**C**) Total exploration time for both objects in the first minute of probe phase (One-way ANOVA, p = 0.2164, n = 16).

We hence decided to separate the data of the two cohorts and analysed the second cohort on its own. By doing so, we found a striking difference in the DI of the *Fmr1* KO rats of the two groups. Whereas the first cohort showed the *Fmr1* KO rats to have impaired discrimination and PAM treatment to rescue it (Figure 6.1A), the second cohort showed the *Fmr1* KOs to significantly discriminate above chances (Figure 6.3A-B), not differently from the WT controls (DI: WT veh = 0.29 ± 0.10, KO veh = 0.39 ± 0.07, WT PAM = 0.29 ± 0.07, KO PAM = 0.28 ± 0.10; seconds of exploration: WT veh, novel = 10.53 ± 0.77, WT veh, familiar = 6.14 ± 1.01, WT PAM, novel = 10.69 ± 1.16, WT PAM, familiar = 6.00 ± 1.03, KO veh, novel = 13.17 ± 1.49, KO veh, familiar = 6.35 ± 1.27, KO PAM, novel = 10.79 ± 1.74, KO PAM, familiar = 6.80 ± 1.38).

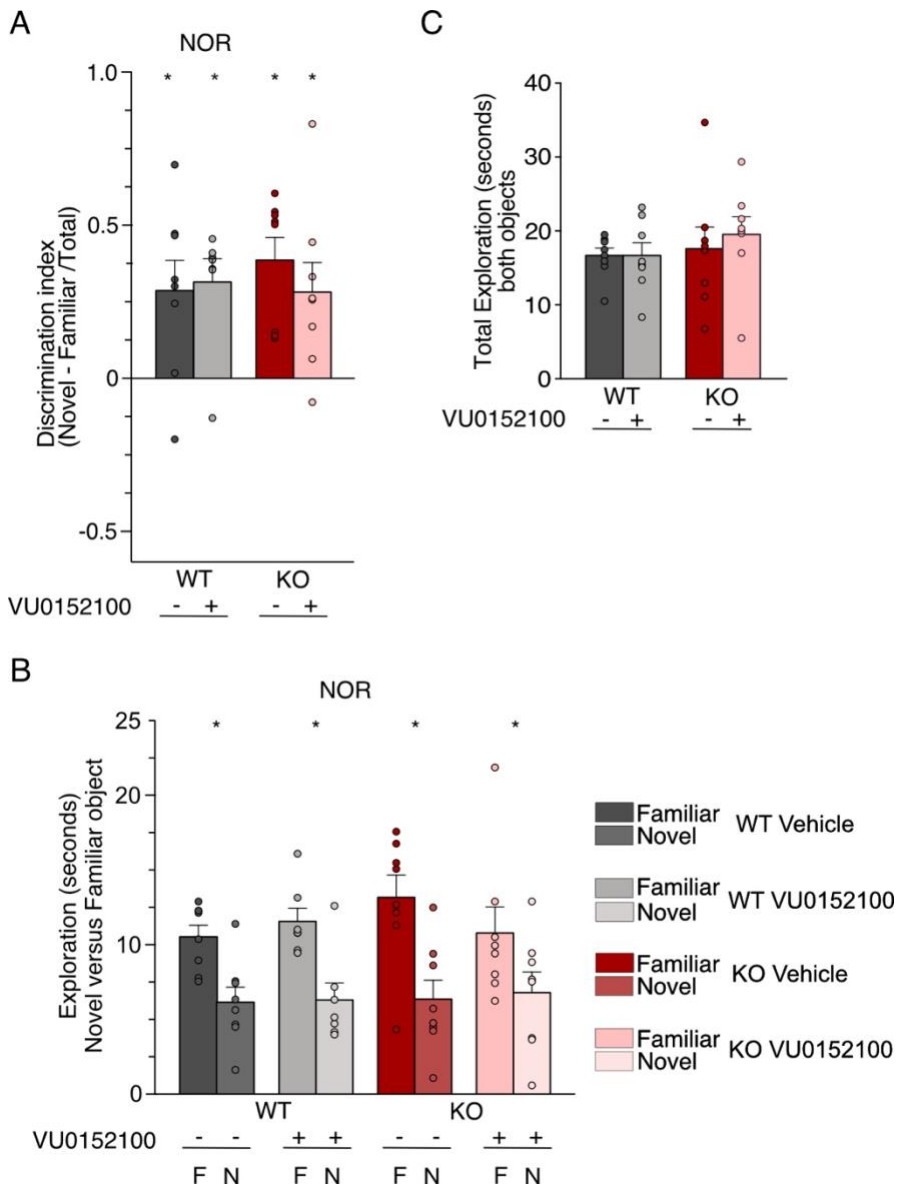

Figure 6.3 Second batch only: *Fmr1* KO rats no longer present a long-term memory impairment for novel object recognition (<100 postnatal days).
(**A**) Discrimination index of novel object versus familiar of four experimental groups. One sample t-test with theoretical mean of 0.0 (WT veh *p = 0.0231, WT PAM *p = 0.0060, KO veh *p = 0.0012, KO PAM *p = 0.0224; unpaired t test: WT vs KO veh p = 0.4349, KO veh vs KO PAM p = 0.4046, n = 8). (**B**) Total seconds of exploration of novel vs familiar object per experimental group in the probe phase (Paired t test, WT veh familiar vs novel *p = 0.0210, WT PAM familiar vs novel *p = 0.0106, KO veh familiar vs novel *p = 0.0018, KO PAM familiar vs novel *p = 0.0093). (**C**) Total exploration time for both objects in the first minute of probe phase (WT veh = 16.67 ± 1.01, WT PAM = 16.68 ± 1.72, KO veh = 19.52 ± 2.39, KO PAM = 17.59 ± 2.93; One-way ANOVA, p = 0.7588, n = 8).

