
THE UNIVERSITY
*of* EDINBURGH

# Modelling Fragile X Syndrome in rats:

# New directions in translational research

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...φύσιν δὲ ἕκαστον ἔχει καὶ δύναμιν ἑφ᾽ ἐωυτοῦ, καὶ οὐδὲν ἄπορόν ἐστιν οὐδὲ ἀμήχανον.

Each (disease) has a nature and power of its own; none is hopeless or incapable of treatment

Hippocrates, The Sacred Disease, 400 BCE

# Abstract

Fragile X syndrome (FXS) is the leading single gene cause of intellectual disability and Autism Spectrum Disorder (ASD). It is caused by epigenetic silencing of the fragile X mental retardation gene (*FMR1*), causing a loss of Fragile-X Mental Retardation Protein (FMRP). Over the last 2 decades, much has been learned about the pathophysiology related to the loss of FMRP from mouse models of FXS. The recent generation of a rat model of FXS opens the door to: validate phenotypes across mammalian species, address cognitive dysfunction using paradigms that are more difficult to address in mice and explore candidate therapeutics more accurately.

This thesis explored the validity of a new rat model for FXS (*Fmr1* KO rat). I showed that Fmr1 KO rats exhibit normal spatial navigation memory, social interactions and anxiety levels. On the contrary, when subjects were tested in a battery of spontaneous exploration tasks: object recognition (OR), object-context (OC), object-place (OP), and object-place-context (OPC) recognition, which assess associative memory, Fmr1 KO rats showed a severe deficit in remembering the most complex (episodic-like) associations.

Following these results, I sought to explore the development of associative memory from postnatal day 25 (P25) to adulthood (P71). Subjects were tested in the four spontaneous exploration tasks, previously mentioned, 8 times between P25 and P71 to assess the development of their ability to discriminate novel from familiar associations between objects, contexts and places. Fmr1 KO rats' ability to discriminate novel from familiar object-place (spatial) and object-place-context (episodic-like) associations was significantly impaired (OP was delayed, and OPC ability did not develop).

In the last part of this thesis I examined whether early therapeutic intervention with lovastatin can restore the cognitive deficits I observed. Subjects were fed either a diet containing lovastatin ("lovachow") or an identically looking control diet, between P29 and P64, and tested in the four spontaneous exploration tasks, previously mentioned. *Fmr1* KO rats demonstrated a developmental profile of associative memory indistinguishable from that of WT animals. At P64, lovachow was replaced with standard laboratory chow and the animals were tested 1 and 3 months later. Surprisingly, lovastatin treated *Fmr1* KO animals maintained the ability to perform the OPC task even at 3 months after the end of treatment, whereas Fmr1 KO animals on control chow showed no improvement with age.

The findings of this work indicate that transgenic rats can complement existing mouse models of FXS, providing valuable insights into the effects of FMRP loss on cognitive function. Furthermore, the results from the treatment study show that not only can lovastatin treatment prevent the emergence of cognitive deficits associated with Fragile X Syndrome but also that lovastatin (and perhaps pharmaceutical interventions more generally) may prevent the developmental deficits in neuronal circuit formation which can be maintained into adulthood.

# Lay Summary

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and the most frequent single gene cause of autism, affecting approximately 1 in 4,000 males and 1 in 6,000 females. Over the last 2 decades, much has been learned about the disease mechanism using mouse models of FXS. This thesis focusses on a new rat model of FXS (*Fmr1* KO rat), and examines whether it can model FXS efficiently. Furthermore, it examines whether therapeutic intervention early in life can prevent emergence of cognitive deficits and whether any benefits are dependent on ongoing treatment.

*Fmr1* KO rats appear to have normal cognitive performance in various tests but they are severely impaired in a complex type of memory called episodic memory (memory of events). This is a novel finding which offers valuable insights into the cognitive impairments associated with FXS but also provides a good way to test candidate therapies.

Following my previous results, I sought to explore how episodic memory and other forms of associative memory develop in juvenile rats and, since FXS is a neurodevelopmental disorder, examine if *Fmr1* KO rats experience any developmental delays. I found a certain type of associative memory (association of an object and its position) takes longer to appear in this rat model of FXS and episodic memory, as previously observed, doesn't develop at all.

Finally, I investigated if early therapeutic intervention can restore normal development of learning and memory. Treated *Fmr1* KO rats demonstrated a developmental profile of associative memory indistinguishable from that of normal animals. I then tested if the beneficial effects of this treatment could be maintained into adulthood without ongoing drug application. After 5 weeks of treatment, drug-containing diet was replaced with standard laboratory diet and the animals were tested 1 and 3 months later. Surprisingly, treated Fmr1 KO animals maintained normal learning behaviour even at 3 months after treatment had been terminated, whereas Fmr1 KO animals on control diet showed no improvement with age.

The findings of this thesis indicate that rat disease models can expand our knowledge of FXS, thus complementing existing mouse models. Furthermore, the results from the treatment study show that not only can appropriate treatment prevent of cognitive

delays associated with Fragile X Syndrome but also that pharmaceutical interventions during potentially critical developmental windows can have long lasting or even permanent effects.

# Acknowledgements

Making the transition from chemistry to neuroscience is not a trivial task. I've been very lucky to be part of a team which made it look so simple. Firstly, I would like to thank my supervisor Dr Emma Wood who was patient, strict when she had to be, supportive and understanding all in the right amounts; a true mentor. She taught me to focus on the right question since my mind has a tendency to drift from the main idea to smaller questions of minor importance; she taught me how to think as a behavioural neuroscientist.

I would like to thank Dr Vassilis Beglopoulos and Prof Richard Morris. Even though they weren't directly involved in the work described in this thesis, they were the ones who brought me in Edinburgh and gave me a chance to make the transition; if it wasn't for them to open the door for me to the beautifully complex world of neuroscience I wouldn't have been in this position today.

Question! Probably a third of all the sentences I've formulated during the last 5 years, started with this word. I have asked thousands of questions, so having people around who were willing to give me answers, or help me find these answers, was amazing. From people in the PhD student office: Adrian, Roddy, Tizzy, Fitzy, Daisy and Rachael, Rosie and Ellie to people in the postdoc room Lisa, Tomo, Mio, Janine, Dorothy, Dave and Bruce, everyone has spent time answering weird and not so weird questions, helping me design experiments and expand my knowledge in neuroscience.

I've been amazingly lucky to have a great thesis committee; Emma, Peter, Sally and David were the best group of people I could have, to guide me through this process. Every thesis committee meeting was a bit intense for me for the simple reason I had nowhere to hide, there was no room for arguments not supported by data; between the four of them, they cover all aspects of neuroscience. This was the best way to improve the way I think about experiments and interpret data.

I would also like to thank my parents Nikos and Dimitra who let me "do my thing" and supported me, even though they didn't always agree with my decisions. I want to say a very big thank you to Dennis, my soon to be father in-law, who proofread a big part of this work and provided invaluable comments on my writing.

Last but not least I want to thank my fiancée Jude. She has been very patient and supportive throughout my PhD research and the writing process and I feel lucky to be with her.

This is definitely not the end; this is just the beginning. Completing a PhD is the first step of a career I've always dreamt of. The reason I decided around 20 years ago, while I was still in primary school, to pursue a career in science is because I saw the passion of two people who are very special to me; Prof "Lola" Theodora Choli-Papadopoulou and Dr "Biskotos" George Papadopoulos; my first mentors. They taught me why and how to love science, to chase my dreams but at the same time always plan ahead and be prepared for challenges and changes in my research direction.

I declare that all presented work is my own except if stated otherwise; and I composed this thesis myself. The work in this thesis has not been submitted for any other degree or professional qualification.

Antonis Asiminas

Chapter 1: Data collection for open field, light/dark box and reference memory and reversal task in watermaze was performed in collaboration with Dr Sally M Till. USVs data collection was performed in collaboration with Prof Maria Louisa Scattoni, Dr Caterina Michetti and Dr Sally M Till. Due to the special nature of the dataset, data analysis in the same experiment was primarily carried out by Dr Caterina Michetti Prof Maria Louisa Scattoni who are experts in the field.

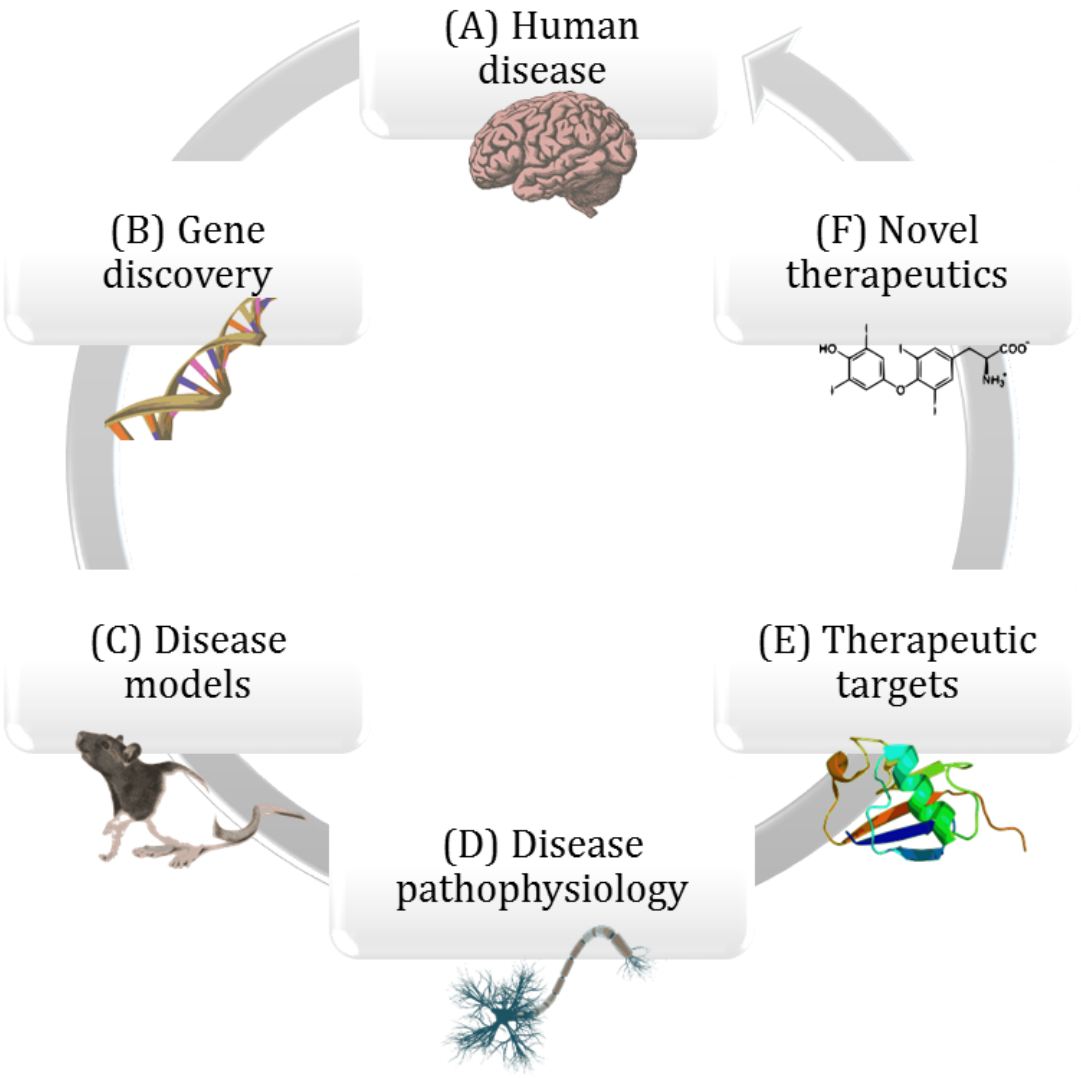
Chapter 3: Data collection for Object exploration tasks and daily food/ weight monitoring of the animals was performed by Athina Aruldass, Kimberley Reed and Cecilia Neill-Edwards during summer placement (AA) and honours project (KR & CNE); all data analysis was performed by the author.

# Table of Contents

# Overview

Animal models of Fragile X Syndrome (FXS) have proven invaluable to advance our understanding of the pathogenesis and pathophysiology of FXS. Until the development of the first *Fmr1* knockout mouse (*Fmr1* KO) model of FXS (Bakker et al., 1994) it was not feasible to study the effects of FMRP loss in vivo. The discoveries made using this model have undoubtedly changed the way we think about, not only FXS, but also neurodevelopmental disorders in general (Wijetunge et al., 2013). Although there is currently no effective therapy against the core symptomatology of FXS, our increasing understanding of the disease pathophysiology has created multiple lines of investigation for targeted therapies. Even though many phenotypes associated with the loss of FMRP have been shown to improve in preclinical treatment studies, attempts to translate these animal-model success stories into successful treatments for human patients have so far been unsuccessful. The recently created rat model of FXS offers a great potential for the study not only FXS but also ASD and other neurodevelopmental disorders. This thesis aims to introduce this new rat model into the research community and highlight its importance as an exciting new alternative to the established mouse model on 3 different levels: (1) the validation of phenotypes across mammalian model species carrying the same genetic lesion, (2) the exploration of pathophysiology and behaviour associated with FXS, using assays which are quite challenging for the mouse model and (3) bridging the gap between preclinical and clinical research.

In the three introductory chapters of my thesis, I will discuss FXS, the most frequently encountered singe gene cause of inherited intellectual disability and the most common monogenic cause of autism. I will then review reseach on the mouse model of the syndrome which carries an analogous genetic lesion and expresses similar physiological and behavioural phenotypes. This mouse model of FXS has been an invaluable tool for understanding pathophysiology of the disease on multiple levels, but my review is mainly focussed on the behavioural phenonype. Drug discovery is a challenging but absolutelly critical process in translational research. The empirical approach to drug discovery - focussed only on safety and efficacy in humans, but not connected to the underlying biological mechanisms – is gradually being replaced by a mechanism-based, targeted approach. Thus, in the second introductory chapter, I will summarise the most recent findings in FXS drug development and summarise current mechanism-based FXS




**The era of mechanism-based treatments for neuropsychiatric disorders.** The process starts with a thorough characterisation of the neuropsychiatric disease and genomic investigations in affected individuals (**A**). Once affected genes, which confer a risk or cause the disease, have been identified (**B**), animal models of the disorder which carry the same genetic lesion(s) can be created (**C**). Once we have these animal models, basic knowledge of neurobiology has to be applied in order to study of the cellular, physiological, and behavioural consequences, known as the disease pathophysiology (**D**). We may then be able to identify target(s), parts of core processes in the brain, which have gone awry and are amenable to pharmacological (or other) manipulations (**E**). The final step is to validate novel therapeutics created to interact with our identified targets, in clinical trials (**F**).

therapeutic strategies and their translation from animal models to humans. Finally, in the last introductory chapter, I will reflect on the behavioural differences between rats and mice and I will attempt to highlight the huge potential impact of the newly generated rat models of FXS and autism.

The first series of experiments described in Chapter 4 focus on the initial behavioural characterisation of *Fmr1* KO rats. The main aim is to determine whether this rat model recapitulates behavioural phenotypes seen in the mouse model. The tasks are divided into three categories, highlighting the three main behavioural domains affected in FXS: elevated anxiety/repetitive behaviours, social interaction abnormalities and cognitive deficits. Although the main body of work in this chapter makes use of the commercially available *Fmr1* KO rat, on an albino Sprague-Dawley background strain, the last section of the chapter, I introduce a custom made model of FXS in a Long-Evans Hooded background strain, in an attempt to examine whether there are strain specific behavioural deficits as observed in the mouse model, or not.

The study described in Chapter 5 attempts to build upon the findings of Chapter 4. We aim to investigate the development of associative object memory in *Fmr1* KO rats and their wildtype littermates. The rationale behind this study is to identify possible developmental delays in this model of FXS, which would be consistent with the human phenotype since FXS is a neurodevelopmental disorder (children with FXS meet almost all developmental milestones later in life).

The final experiment (Chapter 6) described in this thesis goes a step further towards closing the loop of molecular medicine. It investigates the effect of early pharmacological treatment with lovastatin (a candidate therapeutic also discussed in Chapter 2) on two levels: (1) restoring normal cognitive development (described in Chapters 5), and (2) resulting in robust long lasting effects.

# 1. Studying Fragile X Syndrome

## 1.1 From Martin and Bell to Fragile X

In 1943 Martin and Bell described an X-linked form of intellectual disability in a family with 11 affected boys; they had below average IQ and specific morphological characteristics (Martin & Bell, 1943) (Fig. 1.1A). Several years later, in 1969, Lubs (1969) revealed the existence of a "fragile site" on the long arm of chromosome X in Xq27.3 (FRAXA site)(Fig. 1.1B), when they cultured lymphocytes derived from 4 boys with intellectual disability. In 1977, Sutherland noticed that this fragile site was more easily observed if folic acid is not added to the culture medium. The discovery of the fragile site in the members of the family that Martin and Bell first studied lead to the connection between the chromosomal abnormality and the clinical image of the syndrome, known today as Fragile X syndrome (FXS). Its known genetic aetiology, prevalence, and neurobiological commonality with less well understood neurodevelopmental disorders - including autism spectrum disorder (ASD), attention deficit and hyperactivity disorder (ADHD), and other forms of intellectual disability - make FXS a valuable model for understanding the neurobiology of these diseases and developing targeted therapeutic interventions (Elizabeth Berry-Kravis, Knox, & Hervey, 2011).

## 1.2 Genetics of FXS

Fragile X Syndrome is the most prevalent single-gene cause of mental retardation (Turner et al., 1996). Recent estimates indicate that approximately 1 in every 4000 males and 1in every 8000 females are affected (Crawford, Acuña, & Sherman, 2001; P. J. Hagerman, 2008; Turner et al., 1996). FXS arises from an inter-generational trinucleotide expansion (cytosine, guanine and guanine) in the 5' untranslated region (UTR) of the Fragile X Mental Retardation 1 gene (*FMR1*) (Verkerk et al., 1991)(Fig. 1.1C). This gene encodes FMRP, a protein involved in the regulation of mRNAs at the post-transcriptional level, playing key roles in synaptic structure and function, and in underlying cognitive processes. In typically developing individuals, the number of trinucleotide (CGG) repeats varies between approximately 5 and 50 with an average number of 30 trinucleotide units (Cunningham et al., 2011; Fu et al., 1991; Snow et al., 1993). However, individuals

affected with FXS typically have more than 200 CGG repeats (Chiurazzi, Neri, & Oostra, 2003)(Fig. 1.1C). A two-step process leads to the full mutation of the *FMR1* gene. The first mutation results in the expansion of the CGG trinucleotide to 55 - 200 repeats (called a premutation); the person is considered a carrier at that point and can have only up to mild clinical symptoms. The second step of the mutation, results in an affected individual with more than 200 repeats (called a full mutation). The premutation only develops into a full mutation when passed by a female to her offspring, because a recombination event with the other X chromosome facilitates the expansion process. That expansion results in an epigenetic silencing of the *FMR1* gene on the X chromosome (Sherman et al., 2002) through over-methylation of the promoter, which in turn prevents expression of Fragile X Mental Retardation protein (FMRP) (Verkerk et al., 1991). Although this increase in the number of trinucleotide (CGG) repeats accounts for the vast majority of cases of FXS, rarely an individual can also be affected by point mutations or deletions within *FMR1* gene which can result in its silencing (Grønskov, Hallberg, & Brøndum-Nielsen, 1998; Santoro, Bray, & Warren, 2012).

While FXS was originally thought to be a recessive genetic disorder, scientists noticed that approximately 1/3 of carrier females exhibited mild clinical symptoms including cognitive difficulties, emotional and social deficits and occasionally depression (Kim Cornish et al., 2005; F. Tassone et al., 2000). This points to the fact that FXS is not recessive, but rather an X-linked dominant disorder with reduced penetrance in females due to the normal process of X-inactivation (Sherman et al., 1985). Female premutation carriers - even those not experiencing any cognitive and emotional abnormalities - may experience problems later in life. Almost twenty-eight percent of female premutation carriers suffer from premature ovarian failure and early menopause, a condition called Fragile X associated primary ovarian insufficiency (FXPOI) (Cronister et al., 1991; Sullivan et al., 2005). Male premutation carriers are at high risk of developing a neurological disorder known as Fragile X tremor/ataxia syndrome (FXTAS) - characterized by ataxia, tremor, cognitive and autonomic dysfunction, and Parkinson's-like symptoms - usually by the age of 50 (Hagerman et al., 2004; Moore et al., 2004).