After in depth analysis of the data from the two cohorts, we hypothesized that the difference in discrimination between the first 16 and last 16 *Fmr1* KO rats to be due to their different average ages. The overall cost of running a NOR recognition assay with M_4 PAM treatment is high, the rats need to grow to about three months of age, and the VU0152100 compound is expensive, with a single vial of 25 mg costing about £156. We had to inject 56 mg/kg of PAM to half those rats, which in the first cohort weighted between 700 grams and 1 kg each. That is why we decided to start some weeks of age earlier with the second cohort. We thought that, if the rats had reached adulthood, a wide age range of 70-140 days would not matter significantly on the phenotype, but it would on the weight of the animals. The second cohort of animals had therefore an average age range of 70-90 postnatal days. Unfortunately, our assumption proved wrong.

When we went back to confront the performance of the two cohorts in their first experience of the NOR assay, without M_4 PAM treatment, we confirmed that the performance of the *Fmr1* KO rats differed from the beginning. Their difference in performance was not due to treatment or vehicle administration (Figure 6.4A-B) (rats <p100: WT DI 1min = 0.33 ± 0.08, KO DI 1min = 0.28 ± 0.09, WT DI 2min = 0.29 ± 0.04, KO DI 2min = 0.25 ± 0.08, WT DI 3min = 0.18 ± 0.05, KO DI 3min = 0.17 ± 0.09). Very likely, the difference found in their performance is due to their age.

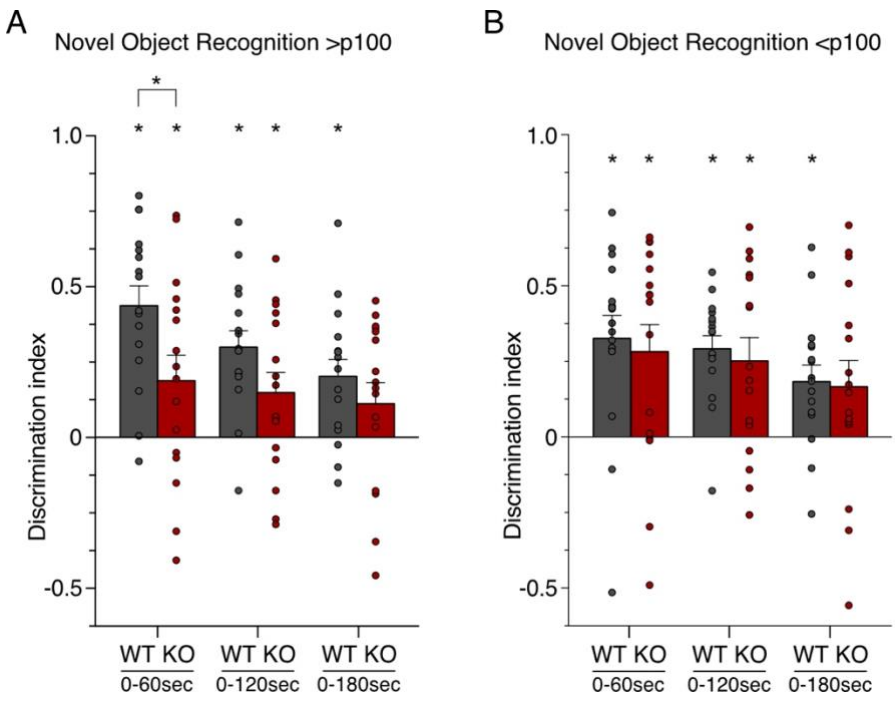

Figure 6.4 *Fmr1* KO rats show a deficit in long-term NOR paradigm only at older adult ages (>120 postnatal days).
(**A**) Discrimination index of novel versus familiar in older (>p100) *Fmr1* KO and WT rats as previously shown in Figure 5.7A (see there for statistics data). (**B**) Discrimination index of novel versus familiar in younger (<p100) *Fmr1* KO and WT rats. One sample t-test with theoretical mean of 0.0 (WT 1min *p = 0.0007, KO 1min *p = 0.0069, WT 2min *p = <0.0001, KO 2min *p = 0.0051, WT 3min *p = 0.0045, KO 3min *p = 0.0757; unpaired t test: WT 1min vs KO 1min p = 0.7130, WT 3min vs KO 3min p = 0.8711, n = 16).