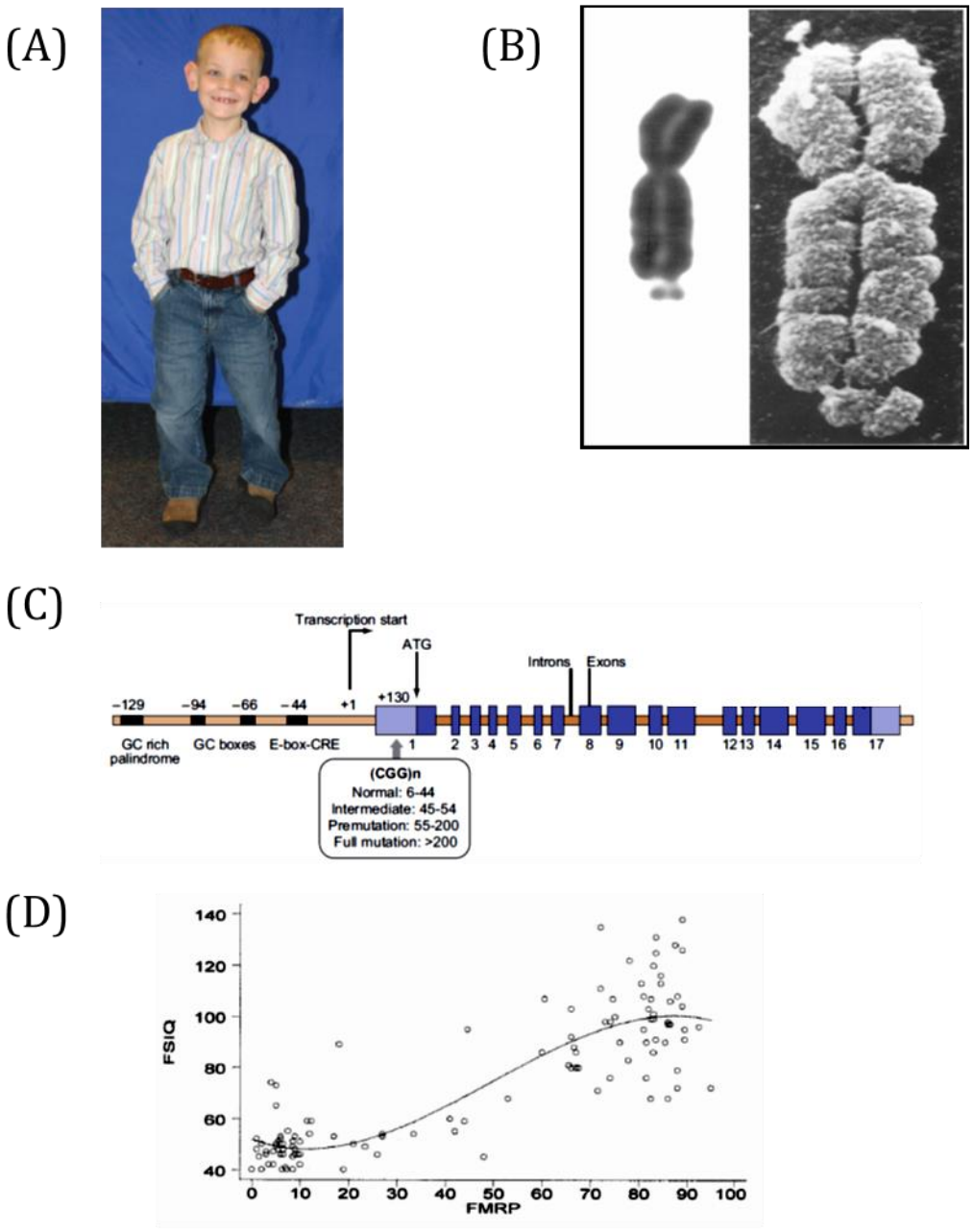



**Figure 1.1 The fragile X phenotype. (A)** Affected individual: the characteristic facial features of the syndrome include long, narrow face, prominent forehead, jaw, and ears. **(B)** The "marker X" chromosome. This peculiar constriction at the end of the long arm of metaphase chromosome X is characteristic in fragile X (FX) individuals. **(C)** Schematic representation of the fragile X mental retardation 1 (*FMR1*) gene. The CGG repeat is located within the untranslated region of the first exon. Expansion over 200 repeats leads to overmethylation of the promoter and transcriptional silencing. **(D)** Scatterplot of full-scale intelligence quotient (FSIQ) on FMRP levels (percentages of normal expression) showing high correlation between the two paprameters. Figures modified from Penagarikano, Mulle & Warren 2007 and Loesch, Huggins & Hagerman 2004

The fact that FXS has single gene aetiology enables us to establish a direct link between the *FMR1* gene mutation and corresponding FXS phenotype (Fig. 1.1D). In typically developing individuals, FMRP is expressed in most cells with high levels of FMRP in both foetal and adult brains (Abitbol et al., 1993; Devys, Lutz, Rouyer, Bellocq, & Mandel, 1993). In individuals with FXS, the lack of expression of *FMR1* in somatic cells (Pieretti et al., 1991) leads to a well-defined phenotype that is characterized by a range of physical, neuroanatomical, behavioural and cognitive deficits.

## 1.3 Clinical symptoms of Fragile X Syndrome

### 1.3.1 Cognitive symptoms

Intellectual disability is the main symptom of Fragile X syndrome, and can vary in severity even between members of the same family. The vast majority of patients (>90%) have IQ between 20 and 70 (Dykens et al., 1988; Cornish et al., 2001). While preschool children with FXS show an IQ close to the lower end of the average, cognitive development is significantly delayed in childhood and adolescence relative to unaffected individuals. Therefore, it is common for boys with FXS to progressively fall behind in school. By adulthood, most FXS men have an IQ well below the average of 100- around 40 - with specific deficits in visuospatial skills, attention, and executive function (Van der Molen et al., 2010). Females carrying the full mutation tend to experience learning difficulties, with 25% having cognitive defects severe enough to be characterized as intellectual disability (de Vries et al., 1996). Males with the fully mutated gene and extensive over-methylation have a mean IQ of 41, while men with the full mutation but less than 50% methylation have a mean IQ of 88 (Merenstein et al., 1996). It is thus obvious that the IQ of an individual heavily depends on the methylation pattern of his DNA, which impacts expression of FMR1 and production of Fragile X Mental Retardation Protein (FMRP) (Warren & Ashley, 1995).

### 1.3.2 Physical Symptoms

In addition to the cognitive impairments associated with FXS, which are the hallmark feature of the syndrome, a number of physical symptoms are common. The typical triad of features in FXS adult males includes macroorchidism (enlarged testicles), elongated faces and large prominent ears (Hagerman, 1997) (figure 3). Also common are flat feet,

high arched palate, and connective tissue abnormalities like hyper-extensible joints (Beckel-Mitchener & Greenough, 2004). Amongst all the aforementioned physical features, it has been suggested that large ears are a feature particularly associated with FXS and no other forms of intellectual disability (Bagni et al., 2012).

### 1.3.3 Behavioural Symptoms

Males with FXS have distinct behavioural features. Hyperactivity and attentional deficits manifest in almost 9 out 10 affected individuals, while restrictive language and, repetitive, compulsive behaviours such as hand-flapping occur in 95% of boys (Hagerman, 1997; Merenstein et al., 1996). FXS males display a pronounced eye gaze aversion, excessive shyness, and anxiety. Anxiety disorders are often seen in both male and female affected individuals and include selective aphonia (lack of speech), social seclusion, specific phobias, as well as generalized anxiety (de Vries et al., 1996; Hagerman et al., 2009; Sullivan et al., 2007). Aggressive behaviour occurs in approximately 30 to 50% of males and can also include impulsivity, tactile defensiveness and hand biting, (Hagerman et al., 2009). Females with FXS tend to have less intense and widely variable symptoms. Quite often, they display executive function and attention deficits, even when their IQ is within the normal range (de Vries et al., 1996; Hagerman et al., 2009). Visual perception deficits are also markedly affected in females with the full mutation. Finally, social deficits and social anxiety are problematic in FXS females and can lead to general shyness and selective aphonia (Elizabeth Berry-Kravis et al., 2011).

### 1.3.4 Neurological Symptoms

Affected individuals experience profound deficits in sensory processing. The pronounced hyperarousal and hyper-responsivity to auditory, tactile, visual, and olfactory stimuli in the environment, can lead to sensory defensiveness - defined as aversive and out of proportion behavioural response, to certain types of stimuli of any sensory modality, that most people would find to be non-painful (Hagerman & Hagerman, 2002). Hypersensitivity to visual stimuli or visual avoidance is evident in more than 9 out of 10 males with FXS, including high functioning males of normal intelligence (Merenstein et al., 1996). Auditory hyper-responsivity is also a common sensory integrative dysfunction in individuals with FXS as well as other types of intellectual disability. Its prevalence is quite variable, ranging from 15 to 100% in patients on the autism spectrum and is heavily dependent on the way of assessment (in

clinical research tests 15 - 40%, in questionnaires for parents 16 - 100%, in questionnaires for teachers approx. 30%) (Sullivan et al., 2007).

The prevalence of epilepsy in people with an intellectual disability (ID) is apparently higher than in the general population (McGrother et al., 2006), therefore is not a surprise that seizure susceptibility is high amongst males with FXS with a prevalence of 13 to 18%. Seizures gradually stop in adulthood (Elizabeth Berry-Kravis, 2002). Many seizures are generalized tonic-clonic in which the whole brain is involved from the onset and include a loss of consciousness and violent muscle contractions, while other episodes can be subtler, partial complex or partial motor seizures which start at a specific focal area (Musumeci et al., 1999). Seizures are characterized by muscle tension followed by convulsions, a types of seizure called tonic-clonic. If not controlled properly, status epilepticus (a state of persistent seizure) can under certain conditions lead to death. While the pathophysiology underlying seizures in FXS is not adequately understood, these seizures can be triggered by environmental stimuli (Hagerman & Hagerman, 2002). Sudden aggressive attacks not caused by such stimuli may have temporal lobe origin or be partial complex seizures (Hagerman & Hagerman, 2002).