6.2.2 M_4 PAM treatment of elevated protein synthesis phenotype in *Fmr1* KO adult mice and rats

As the deficit in NOR rescued by M_4 PAM treatment appears only at adult ages (>120 postnatal days), we wondered whether M_4 PAM treatment this late in life could also rescue the elevated hippocampal protein synthesis. We tested the compound in both adult rats and mice, using the same 5 μM dose adopted for juvenile mice in Thomson et al. 2017.

We collected hippocampal tissue from both naive and non-naive rats. The non-naive rats were the ones used for NOR testing with single injection of M_4 PAM or vehicle. Before their use for tissue extraction, the non-naive rats were kept in their home cages for about eight weeks to guarantee complete clearing from the previous treatment. Regardless, as a single M_4 PAM injection could have still resulted in permanent changes in the brain chemistry, we run a statistical comparison of the metabolic labelling results obtained with the non-naive rats previously treated with M_4 PAM, non-naive rats previously treated with vehicle, and naive rats. We found no significant difference between the three groups (we run unpaired t tests). The final graph shown in Figure 6.5A is the product of the combination of data from all these three groups of experimental animals.

I found executing the metabolic labelling experiment with elderly rats particularly difficult. Their skin and skull are significantly thicker compared to juvenile rodents, and their brain tissue softer and more easily damageable during the extraction from the skull. For optimal control of result authenticity, we carried out repeats of the experiment several times over the span of over six months, with both me and Dr. Susana R. Louros as experimenters. We consistently found the same results. First, the genotype difference between KO and WT hippocampal slices, however significant, was only about 10% compared to the 15-20% seen in previous studies with mice and rats (Osterweil et al. 2010; Asiminas et al. 2019), perhaps due to the difficulty of the procedure. Second, surprisingly, treatment with 5 μM M_4 PAM not only failed to rescue the increased protein synthesis phenotype of the adult *Fmr1* KO rats, but it exacerbated it, and caused a significant increase in WT protein levels as well (Figure 6.5A) (WT veh = 100 ± 2.32%, WT 5 μM = 110.08 ± 4.45%, KO veh = 108.68 ± 4.49%, KO 5 μM = 117.81 ± 3.45%).

This unexpected result prompted us to repeat the experiment with adult mice, to see whether this difference in M_4 PAM effect on protein synthesis is conserved between rodent species. We show here a preliminary study with an overall low number of experimental animals (n = 7, not all littermate pairs, not all age-matched). While the genotype difference between WT and KO vehicle groups is not yet significant,

treatment is significant with both two-way ANOVA and post-hoc analysis (Figure 6.5B) (WT veh = 100 ± 3.53%, WT 5μM = 127.13 ± 6.55%, KO veh = 110.41 ± 3.11%, KO 5μM = 129.88 ± 9.52%). Consistently with the previous results (Figure 6.5A), M_4 PAM treatment of hippocampal slices seems to have opposite effects on *Fmr1* KO rodent models at juvenile and adult ages, failing to show beneficial properties in adulthood (Figure 6.5A-B).

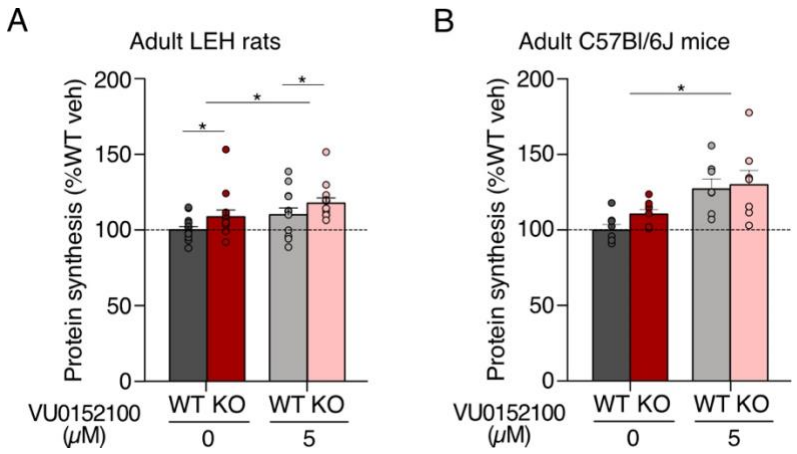

Figure 6.5 M_4 PAM VU0152100 significantly increases protein synthesis in *Fmr1* KO and WT adult rat and mice hippocampus
(A) Graph shows treatment with M_4 PAM (5 μM) in adult WT and *Fmr1* KO rat slices, **Dr. Louros contributed to this dataset** (ANOVA treatment *p = 0.0402, genotype p = 0.0842; Sidak WT veh vs KO veh *p= 0.0220, WT PAM vs KO PAM *p = 0.0399, WT veh vs WT PAM *p = 0.0093; KO veh vs KO PAM *p = 0.0167; n = 12). **(B)** Graph shows preliminary results with M_4 PAM treatment (5 μM) in adult WT and *Fmr1* KO mouse slices (ANOVA treatment *p = 0.0036, genotype p = 0.4659; Sidak WT veh vs KO veh p= 0.1434, WT PAM vs KO PAM p = 0.8314, WT veh vs WT PAM *p = 0.0027; KO veh vs KO PAM *p = 0.0136; n = 7). Error bars = SEM. N = not all littermate pairs, not all age-matched.

6.2.3 M_4 expression in *Fmr1* KO adult rats

If M_4 PAM treatment at adult ages exacerbates the already elevated hippocampal protein synthesis in both mice and rats, then what are the levels of M_4 expression at this age? Here, we investigated the expression levels of M_4 receptor in the *Fmr1* KO rat. Unfortunately, we encountered several technical difficulties in the process and the

obtained immunoblots could not be reliably quantified because of the high non-specificity of the antibody binding to the M_4 protein.

When probing the membranes with adult rat hippocampal proteins for M_4 expression, we found the two antibodies tested to be very non-specific for M_4 protein binding. The first antibody was from Abcam, the same used in the study by Thomson and colleagues, the second one from Elabscience. We tried both on three identically run 10% gels and transferred membranes, blocking them prior to primary incubation with either 5% BSA, 5% milk or 5% fish gelatine. Despite trying all these different protocols, we found the binding of the antibodies to be highly non-specific, with tenths of bands appearing all over the probed membranes, particularly one non-specific band at about 85kDa. Knowing from previous studies on mouse tissue that M_4 is expressed as two bands around 53kDa (Thomson et al. 2017), to prevent exaggerated binding of the primary antibody to non-specific sites, we cut the membranes at molecular weights of <75kDa and >35kDa. We blocked the membranes in 5% BSA, which worked the best at reducing non-specific primary antibody binding, and again tried both primary antibodies (Figure 6.6A). The blot probed for M_4 with the Elabscience antibody showed perhaps even more non-specific bands that the one from Abcam, and the bands of the two blots did not seem to correspond either. Because of these unreliable results, we were not able to quantify the expression levels of M_4 in rat hippocampal samples.

To further corroborate our decision of discarding these results out of our conclusions, we obtained a full brain sample of a mouse with the *CHRM4* gene for M_4 expression fully knocked down (M_4 KO). To test the validity of the primary antibodies, we run this sample in an 8% gel along with WT and *Fmr1* KO rat samples. To our disappointment, we found that the bands appearing above 50kDa were still present in the M_4 KO sample, proving the non-specificity of the primary antibodies. We show here an example blot probed for M_4 signal with the Abcam antibody, compared to previous total protein staining (Figure6.6B). It is likely that although the molecular weight we looked at for M_4 expression is correct, there are other muscarinic receptors of similar weight being detected by the primary antibody. For example, M_1 has a predicted molecular weight of 51kDa.

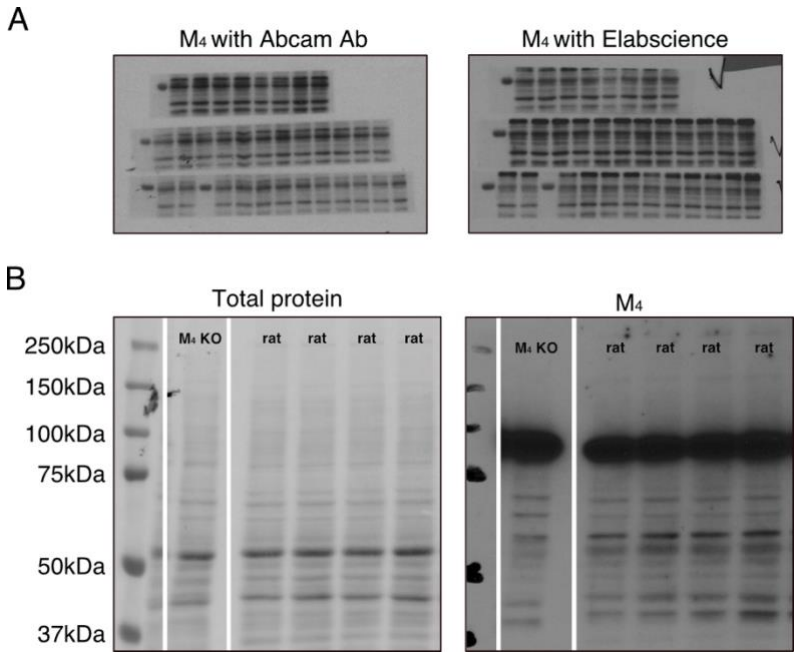

Figure 6.6 M_4 expression cannot be confidently measured in hippocampal samples from adult rats.
(**A**) Example of membranes cut at <75 and >35kDa, probed for M_4 expression with antibodies from Abcam (left) and Elabscience (right). (**B**) Example of full membrane with samples from WT and *Fmr1* KO rats, and M_4 KO mouse. On left, membrane with non-specific staining for total protein, on right, same membrane probed for M_4 expression with antibody from Abcam.