### 1.4 Fragile X Syndrome and Autism Spectrum Disorders

Autism Spectrum Disorders (ASD) are extremely heterogeneous and their aetiology is characterised by increased biological complexity. While the main cause of most cases of ASD remains elusive, a growing body of literature suggests that ASD has a strong genetic basis. In families with a child on the autism spectrum, the risk that a future sibling will be affected is 25 to 50 times greater than for the general population (Abrahams & Geschwind, 2008; DiCicco-Bloom et al., 2006). Studies of twins yield even more powerful evidence that autism has a strong genetic component: concordance rates are between 70 and 90% for monozygotic twins and 0 - 10% for dizygotic twins (Abrahams & Geschwind, 2008). This evidence has led many researchers to search for genes associated with increased risk of ASD.

| Category of Characteristics | Genetic background |
|---|---|
| **Facial** | Large and prominent ears (75-78%) |
| | Long face |
| | Mandibular prognathism (80% adult men) |
| | Cleft palate |
| | Macrocephaly |
| **Ophthalmological** | Strabismus (8%) |
| | Refractive errors |
| **Neurological** | Seizures (23%) |
| | Hypotonia (children) |
| | Clonus (adults) |
| | Positive palmomental reflex |
| **Psychiatric** | Poor eye contact |
| | Attention deficit hyperactivity disorder |
| | Anxiety |
| | Repetitive behaviour |
| | Aggression and distress crisis |
| | Social anxiety |
| **Cognitive** | Mental retardation |
| | Cognitive and language deficit |
| **Orthopaedic** | Flat feet |
| | Hyper extensibility in the metacarpophalangeal joint |
| | Scoliosis |
| | Double jointed thumbs |
| **Genitourinary** | Macroorchidism (95% of adult men) |
| **Cardiovascular** | Cardiac abnormalities (mitral valve prolapse) |
| **Other** | Obesity, cramped teeth, tall or short stature |

**Table 1.1** Clinical characteristics of patients with Fragile X Syndrome. The majority of the phenotypic characteristics have been described in males with FXS, women typically have similar features although often less severe. Modified from Saldarriaga et al., 2014

FXS is the most common known single gene cause of autism spectrum disorders, responsible for approximately 4% of all cases (Hagerman et al., 2010). Interestingly, not all individuals with FXS meet all the criteria of ASD: 30% of males with FXS will be diagnosed at some point in their lives with full-blown autism and an additional 30% have pervasive developmental disorder not otherwise specified (PDD-NOS) (Hagerman et al., 2010). PDD-NOS is still in the autism spectrum disorders; it includes cases where the criteria for full autism have not been met due to late age of onset, atypical symptomatology, or sub-threshold symptomatology (De Bruin et al., 2007). FXS patients who do not meet the criteria to be include in the previous two groups, have at least one autistic feature, like eye gaze aversion, hand flapping etc. Comorbidity of FXS with additional neurological medical problems such as seizures, leads to an increased risk of having full-blown autism compared to patients with FXS alone (Garcia-Nonell et al., 2008). Moreover, FXS comorbidity with autism increases in male and female FXS patients who have low IQs compared to individuals on the upper end of the IQ range (Hagerman et al., 2010). This high chance of co-occurrence between FXS and ASD has led many researchers to propose that FXS, being an aetiologically better defined (i.e., a single-gene) disorder, will provide valuable insights into the aetiology of non-syndromic ASD (Belmonte & Bourgeron, 2006).

Both ASD and FXS are highly heterogeneous disorders in terms of symptom severity and manifestation amongst affected individuals. This variability is likely to be the outcome of differences in genetic background, prenatal and postnatal environmental factors, and the interplay of the two. It is obvious from the above that environmental influences and additional genetic anomalies work in an intertwined fashion to modulate the interrelationships between behavioural, cognitive, and attentional deficits in FXS (Hagerman et al., 2010). There are only a few examples of FXS patients who have additional pathological mutations - such as Down or Tourette syndrome, other sex chromosome disorders, allelic variants of the serotonin transporter that lead to increased susceptibility to depression, and gene expression changes related to Prader-Willi phenotype (Garcia-Nonell et al., 2008; David Hessl et al., 2008; Nowicki et al., 2007). Their symptoms are noticeably more severe or more multifaceted than patients with FXS alone. Males with the later condition (Prader-Willi phenotype and FXS) are usually severely obese, show excessive hunger, hypogenitalia, and a higher comorbidity with autism than patients with FXS alone (Nowicki et al., 2007). Concluding this section, we should keep in mind that the prenatal and postnatal environment is likely to impact

disease phenotype in a much greater degree than previously thought. Environmental influences on the pathophysiology of FXS and the severity of the symptoms associated with the disease, are just beginning to be explored and are likely to include exposure to toxins, abnormal immune response, and abuse or neglect (Hagerman et al., 2010).

## 1.5 The mouse model of FXS

### 1.5.1 Mouse model generation

The FMR1 gene is highly conserved across different mammalian species; Fmr1 gene in mice has a 95% homology with the human gene including the upstream regulatory region which contains the trinucleotide repeats. Moreover, the mouse homologue of FMRP shares 97% homology with human FMRP in their amino acid sequence (Ashley et al, 1993a; Ashley, et al., 1993b). Furthermore, the expression profiles of Fmr1 mRNA are very similar in terms of tissue distribution and developmental time course between humans and mice (Hergersberg et al., 1995; Hinds et al., 1993). However, possibly due to differences in epigenetic responses between mice and humans, when mice are engineered with the pathological CGG trinucleotide expansion, the Fmr1 gene does not become over-methylated and so fails to be silenced (Brouwer et al., 2007; Santoro et al., 2012).

More than 20 years ago, the first and most widely used KO (KO) mouse model was generated with a neomycin cassette interruption in exon 5 of the Fmr1 gene which results in translational silencing and loss of FMRP expression (C E Bakker et al., 1994). This outcome has a good construct validity for the full mutation that occurs in FXS patients. Since its creation, this mouse model has been proven to be invaluable in our understanding of the disease pathophysiology, the normal function of the product of the Fmr1 gene, fragile X mental retardation protein (FMRP) in health and in disease, and designing novel therapeutic strategies.

### 1.5.2 Effects of background genetics in phenotype

In the previous section about the human phenotype (section 1.2.4) it was mentioned, that there is significant heterogeneity in the symptom manifestation of complex disorders like autism and FXS, possibly due to genetic background effects, prenatal and

postnatal environment stimuli, and the interplay between the two (Hagerman et al., 2010). Therefore, not all affected individuals express the same constellation of symptoms, nor do these symptoms manifest themselves with the same severity. This heterogeneity is also evident in the mouse model of FXS (Table 1.3). Taking into account the research in the field so far, most behavioural studies on the mouse model of FXS have been conducted on a pure B6 or FVB strain (Kazdoba, Leach, & Crawley, 2016; Santos, Kanellopoulos, & Bagni, 2014), though relatively recent studies have explored how behavioural phenotypes are modified by genetic strain (Corinne M. Spencer et al., 2011). The effects of genetic background on behaviour and physiology have been documented previously (Sittig et al., 2016) but it is plausible that the diversity seen in the Fmr1 KO mouse phenotype echoes the range of clinical symptoms amongst individuals with FXS, rather than being due to subtle differences in experimental procedures or genetic background influence alone. The inconsistency in the magnitude and direction of phenotypic differences seen in the Fmr1 KO mouse may at first seem discouraging and may suggest the idea of disregarding the model altogether. However, the heterogeneity of FXS is so notable that affected individuals show a range of cognitive impairments, with affected males displaying mild to severe cognitive symptoms (Hessl et al., 2009; Schneider et al., 2009). This poses an obvious challenge for FXS animal models, but it might also be considered an advantage.

### 1.5.3 Pathophysiology of the *Fmr1* KO mouse

#### Dendritic spine morphology and neurotransmission

Fragile X mental retardation protein (FMRP) functions primarily as an RNA-binding protein and is highly expressed in neurons, and more specifically in the cell soma, the dendrites and postsynaptic terminals (Antar et al., 2004; Bakker et al., 2000; Feng et al., 1997). Over the last years, the functional characterization of FMRP has revealed its specific dynamics: FMRP enters the nucleus and interacts with pre-messenger ribonucleoprotein (pre-mRNP) complexes in order to escort them to the cytoplasm (Fig 1.2). FMRP-containing mRNPs are largely associated to polyribosomes and involved in translational control both in soma and in dendritic spines (Bardoni et al., 2006; Dury et al., 2013). Furthermore, in neurons, some of the FMRP-mRNP complexes can be translocated to distant locations, such as dendrites, as component of RNA-granules, where they mediate the binding between mRNAs and molecular motors, such as

kinesins, promoting transport upon specific stimuli (Bassell & Warren, 2008; Davidovic et al., 2007). It is likely that this mechanism also influences abundance of a subset of mRNAs in synapses (Dictenberg et al., 2008). Decreased capacity to transport mRNA and control local translation into distal processes may result in an abnormal level of their protein products with consequences on the structure and synaptic plasticity, as observed in FXS patients and existing animal models (Maurin, Zongaro, & Bardoni, 2014).

Dendritic spines, small protuberances along the neuronal dendrites, are loci of excitatory and inhibitory synaptic input, where a large number of ligand receptors and various signalling molecules that are essential for synaptic function are located (Esther a Nimchinsky, Sabatini, & Svoboda, 2002). Post-mortem analysis of human brain tissue revealed that individuals affected by FXS have an elevated density of dendritic spines compared to age-matched unaffected subjects, with the vast majority of dendritic spines appearing lengthened and immature (Greenough et al., 2001; Hinton et al., 1991; Rudelli et al., 1985; Wisniewski et al., 1991). *Fmr1* KO mice bred on both the B6 and the FVB genetic background exhibit directly analogous deficiencies in spine quantity and morphology (Galvez et al., 2003; Galvez & Greenough, 2005; McKinney et al., 2005; Nimchinsky et al., 2001), contributing additional face validity to the *Fmr1* mouse model. Analysis of the developing barrel cortex of young (1 week old) *Fmr1* KO mice revealed enhanced spine density and increased spine length in mutant mice compared to control littermates; this difference was not evident at 4 weeks of age (Nimchinsky et al., 2001). This lack of spine irregularities in 4 weeks' old mice was also seen in the developing somatosensory cortex of *Fmr1* KO mice by the Galvez and Greenough (2005). In the same study, adult *Fmr1* KO mice were shown to exhibit increased density of immature, thin elongated spines compared to control littermates (Galvez & Greenough, 2005). This leads us to hypothesise that there may be a short time period in postnatal synaptic development, during which dendritic spine morphology normalises in the absence of FMRP, but is not maintained throughout life. Similar structural deficits in dendritic spines have been observed in various other brain regions of older *Fmr1* KO mice. For example, *Fmr1* KO mice possess higher densities of lengthened spines in the visual cortex at 16 weeks of age compared to wildtype littermate controls (Comery et al., 1997). These studies suggest that the presence of functional FMRP is essential for the development of healthy dendritic spine morphology, and that the loss of FMRP effects adversely the normal structure of the synapse.