6.3 Discussion

First part of this chapter showed the effects of M_4 PAM treatment in NOR memory task. As described, at the time of treatment we did not realize that the deficit displayed by the *Fmr1* KO rats is age-dependent: only rats above the age of 120 postnatal days seem to display it, while younger rats perform normally. This lack of knowledge prevented us to finalize the dataset with NOR and M_4 PAM treatment. Nevertheless, although the results just discussed are of preliminary nature, we believe they are a good indication of the M_4 PAM ability to rescue the age-dependent memory deficit of *Fmr1* KO rats.

In fact, there is evidence for involvement of muscarinic receptors in FXS. In over six-months old human foetuses, the expression of FMR1 mRNAs is highly diffused in almost all brain structures, but its highest levels are in the cholinergic neurons of the nucleus basalis and in the hippocampal pyramidal neurons (Abitbol et al. 1993). The cholinergic system in the cortex has been associated to input filtering and attention, with its integrity being necessary for attentional performance. Moreover, cholinergic projections from the cortex to the hippocampus are thought to work jointly in the processes of memory, and acetylcholine levels in the hippocampus have been shown to increase during memory formation (Sarter et al. 2003). Although it is not clear how much this system is necessary for memory acquisition, consolidation or retrieval, and through which specific receptors, there is certainly evidence for some involvement. For example, females with FXS were found to have reduced activation of hippocampal and basal forebrain areas during a visual memory task (Greicius et al. 2004). Furthermore, although the data on overall choline levels in the brain of FXS individuals is controversial, with one study showing decreased ratio of choline/creatine compared to controls (Kesler et al. 2009) and another finding no difference in acetylcholine levels (Scremin et al. 2015), a six-week open-label trial with administration of an acetylcholinesterase inhibitor to FXS subjects improved their scores of aberrant behaviour, hyperactivity and measures of working memory and adaptability (Kesler et al. 2009). Thus, it is not much of a surprise to find that modulation of muscarinic receptors can influence some of the phenotypes of the *Fmr1* KO mouse model (D'Antuono et al. 2003; Neuhofer et al. 2018; Thomson et al. 2017).

After finding that M_4 PAM treatment immediately after memory acquisition could rescue its deficient consolidation in adult *Fmr1* KO rats, we moved on investigating whether M_4 PAM treatment could rescue the elevated hippocampal protein synthesis at adult ages. Surprisingly, we found M_4 PAM treatment to further exacerbate the elevated hippocampal protein synthesis in both adult *Fmr1* KO mouse and rat, and to increase normal hippocampal protein synthesis in the WT controls.

The increased M_4 expression in the CA1 area of juvenile *Fmr1* KO mice is hypothesized to be a result of a compensatory mechanism for helping reduce the

increased translation caused by FMRP loss (Thomson et al. 2017); and the M_4 PAM rescuing of AGS and the other biochemical and physiological phenotypes in juvenile *Fmr1* KO mice is certainly directly related to FXS pathology.

Finding that positively modulating M_4 at adult ages no longer ameliorates the core protein synthesis phenotype, could suggest a change in this compensatory translational mechanism from juvenile to adult ages. This brings uncertainty to why the M_4 PAM still rescues the deficit in NOR assay. This phenotype, differently from the mGluR-dependent LTD, does not seem to be associated to the elevated hippocampal protein synthesis of *Fmr1* KO rodents. However, this does not mean for it to not be associated to FXS pathology.

We explored earlier the numerous findings hinting at a major role for muscarinic receptors in long-term memory and learning, and we know the processes of memory and the areas of hippocampus and basal forebrain to be significantly affected in FXS (Greicius et al. 2004; Baratti et al. 1979; Kopf et al. 1998; Carballo-Márquez et al. 2007; Power et al. 2003). Therefore, a dysfunctional cholinergic system due to absence of FMRP could have a major role in the development of FXS phenotypes, even in adulthood. It could also be that FMRP loss predisposes for an early weakening of normal processes for memory consolidation, and positive modulation of M_4 allows for compensation of this disease- and age-related losses. Indeed, acetylcholine levels in the hippocampus and prefrontal cortex have been shown to increase during memory formation, retrieval, and food reinforcement of memory. Hence, the rescue of the poor performance of older *Fmr1* KO rats in NOR could be simply due to strengthening of the M_4 receptor response to acetylcholine signalling, directly implicating the M_4 receptor in memory consolidation (Iso et al. 1999; Hironaka et al. 2001; Chang & Gold 2003).