As a negative regulator of mRNA translation, FMRP heavily influences protein synthesis and amongst other things can affect the fine balance of components in the synaptic machinery located in dendritic spines (Fig. 1.3). Long term potentiation (LTP) and depression (LTD) are two basic plasticity mechanisms which lead to long lasting enhancement and decrease, respectively, of signal transduction between two neuronal synapses (Buffington et al., 2014; Malenka & Bear, 2004; Whitlock et al., 2006). These activity-dependent cellular events rely heavily on transcriptional and translational regulation of synaptic proteins in order to rapidly and accurately respond to synaptic activity modulation and support cognitive function. Studies focussed on plasticity mechanisms such as LTP and LTD, which are considered to express the electrophysiological correlates of learning and memory processes (Malenka & Bear, 2004; Takeuchi, Duszkiewicz, & Morris, 2014), have revealed defects in various brain structures of mice lacking the *Fmr1* gene (Table 1.2). A specific form of LTD, which is dependent on protein synthesis and metabotropic glutamate receptor (mGluR) activation (mGluR-LTD), has been shown to be enhanced in hippocampus *Fmr1* KO mice and cultured hippocampal neurons where FMRP was reduced with the use of small interfering RNAs (siRNAs) (Huber et al., 2002; Nakamoto et al., 2007; Nosyreva & Huber, 2006). LTP, along with decreased AMPA receptor surface localisation and selective increases in NMDA receptor subunit protein expression, has been shown to be impaired in *Fmr1* KO mice (Krueger et al., 2011; Li et al., 2002; Nosyreva & Huber, 2006; Schütt et al., 2009; Seese et al., 2012; Shang et al., 2009). Two different FMRP deficient mouse models also display abnormal synaptic plasticity. The first is *Fmr1* KO2 mice, an *Fmr1* null mouse model which lacks both FMRP and *Fmr1* mRNA due to deletion of the *Fmr1* promoter and first exon (Mientjes et al., 2006). The second is called *Fmr1* I304N, a relatively new FXS mouse model in which the endogenous *Fmr1* gene harbours an isoleucine to asparagine mutation (I304N) which leads to a non-functional FMRP (Zang et al., 2009). In the hippocampus of *Fmr1* KO2 mice, a lower ratio of AMPA to NMDA receptors was detected early in postnatal development compared to wildtype littermate controls (Pilpel et al., 2009b). The upregulation of NMDA receptors in the *Fmr1* KO2 hippocampus resulted in increased NMDAR-dependent LTP. Taken together, this data confirms that the absence of *Fmr1* results in anomalies in normal synaptic development and activity, which is likely to contribute to the FXS behavioural and neurological phenotype. Given the importance of FMRP for the regulation of proteins vital to synaptic function, it is not surprising that FMRP deficiency results in defects in the structure and function of synapses.

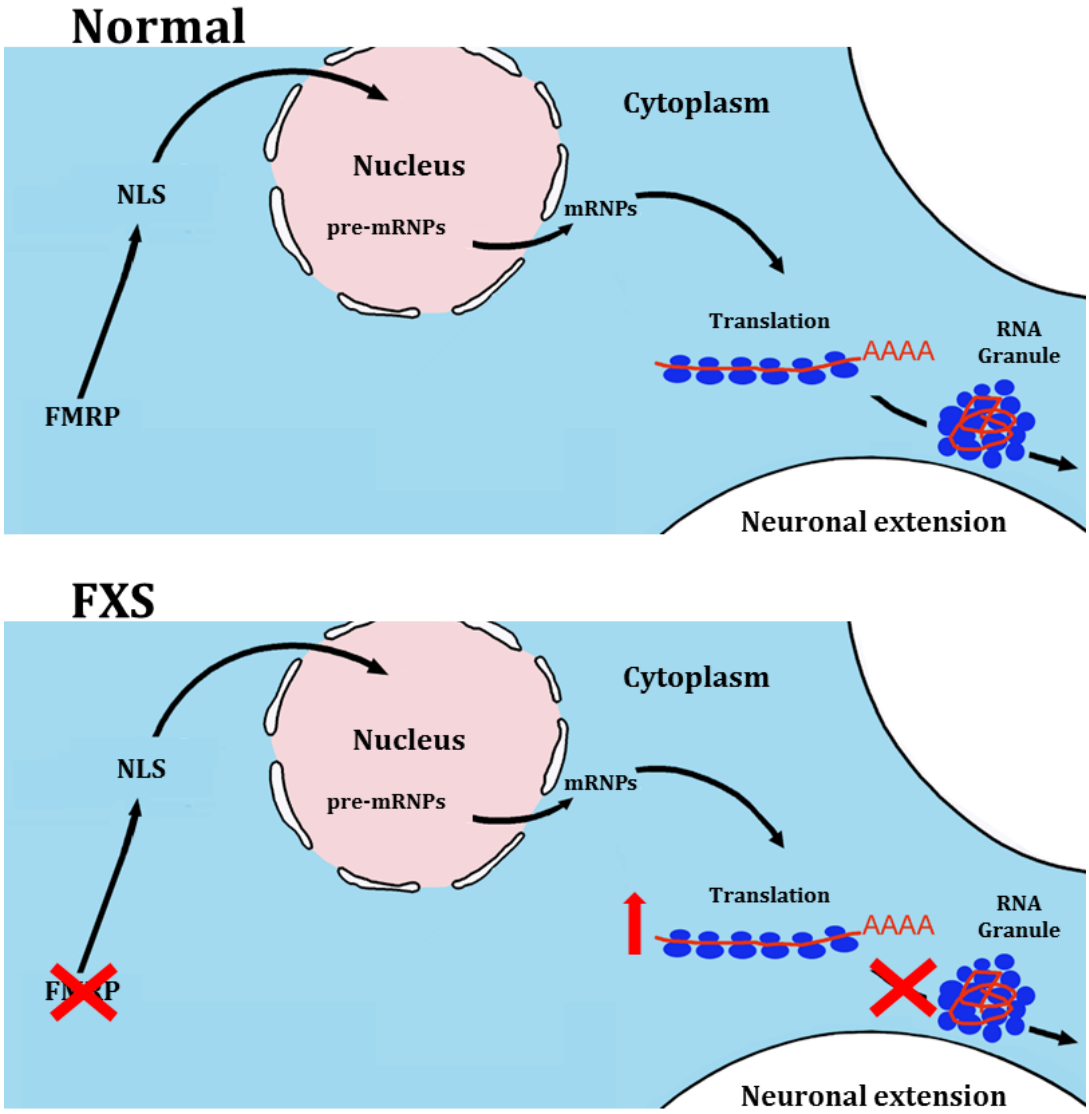



**Figure 1.2 Schematic representation of FMRP function.** (A) FMRP interacts with protein partners that lock the Nuclear Localisation Signal (NLS) domain and let it enter the nucleus and join the nascent messenger ribonucleoproteins (mRNPs) complexes emerging from the nuclear pores. In the cytoplasm the FMRP-mRNPs complexes either associate with the translation machinery (when needed) or are transported in RNA-granules to distal parts of the neuron staying translationally repressed until needed. (B) In the absence of FMRP, mRNPs cannot be regulated and the binding of RNA granules to transport proteins cannot take place, since FMRP normally acts as an adaptor. As a results mRNA translation is not regulated properly and protein synthesis is increased. Figure modified from Dury et al., 2013