Thus, there are still questions to be answered to complete this study. We showed that M_4 positive modulation at adult ages exacerbates the elevated hippocampal protein synthesis in both mouse and rat FX models. Crucial problem in the follow up investigation was the lack of appropriate primary antibodies to adopt for measuring M_4 hippocampal expression in the adult rat. The development of M_4-specific high quality antibodies for M_4 expression in western blotting and immunostaining assays

would be of primary importance for future studies in adult FXS models. However, we believe that before tackling the details of M_4 PAM treatment in adult ages, we should first focus on uncovering the mechanisms underlying its beneficial effects in juvenile ages. The discussion paragraph at the end of Chapter 4 describes the results obtained in that investigation and lists future experiments. Identifying the activity mechanisms of M_4 in juvenile *Fmr1* KO mice would certainly help direct the follow up experiments to be conducted in adult *Fmr1* KO rodents. Pursuing these further investigations would be extremely important to elucidate the mechanisms of this hypothesized compensatory translation shift, and analyse its development along age. The changes of a compensatory change along development and aging can provide invaluable information on the disease pathology itself.

Finally, although the rescue of NOR deficit at adult ages could be not directly related to FX pathology, we believe it would still be of valuable knowledge to finalise that dataset and pursue further experiments to uncover M_4 properties in adulthood.

Overall, this research on M_4 positive allosteric modulation illustrates a second potential treatment strategy for FXS. Traditionally, $mGluR_5$, ERK1/2 and other protein synthesis regulators such as the mTOR-p70S6K pathway would be targeted for inhibition to reduce the *Fmr1* KO elevated protein synthesis and consequently ameliorate the other protein synthesis-dependent phenotypes. However, the study by Thomson et al. in 2017 and the follow up research illustrated in these chapters shows how strengthening some already augmented processes could also be helpful in FXS. In this case, M_4 signalling. Augmenting the magnitude of homeostatic shifts could be a differently way to approach the disease therapeutically.

Chapter 7

Conclusions

Conclusions:

The neurodevelopmental Fragile X Syndrome is characterized by intellectual and learning disability, childhood-associated seizures, dysmorphic features, anxiety, hypersensitivity to sensory stimuli, ADHD and autism (Berry-Kravis 2002; Hagerman et al. 2008; Loesch et al. 2003; Munir et al. 2000; Turk 1998). It arises from transcriptional loss of the *FMR1* gene for the FMRP protein, a negative regulator of protein synthesis in neuronal cell bodies and synapses (Antar et al. 2004). When FMRP mRNA was found to be rapidly synthesized at synapses downstream activation of $mGluR_5$, and the FMRP protein to associate and inhibit the translation of specific mRNAs (Weiler et al. 1997), the mGluR theory was formulated. The protein synthesis-dependent long-term effects of $mGluR_5$ activation were found significantly exacerbated in the *Fmr1* KO animal models compared to WT (Huber et al. 2002), and basal levels of protein synthesis itself were found significantly elevated in numerous areas of the *Fmr1* KO brain (Qin et al. 2005). Thus, the mGluR theory of FXS indicates the loss of FMRP downstream $mGluR_5$ activity as responsible for all exaggerated $mGluR_5$ protein synthesis-dependent effects in the *Fmr1* KO rodent models, and postulates these as the underlying cause of most FXS cognitive and behavioural phenotypes (Bear et al. 2004). To correct the core FXS deficits, the theory proposes the search for therapies targeted at reducing the activation of either $mGluR_5$ itself or its downstream effectors, including the ERK1/2 signalling pathway.

This prompted the investigation of statin drugs, which by targeting cholesterol synthesis in the mevalonate pathway also decrease the production of so-called isoprenoids intermediates (Istvan 2003), necessary for the posttranslational modification and activation of proteins such as the GTPase Ras (Ling & Tejada-Simon 2016). Being the GTPase Ras upstream the ERK1/2 pathway, known to be involved in protein synthesis regulation downstream $mGluR_5$ (Gallagher et al. 2004; Osterweil et al. 2010), the statin lovastatin was tested for FXS treatment in the *Fmr1* KO mouse and rat models with successful results (Osterweil et al. 2013; Asiminas et al. 2019). However, lovastatin is not available for medical prescription in several countries worldwide, including the UK, and the more potent and widely licenced statin

simvastatin has been proposed as an alternative. First part of this PhD project was to test and compare the effects simvastatin and lovastatin on core phenotypes of the *Fmr1* KO mouse: the elevated hippocampal protein synthesis and the increased susceptibility to AGS. After finding no ameliorating effects of simvastatin treatment on either of these phenotypes and a full rescue of both with lovastatin, we investigated whether simvastatin could reduce ERK1/2 activation similarly to lovastatin. Consistently with the lack of positive effects of simvastatin treatment in the *Fmr1* KO mouse, we found simvastatin to not influence ERK1/2 activity, contrarily to a significant decrease in its activation with lovastatin stimulation. This study showed how targeting ERK1/2 activity downstream $mGluR_5$ signalling is highly beneficial in treating core *Fmr1* KO phenotypes, and it showed how drugs of the same family should not be assumed to share all targets. Although all statins are known to target mevalonate production and hence consequently influence isoprenoids synthesis, we are unaware of how much and to what extent each statin specifically influences the production of these secondary products of the pathway. Indeed, we showed that simvastatin, at the doses tested, does not affect Ras-ERK1/2 as lovastatin does. However, targeting ERK1/2 activity is still proven to be an effective way to treat FXS, and clinical trials for lovastatin treatment in FXS patients have been promising.