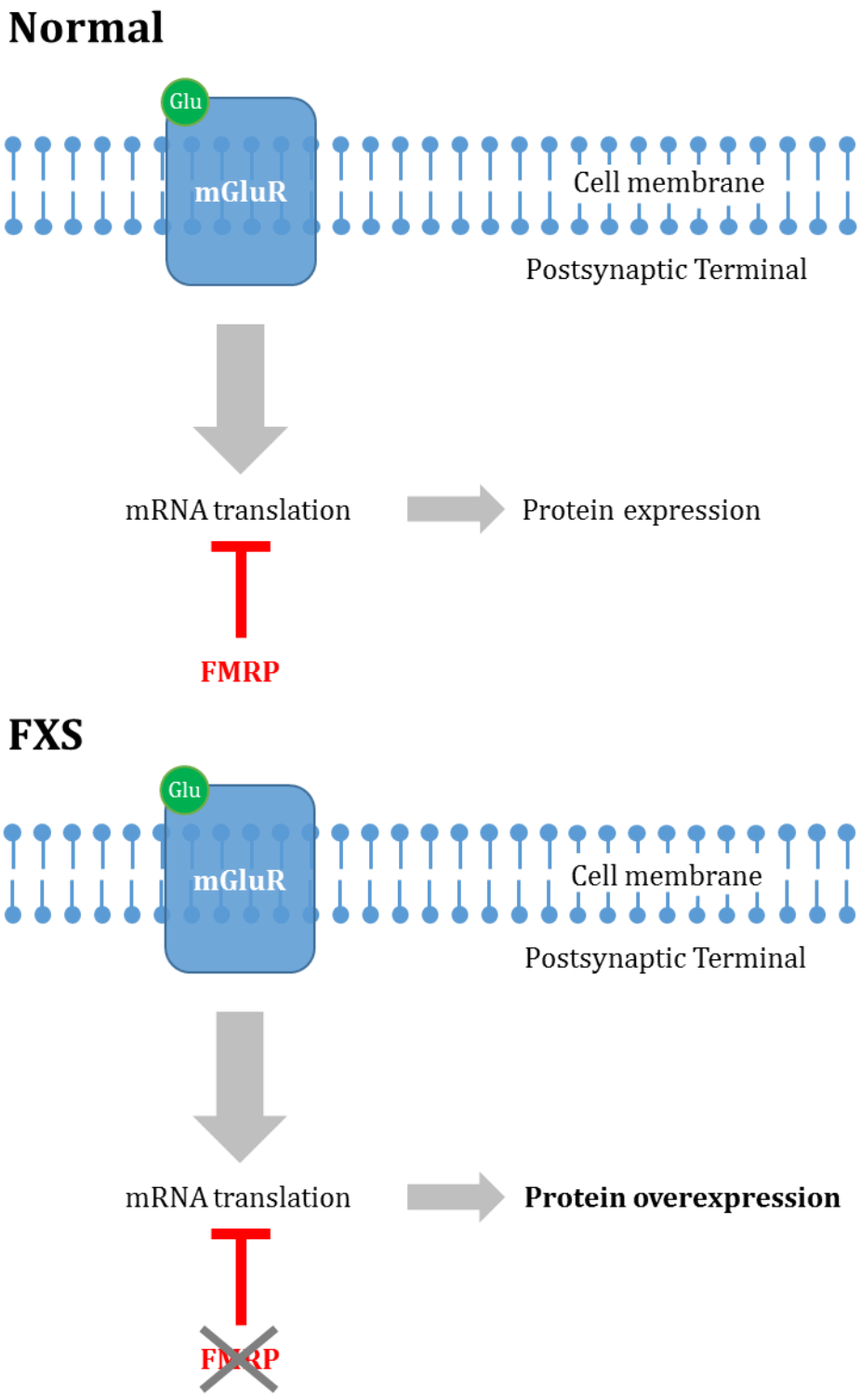



**Figure 1.3 Role of Fragile X mental retardation protein (FMRP) in modulating synaptic plasticity.** The activation of group I metabotropic receptors (mGluR) stimulates translation of specific mRNAs at synapses. FMRP normally acts as a translational repressor regulating such expression; in the absence of FMRP, these mRNAs are over-translated leading to abnormalities in synapse structure and function.

| Plasticity mechanism | Brain region | Observed phenotype | Age | Reference examples |
|---|---|---|---|---|
| **mGluR LTD** | Hippocampus | Enhanced | P25-30 | Bhattacharya et al., 2012 |
| | Hippocampus | Protein synthesis independent | 4-12 wk | Nosyreva and Huber, 2006 |
| | Cerebellum | Enhanced | 3-7 wk | Koekkoek et al., 2005 |
| | Hippocampus | Enhanced and PS indipendant | 3-7 wk | Volk et al., 2007 |
| **LTP** | Hippocampus | Normal | 8-10 wk | Li et al., 2002 |
| | Hippocampus | Deficient with weak stimulus; normal with strong | 2-3 month | Lauterborn et al., 2007 |
| **Late-LTP** | Hippocampus | Normal | 5-7 wk | Paradee et al., 1999 |
| **LTP** | Anterior cingulate cortex | Deficient | 6-8 wk | Zhao et al., 2005 |
| | Somatosensory, temporal cortex | Deficient | 8-10 wk | Li et al., 2002 |
| | Somatosensory cortex | Delayed window for plasticity | P3-10 | Harlow et al., 2010 |
| | Amygdala | Impaired (mGluR-dependent) | 6-8 wk | Zhao et al., 2005 |
| **STD-LTP** | Prefrontal cortex | Deficient with weak stimulus; normal with strong stimulus | P14-23 | Meredith et al., 2007 |
| | Somatosensory cortex | Deficient with weak stimulus | P10-18 | Desai et al., 2006 |

**Table 1.2 Synaptic plasticity phenotypes observed in the mouse model of FXS.** Modified from Sidorov, Benjamin Bear 2013).

**Physical Symptoms**

Like males with FXS *Fmr1* KO mice have notably enlarged testes compared to wildtype littermate controls, but overall normal structural morphology (Ce E Bakker et al., 1994; Slegtenhorst-Eegdeman et al., 1998). It has been shown that this difference in testicle size is due to elevated proliferative activity of Sertoli cells which help in the process of spermatogenesis; This increased activity increases the number of germs cells in the testicles, and therefore, their weight and size (Slegtenhorst-Eegdeman et al., 1998). The presence of enlarged testes echoes the macroorchidism seen in male individuals with FXS, and therefore extends the face validity to the *Fmr1* KO mouse model in this aspect of the clinical disorder. Other physical characteristics, such as body temperature, body weight, and neurological reflexes do not differ between KO and wildtype mice, suggesting an otherwise typical general physical development (Ce E Bakker et al., 1994; Peier et al., 2000). There is also no prominent effect on any facial features or connective tissue as is observed in individuals affected by FXS, at least none that are perceivable to human observers (Hagerman, 1997).

### 1.5.4 Cognitive deficits of the *Fmr1* KO mouse

I mentioned earlier that intellectual impairments are a prominent feature of FXS in affected individuals and can range from mild to severe. The difference in IQ scores between human patients and neurotypical individuals widens over time until around 12 years of age, which is probably due to the delayed development individuals with FXS experience rather than a true deterioration in overall intellectual functioning (Hall et al., 2008; Skinner et al., 2005). Novel approaches in cognitive testing suggest that conventional IQ tests can be adapted in order to reveal subtle irregularities within a selected population (Hessl et al., 2009). The hippocampus, an important brain structure for cognition, has been found to be larger in individuals with FXS (Kates et al., 1997; Reiss, Lee, & Freund, 1994); additionally, functional deficits in hippocampus-dependent tasks in subjects with FXS (Cornish, Munir, & Cross, 1998, 1999) would suggest that any hippocampus dependent task should reveal a deficit in mouse models of FXS. Furthermore, while no volume or morphology abnormalities in amygdala and prefrontal cortex of individuals with FXS have been reported, FXS patients have abnormal behavioural responses in tasks requiring the amygdala (S. Y. Kim et al., 2014), frontal lobe (Mazzocco et al., 1992) and prefrontal cortex (Kwon et al., 2001). Therefore, larger

hippocampal volumes and hippocampal morphology differences seen in FXS patients (Jäkälä et al., 1997; Reiss et al., 1994) may or may not relate to the deficits in hippocampal-dependent memory. Keeping in mind that no cognitive task so far depends on a single brain structure; the aforementioned discrepancies could represent another instance in which behavioural tasks that require functional circuits (*i.e.*, the limbic system) may lead to unpredictable outcomes when multiple neural components within that system are impaired (*i.e.*, prefrontal cortex, amygdala, and hippocampus).

Decades of research focussed on the characterisation of the cognitive abilities in individuals with FXS predict that deficits in a FXS mouse models should occur in short-term (visuospatial) memory, visuospatial abilities, sequential information processing, associative learning, executive function and attention (Cianchetti et al., 1991; Kemper, Hagerman, & Altshul-Stark, 1988; Maes et al., 1994; Reiss & Freund, 1992) Starting from the Dutch-Belgium Fragile X Consortium (1994), many researchers over the last 22 years have conducted systematic characterizations of *Fmr1* KO mice and compared the observed phenotypes to the intellectual disabilities exhibited by individuals with FXS.

**Spatial and Working memory**

The Morris watermaze (MWM) is a setup commonly used to evaluate hippocampal-dependent spatial learning in rodents, using a variety of tasks. In the simplest task version (Spatial Reference Memory-SRM task), subjects are trained over several days (usually 1 week) to locate a submerged platform using spatial cues. The latency to find an escape platform from a pool of opaque water decreases with the number of training trials indicating learning. This test, which was initially developed by Richard Morris, is used as a robust readout for spatial learning and memory (Morris et al., 1982). The SRM task was used from the first study describing the creation of the *Fmr1* KO mouse, to evaluate its visuospatial learning and memory (Bakker et al., 1994). The study did reveal mild genotype differences, such as that *Fmr1* KOs' performance was significantly poorer in reversal training (*i.e.*, a change of platform location to the opposite position in the pool) than their wildtype littermates, specifically during the first few trials after location-switching. This difference may reflect an impairment when dealing with alternating reinforcement parameters. Surprisingly however, when the platform was removed from the pool and mice had search for 60 seconds to locate it (probe trial), there were no performance differences between groups; this suggests that visuospatial memory is unimpaired in *Fmr1* KO mice. When Kooy and colleagues (1996) repeated the original

Consortium study (Bakker et al., 1994) using a larger sample size, they observed very similar results in the watermaze reference and reversal task, with the additional finding of a genotype difference during the initial spatial memory acquisition. Again however, no significant differences in the time spent looking in the right platform area, during the probe trial, were observed; this could mean that while there are some differences in watermaze performance, these may not be functionally relevant to the cognitive deficits associated with FXS. Despite the deficit *Fmr1* KOs exhibit in reversal training in watermaze, a different reversal learning task using an E-shaped maze filled with water revealed no difference between genotypes (Kooy et al., 1996). However, even though *Fmr1* KO mice did not show a robust perseveration phenotype across slightly different cognitive modalities (i.e., impaired reversal in Morris water maze, but not E-shaped maze), a cross-shaped watermaze replicated the watermaze acquisition deficit, even if that was in just one of two used background genetic strains (Dobkin et al., 2000; Van Dam et al., 2000). The aforementioned initial learning deficits have been replicated by some (D'Hooge et al., 1997), but others found no differences between groups (Paradee et al., 1999; Uutela et al., 2012). Similarly, the perseveration deficits *Fmr1* KO mice exhibit in reversal learning were replicated by some studies (K. B. Baker et al., 2010; D'Hooge et al., 1997) but not others (Paradee et al., 1999).

This inconsistency in results across laboratories, points to the idea that the spatial learning deficits observed in the first studies may be due small changes in experimental conditions that cannot be systematically controlled. The vast majority of published studies -recently Baker and colleagues (2010) reported some probe trial differences- agrees that the probe trial analyses do not yield any differences between *Fmr1* KO and wildtype mice, indicating limited deficits in spatial learning and memory. To further understand the role of FMRP in spatial learning and memory, the performance of *Fmr1* KO mice has also been analysed in other dry-land mazes. In the Barnes maze (BM) for example, rodents are trained to find an escape hole based on distal extra-maze cues, and contrary to what has been found in the MWM, in the BM the *Fmr1* KO mice show significant differences in retrieval and memory consolidation compared to wildtype littermates (Yan et al., 2004). In addition, a relatively recent study (Guo et al., 2012) showed that *Fmr1* KO mice exhibit a decreased ability to preserve spatial information after food reward, in a radial arm maze apparatus, compared to control subjects. We have to remember that some researchers have reported task-specific impairments in spatial cognition rather than global impairments (Cornish et al., 1998, 1999), although global

cognitive impairments in individuals with FXS have also been reported (Hall et al., 2008; D Hessl et al., 2009; Skinner et al., 2005). The mild deficits in spatial learning and memory reported in *Fmr1* KO mice could support the hypothesis of task-specific cognitive deficits and not global cognitive dysfunction.

Working memory has been shown to be affected in FXS and it is suggested to be amongst the core features of the syndrome (Baker et al., 2011). Several human clinical studies have shown that individuals with FXS exhibit low performance on specific working memory tasks under low-control conditions -verbal and visuospatial (K. Cornish et al., 2001; Dykens, Hodapp, & Leckman, 1987; Jäkälä et al., 1997; Munir, Cornish, & Wilding, 2000). Lanfranchi and colleagues (2009) recently reported working associative memory deficits under high-control conditions (i.e., a dual task requirement; for example, specific word recall only upon the presentation of a stimulus with particular properties) in affected individuals; these deficits were specific to another component of working memory, central executive functioning. While these studies and others suggest that human cognition deficits in FXS are task-specific and not global in nature, additional research has revealed impairments in all components of working memory in FXS regardless of task complexity and modality (Baker et al., 2011; Munir et al., 2000). The conflicting results related to specific and general working memory abnormalities in individuals with FXS could be due to task-specific contingencies (e.g., the type of stimuli used), as FXS patients shown more accurate recall with familiar stimuli rather than with abstract novel material (Maes et al., 1994). In *Fmr1* KO mice, different working memory tasks, such as olfactory working memory and visuospatial working memory in a radial arm maze, can rely heavily on different brain regions (i.e., olfactory bulb or hippocampus, respectively). In several tasks, including those in the radial arm maze, *Fmr1* KO mice did not show to exhibit robust working memory deficits (Yan et al., 2004), although others have reported working memory impairments in *Fmr1* KOs in a version of a serial reversal task in the watermaze (Baker et al., 2010). Furthermore, *Fmr1* KOs performed similarly to the wildtype littermates in the "olfactory discrimination task" (Guo et al., 2011; Mineur et al., 2002; Moon et al., 2008; Yan et al., 2004), suggesting that their working memory (which correlates with IQ in humans) is not affected. It is plausible that the olfactory bulb, hippocampus or other brain structures are compensating for deficiencies in working memory in some of these tasks. Therefore, behavioural tasks which are less dependent on multiple brain regions are necessary in order to examine whether *Fmr1* KO mice

exhibit robust working memory deficits, as this would expand the face validity of the model.

The conflicting results in cognitive assays to date have sparked a debate as to whether the *Fmr1* KO mouse is a reliable and sufficient model of FXS in humans, since the core symptom of intellectual impairment is not prominent enough in the mutant mouse model.

**Associative memory**

Fear conditioning studies have been used to further elucidate whether other specific cognitive domains (i.e. associative learning) are affected in *Fmr1* KO mice (Phillips & LeDoux, 1992). Fear conditioning can be divided into several distinct subtypes that dependent on the amygdala, hippocampus, and prefrontal cortex, each to a different extent. Contextual fear conditioning requires both the amygdala and hippocampus, while delay-cued fear conditioning requires the amygdala but not the hippocampus (Fanselow & Kim, 1994; Gould & Leach, 2014; Logue, Paylor, & Wehner, 1997). Contextual and delay-cued fear conditioning can also take place during the same training session and can be assessed using different settings in order to reveal hippocampus-dependent and hippocampus-independent memory effects, respectively. Trace-cued fear conditioning, a more difficult task in which the tone and shock are not simultaneous during training, requires hippocampus and prefrontal cortex (Gilmartin & Helmstetter, 2010; Runyan, Moore, & Dash, 2004) and may or may not be independent of the amygdala (Gilmartin, Kwapis, & Helmstetter, 2012; Raybuck & Matthew Lattal, 2011). In these tasks a conditioned and non-aversive stimulus (a tone, a smell or an environment) is coupled with a harmful unconditioned stimulus. As a result of this pairing, the conditioned stimulus "obtains" the aversive properties of the unconditioned stimulus, leading subjects to react aversively (freezing) to it; this response can be used as a readout of a defensive behaviour and, by association, an expression of memory (Wehner & Radcliffe, 2004). When *Fmr1* KO mice were tested in both cued (tone) and contextual (environment) fear conditioning paradigms, they were shown to freeze less than the control littermates indicating associative memory deficits (Ding, Sethna, & Wang, 2014; Guo et al., 2011, 2012; Hayashi et al., 2007; Olmos-Serrano, Corbin, & Burns, 2011; Paradee et al., 1999). Trace fear conditioning studies have revealed mixed results: some showed *Fmr1* KO mice may have deficits (Zhao et al., 2005) while others showed *Fmr1*

KOs to be identical if not slightly better than their wildtype littermates in acquisition of trace fear conditioning (Baker et al., 2010).

As in the case of spatial and working memory, other research groups did not report any memory deficits in the *Fmr1* KOs compared to littermate controls, using the same behavioural tasks (Peier et al., 2000; Uutela et al., 2012; Van Dam et al., 2000). These inconsistences could, once again, stem from small differences in the experimental protocols and/or from the influence of the background genetic strain (Corinne M. Spencer et al., 2011). Interestingly, Olmos-Serrano and colleagues (Olmos-Serrano et al., 2011) reported decreased freezing time in cued fear conditioning, when the same tone is presented in an altered environment (different context) from the initial chamber where the training took place, suggesting that the amygdala-dependent learning is most impaired.

One cognitive test which was used very early on in the behavioural assessment of the *Fmr1* KO mouse model is the passive avoidance task. This task utilizes the association of a mild foot-shock (just like fear conditioning) with an apparently safer but previously "punished" dark compartment of a maze in order to assess memory for the aversive event. Passive avoidance learning relies heavily on the dorsal hippocampus (Lorenzini et al., 1996) but because it involves fear conditioning, it also requires the amygdala (Slotnick, 1973). This dependence of performance in passive avoidance task on the dorsal hippocampus and amygdala would predict that subjects showing abnormalities in the function of either or both of these brain structures -like *Fmr1* KO mice would show decreased performance in this task, but unfortunately experimental data is inconsistent. Indeed, while control mice take more time or even refuse to enter the dark compartment, because they associate it with the shock to their paws, the *Fmr1* KO mice show a range of behavioural responses. Even though amygdala volume or structure are not typically affected, amongst individuals with FXS, affected individuals with FXS experience difficulties with emotion regulation. Furthermore, a recent study revealed that individuals with FXS displayed deceased activation of the amygdala, relative to healthy age-matched subjects, while viewing fearful faces (Kim et al., 2014); this indicates a difference in the processing of potentially fearful stimuli. Inconsistencies in behaviour have been obvious in this type of task as well; passive avoidance learning appeared to be unaffected in *Fmr1* KO mice in some studies (Bakker et al., 1994; Dolen et al., 2007; Veeraragavan et al., 2012; Veeraragavan et al., 2011) but was found to be disrupted in

others (Ding et al., 2014; Michalon et al., 2012, 2014; Yuskaitis et al., 2010). Interestingly, *Fmr1* KO mice seem to be more susceptible to extinction training than wildtype littermates - as shown by shorter latencies to enter the dark compartment (Dölen et al., 2007; Michalon et al., 2012), a finding which is consistent with the exaggerated extinction *Fmr1* KOs exhibit in other assays (Sidorov et al., 2014). Looking again at the divergence in the behaviour response of *Fmr1* KOs we could speculate that existing cognitive deficits combined with abnormal responses to fearful stimuli are working in opposition, explaining some of the inconsistent results in fear-associated tasks such as passive avoidance.

A different task which was used to assess, amongst other cognitive components, associative memory in *Fmr1* KO mice was the "five-choice serial reaction time" task. This paradigm can be used to measure to main aspects of cognition: visuospatial attention and impulsivity. Mice are required to observe a random light in one of five small holes, located on side of the testing box, and respond in a timely manner with a nose-poke in the correct spatial location, in order to receive a food reward. *Fmr1* KO mice were found to be significantly impaired in the acquisition phase; they showed an increased number of errors per trial during the training period (Krueger et al., 2011). They were therefore slower in reaching a pre-set training criterion and completing the task. However, they were able to complete the task.

In an attempt to implement rodent cognitive tasks with more ethological relevance, more recent studies have included spontaneous exploration tasks including novel object recognition as well as spatial and temporal order object recognition tasks. Novel object recognition, which is typically used as a short-term memory task, exploits rodents' innate behaviour in investigating novelty. In the case of novel object recognition (NOR), a subject is placed into a testing box with two identical copies of an object. After a certain interval, the experimental subject is returned to the box where one of the familiar objects has been replaced by novel one (Bevins & Besheer, 2006). If the mouse remembers the previously seen object, it preferentially investigates the novel object. *Fmr1* KO mice have been shown to have a memory deficit in this task (Busquets-Garcia et al., 2013; King & Jope, 2013; Ventura, Pascucci, Catania, Musumeci, & Puglisi-Allegra, 2004), but as with all the previously discussed cognitive domains, this impairment has not always been replicated successfully (Yan et al., 2004). Lastly a recent study reported that *Fmr1* KO

mice are impaired in a hippocampus-dependent spatial object recognition task, known as object location or object displacement task (King & Jope, 2013).