The second part of this project was based on the recent study by Thomson and colleagues in 2017. The study identified the differently translated mRNAs in the CA1 hippocampal region on the *Fmr1* KO mouse compared to WT littermates. Surprisingly, the known FMRP targets were found slightly downregulated rather than upregulated, suggesting a homeostatic shift in their translation levels to compensate for FMRP loss. Instead, the most upregulated mRNAs belonged to the family of acetylcholine muscarinic receptors, with the subtype 4 being the most significantly translated and expressed in the hippocampus. Interestingly, inhibiting M_4 activity further exacerbated the tested *Fmr1* KO phenotypes, whereas positively modulating it with a M_4-specific PAM rescued all core biochemical, physiological and behavioural phenotypes investigated (Thomson et al. 2017; Chapters 4 & 6 of this thesis). In this PhD project, after successfully testing M_4 PAM treatment for AGS susceptibility in juvenile *Fmr1* KO mouse, we investigated what activity mechanisms were underlying the M_4 PAM

beneficial effects. In juvenile *Fmr1* KO mouse, we showed the M_4 PAM to not influence ERK1/2 activity under the condition tested, and to not affect the elongation factor eEF2 or the mTOR downstream target p70S6K.

At the same time, we wondered whether M_4 PAM treatment would ameliorate memory-associated phenotypes in a different species, the rat. Investigating a promising compound in more than one mammalian species is an important step in the assessment of its treatment potential. Moreover, the higher sociability of the rat and its less dominant nature make it an excellent model for behavioural phenotypes of neurodevelopmental disorders characterized by learning and memory impairments and social interaction deficits. For these reasons, we selected the NOR task to assess the effects of M_4 PAM treatment on the deficit for discrimination for novelty displayed by older *Fmr1* KO rats. Although the obtained dataset is still preliminary, we found M_4 PAM treatment to ameliorate this deficit.

Following this excellent result, we investigated whether the M_4 PAM would rescue the elevated hippocampal protein synthesis also at adult ages. Surprisingly, we found M_4 positive modulation to exacerbate the *Fmr1* KO phenotype at adult ages in both mice and rats. Thus, we finally tried to measure M_4 expression in the adult *Fmr1* KO rat hippocampus. We used the previously adopted antibody from Abcam (Thomson et al. 2017) and a second one from Elabscience. Unfortunately, we found both these antibodies to not be selective enough for M_4 binding in rat samples.

Therefore, we were unable in this study to investigate the M_4 expression levels at adult ages and to identify the mechanisms underlying the positive effects of M_4 treatment in juvenile *Fmr1* KO mice. However, we proved them to be unrelated to ERK1/2 modulation.

In conclusion, as M_4 is yet to be proven a target of FMRP activity, but was found to be expressed downstream $mGluR_5$ activation, there is a strong possibility for its increased expression in *Fmr1* KO mice to be due to a homeostatic response for counterbalancing FMRP loss. This is supported by the positive effects of M_4 PAM treatment and detrimental effects of M_4 antagonisms in juvenile *Fmr1* KO mice (Thomson et al. 2017). If this hypothesis is proven valid, this research on M_4 positive allosteric modulation might illustrate a second potential treatment strategy for FXS:

strengthen already formed homeostatic responses to a disease pathology by positive modulating or direct activation of their mechanisms. Augmenting the magnitude of homeostatic shifts could be a different way to approach FXS therapeutically. In the future, combination therapies of direct pathology targeting and concomitant support of homeostatic responses could be a winning strategy in the initial treatment of developmental disorders.

However, we found this hypothesized homeostatic shift to potentially change at adult ages, as the M_4 PAM no longer rescued the elevated protein synthesis phenotype in adult *Fmr1* KO mice and rats. Because acetylcholine signalling has been shown to be majorly involved in memory formation and consolidation, the beneficial effect of M_4 PAM treatment on NOR long-term memory could be unrelated to FXS pathology. Nonetheless, even if the NOR deficit shown by *Fmr1* KO rats was proven to be FX-related, M_4 PAM treatment at adult ages could be detrimental for the overall FX pathology. This because M_4 PAM treatment at adult ages exacerbated the elevated protein synthesis. It is hence likely that other protein synthesis-dependent phenotypes would be also exaggerated by this treatment at this age (Beat et al 2004). Future studies to elucidate the changes of M_4 activity mechanism during development would be crucial. It is not surprising to find that efficient therapies at young ages could no longer be helpful at older ones. Regardless, gaining further knowledge on this hypothesized compensatory mechanism and its changes in adulthood would bring valuable knowledge to field.

Overall, we show in this thesis two different approaches to ameliorate FXS-associated phenotypes of *Fmr1* KO rodent models. Although only one of the two treatment strategies investigated was shown to target ERK1/2 activity, the ameliorating effects of both compounds suggest that targeting the core FX pathology of elevated protein synthesis is a crucial factor for ameliorating other FX phenotypes, such as the GluR-LTD and increased incidence of AGS (Muscas et al 2019; Thomson et al. 2017).

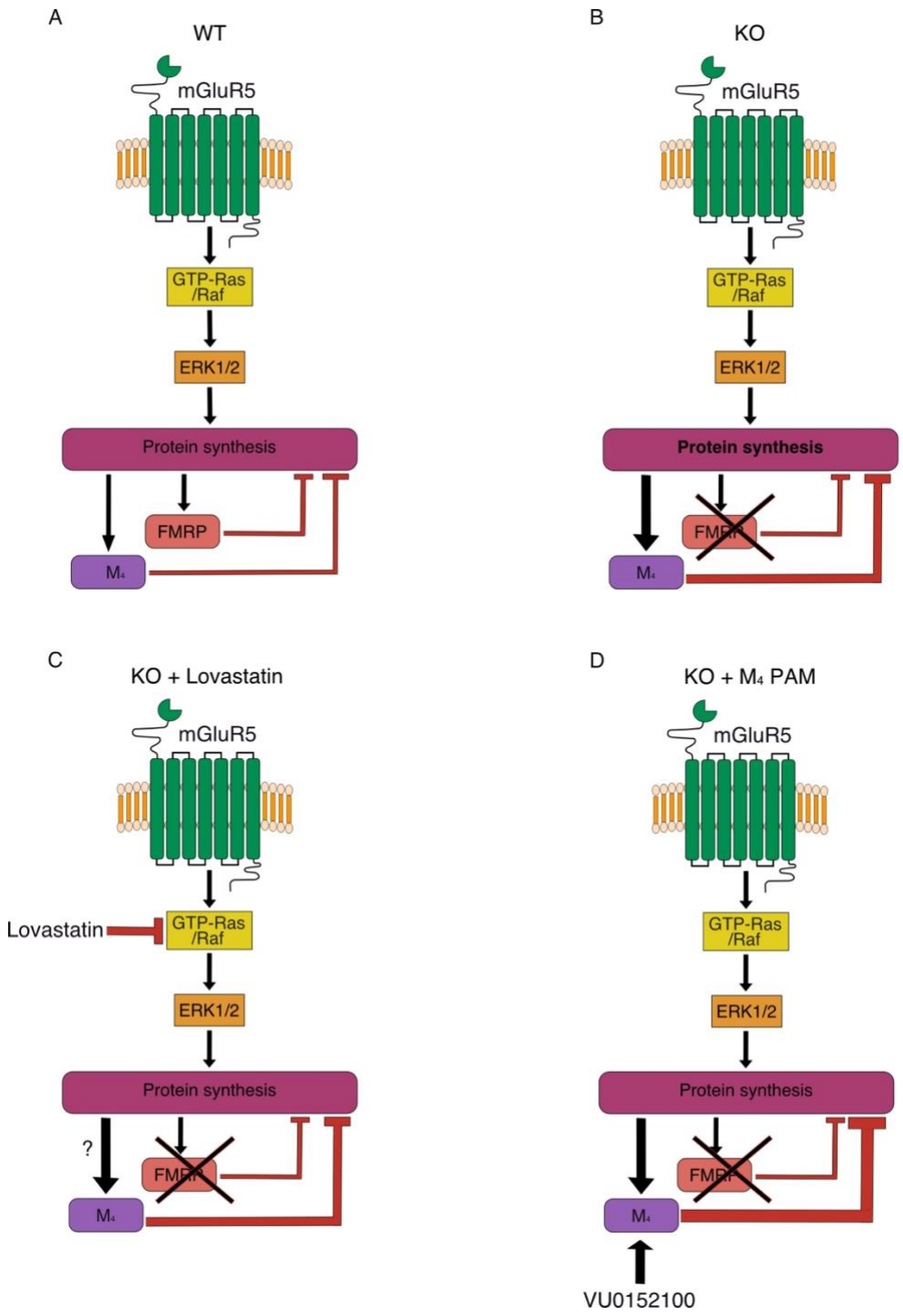

Figure 7.1 Schematics of mGluR5 downstream signalling in WT and *Fmr1* KO juvenile mice. Illustration of the therapeutics strategies discussed in this thesis. (**A**) mGluR5 downstream signalling in hippocampus of WT mice. (**B**) mGluR5 downstream signalling in hippocampus of *Fmr1* KO mice (**C**) Lovastatin treatment negatively targets ERK1/2 signalling downstream mGluR5 in the hippocampus of *Fmr1* KO mice. (**D**) M4 PAM treatment with VU0152100 positively supports FMRP-associated homeostatic responses downstream mGluR5.

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