### 1.5.5 Behavioural abnormalities of the *Fmr1* KO mouse

#### Anxiety, Attention and Hyperactivity

Anxiety is one of the central behavioural features of individuals with FXS, which persists throughout their life (Hagerman & Hagerman, 2002). Anxiety-related behaviour testing in *Fmr1* KO mice, like all previously discussed behaviours, has generated quite inconsistent results, ranging from reduced anxiety-like behaviour in *Fmr1* KOs, to no genotype differences, to increased anxiety-like behaviour on numerous tasks. The elevated plus-maze is an anxiety-related task that utilizes rodents' natural preference for enclosed shady spaces by observing the amount of time and entries made into dark, enclosed (safe) arms as compared to open (exposed-unsafe) arms of an elevated-plus maze (Handley & Mithani, 1984; Lister, 1987; Peier et al., 2000). *Fmr1* KO mice have been shown to spend significantly more time in the open arms and less time in the closed arms, but also have travelled more throughout the maze compared to controls. These findings may simply indicate higher general locomotion or they could be interpreted as decreased fear of exposed areas (Heulens et al., 2012; Liu, Chuang, & Smith, 2011; Yuskaitis et al., 2010). In a very similar task to the elevated plus maze set-up called the zero-maze, *Fmr1* KO mice spent more time in the open quadrants of the maze (Z.-H. Liu et al., 2011). Furthermore, in the open field test, reduced time spent or distance travelled in the centre of an open area is traditionally considered an indicator for anxiety-related behaviour, since wildtype mice prefer to remain in the perimeter (thigmotaxis) when introduced to a novel environment. *Fmr1* KO mice spent a greater portion of their time in the central area of the open field compared to wildtype control mice (Spencer et al., 2005; Yan et al., 2004; Yuskaitis et al., 2010). Taken together, these reports point to a profile of lower anxiety-related behaviours in *Fmr1* KO mice, which is the opposite to the FXS clinical phenotype. In contrast to the findings of the previously discussed studies, others have shown *Fmr1* KO mice to exhibit increased anxiety-like responses in the mirrored chamber task (Spencer et al., 2005), avoidance of the central area of the open field (Restivo et al., 2005) and reduced time spent in the open arms of the elevated plus-maze (T V Bilousova et al., 2009). Another task which has been used to assess anxiety-related behaviours is the light-dark exploration test. The apparatus is divided into two

compartments; a well-lit compartment and dark enclosed one. A typical mouse subject spends more time in a dark part of the testing box than the well-lit (J. Crawley & Goodwin, 1980). It has been shown that mice which received anxiolytic drug treatments increase the number of transitions between compartments (J N Crawley, 1985). When *Fmr1* KO mice were tested in the light-dark test, they made more transitions between the chambers (Ding et al., 2014; Spencer et al., 2011), but did not show any differences in the time they spent in the light chamber compared to wildtype littermates. This could mean that the hyperactivity *Fmr1* KOs exhibit could hide any anxiety-related phenotypes when the tasks used, rely on scoring locomotion. Contrary to the aforementioned studies, several research groups reported no differences between genotypes in the elevated plus-maze (Mineur et al., 2002; Nielsen et al., 2002; Yan et al., 2004), in light-dark test (Spencer et al., 2007), or in the open field (Veeraragavan et al., 2012, 2011). These conflicting results could potentially be explained as a combination of differences in experimental parameters and housing conditions, genetic background, and age at testing (Walf & Frye, 2007). Moreover, elevated locomotion in *Fmr1* KO mice could mask anxiety-like behaviours for the simple reason that all three of these tasks (elevated plus maze, light dark box, zero maze, open-field) use, to some degree, subjects' movement as a measure. Given the sensitive nature of anxiety-related assays, it is important that similar testing protocols are used across labs to determine the robustness of the *Fmr1* KO genotype on anxiety-related phenotypes, but also that novel tasks which tease out these behaviours without taking into account locomotion, should be employed.

It has been mentioned that individuals with FXS exhibit hyperactivity and have difficulties with attention and impulse control (Cornish et al., 2004; Hagerman & Hagerman, 2002; Hatton et al., 2002). Compared to individuals with other forms of severe intellectual disability, individuals with FXS performed better on selective attention, no difference between subjects was found in sustained attention and working memory (Bailey et al., 2001). Moreover, different studies have found that as task difficulty increases, individuals with FXS exhibit more profound attentional deficits, especially when subjects have to inhibit/switch previously learned responses (Wilding, Cornish, & Munir, 2002). In view of FXS clinical symptoms (i.e., its common comorbidity with ADHD), *Fmr1* KO mice were assessed in the previously discussed, "five-choice serial reaction time" task, which is considered the most reliable task for attention and impulsivity in rodents (Winstanley, Eagle, & Robbins, 2006). Although *Fmr1* KO mice were impaired in the acquisition phase of a visuospatial discrimination task, they were

identical to wildtype controls in the five-choice serial reaction time task (Kramvis et al., 2013; Krueger et al., 2011). Specifically, Krueger and colleagues found that *Fmr1* KOs need more training in order to reach a pre-set criterion during initial training (> 50% correct of > 15 trials for 2 consecutive days), when nose-pokes in illuminated holes were marked as correct and nose-pokes in non-illuminated holes were incorrect, but this effect could not be replicated in subsequent studies (Sidorov et al., 2014). Sidorov and colleagues reported a different behaviour instead: they observed enhanced extinction of nose-poke responses in *Fmr1* KOs, which is consistent with the enhanced extinction training seen by some research groups in a passive avoidance paradigm. In a different series of tasks assessing attention, *Fmr1* KO mice were shown to exhibit impaired inhibitory control, exhibiting a higher rate of premature responses than wildtype mice (Moon et al., 2006). This was associated with changes in task parameters, suggesting that inhibitory control in *Fmr1* KO mice could be affected by stress or novelty. Additionally, the incorporation of olfactory distracters in the task significantly disrupted the performance of *Fmr1* KO mice; making more inaccurate responses during distracter presentations (Moon et al., 2006). Perhaps the most consistent behavioural finding in *Fmr1* KO mice is their increased locomotor activity in the open field test compared to wildtype littermate controls (Bakker et al., 1994; Ding et al., 2014; Moon et al., 2006; Peier et al., 2000). It is important to keep in mind that the observed robust hyperactivity phenotype seen in *Fmr1* KOs could easily be a confounding factor for the assessment of sustained attention, given that elevated general activity of mutant mice can easily interfere with task engagement.

**Social interactions and Communication**

Together with high levels of anxiety, individuals with FXS are often diagnosed with social phobias and social avoidance, especially those who are on the autistic spectrum (Cohen et al., 1988; Gross, Berry-Kravis, & Bassell, 2012; Hagerman & Hagerman, 2002). In rodents, a test that assesses preference for social interactions and social novelty is the "three-chambered apparatus", in which the experimental subject is given the choice between exploring a compartment (chamber) containing a stranger/novel mouse or an empty one (alternatively an object/non-social). Usually testing includes a second phase in which the experimental subject can choose between the previously encountered/familiar mouse and a new stranger mouse. The numbers of approaches and the time spent in proximity with each mouse are scored and usually expressed as raw

exploration times or an index expressing the exploration of the novel social stimulus (e.g., novel stranger mouse) versus the novel object or familiar mouse stimulus (Moy et al., 2004). Neurotypical mice will preferentially explore a novel mouse when given the choice between a novel mouse and a novel object or a novel mouse and familiar mouse. Results using the three-chambered social approach with *Fmr1* KO mice to evaluate their sociability are inconsistent in the literature. For example, several research groups have reported that *Fmr1* KO mice have unaffected sociability, preferring the social over the non-social maze compartment (Bhattacharya et al., 2012; Liu & Smith, 2009; McNaughton et al., 2008; Mines et al., 2010; Pietropaolo et al., 2014). On the other hand, *Fmr1* KO mice are affected in social novelty discrimination; they do not show any preference for the unfamiliar stranger mouse in the second phase of the paradigm (Bhattacharya et al., 2012; Mines et al., 2010). Moreover, *Fmr1* KO males show less interest in social interaction with novel female mice (Mineur, Huynh, & Crusio, 2006) as well as impaired social dominance when tested in the "tube-test" with unfamiliar wildtype mice (C. M. Spencer et al., 2005). Such deficits may be partially explained by augmented social anxiety, as seen by the increased rearing and digging behaviour that the *Fmr1* KO mice display in the presence of another mouse (Liu & Smith, 2009; McNaughton et al., 2008; Mines et al., 2010). We have to keep in mind that anxiety phenotypes in *Fmr1* KO mice haven't yielded any robust results over the years. Impaired preference for the unfamiliar mice may indicate lack of interest in general novelty and/or failure to discriminate between familiar and novel mice; although the latter should be supported by robust deficits in olfactory discrimination tasks. As previously discussed, a finding which supports a general lack of interest for novelty is that *Fmr1* KO mice fail to recognize the novel object, in the novel object recognition task (Bhattacharya et al., 2012; Busquets-Garcia et al., 2013; Ventura et al., 2004). These findings suggest that discrimination deficits are not only observed in social tasks and that a deficit in the second phase of the three-chambered apparatus testing is hard to interpret.

In addition to the previously discussed studies, studies using direct social interactions with freely moving juvenile mice of the same sex, or using adult male subjects interacting with females on oestrus, did not reveal any differences between genotype deficits in social behaviour (Pietropaolo et al., 2014; Rotschafer et al., 2012) or they even showed enhanced social interactions for *Fmr1* KO mice, based on the greater engaging duration in affiliative behaviours, such as nose-to-nose sniffing, nose-to-anogenital sniffing and interaction time with a stimulus mouse by *Fmr1* KO mice (Spencer et al., 2005, 2008).

| Behavioural phenotype | Genetic background | Behavioural task | Observed phenotype | Parameter used | Reference examples |
|---|---|---|---|---|---|
| **Anxiety** | FVB/129; C57BL/6J | Open-field | Decreased | Time spent at centre | Peier et al. (2000)<br>Liu et al. (2011) |
| | | | Increased | | Restivo et al. (2005) |
| | C57BL/6J | Elevated-plus maze | Decreased | Time spent in open arms | Yuskaitis et al. (2010) |
| | FVB | | Increased | | Bilousova et al. (2009) |
| | C57BL/6J | Light–dark box | Decreased | Transitions light/dark | Peier et al. (2000)<br>Veeraragavan et al. (2012) |
| **Hyperactivity** | Hybrid FVB/NJ C57BL/6J | Elevated-plus maze | Normal | Time spent in open arms | Yan et al. (2004) |
| | C57BL/6J; FVB | Open field | Increased | Distance travelled; mean speed | Min et al. (2009)<br>Michalon et al. (2012) |
| **Associative learning** | C57BL/6J | Passive avoidance | Normal | Latency to enter dark compartment | Bakker et al. (1994)<br>Dölen et al. (2007) |
| | | | Decreased | | Yuskaitis et al. (2010)<br>Michalon et al. (2012) |
| | C57BL/6J; FVB | Context and cued fear | Decreased | Freezing duration | Paradee et al. (1999)<br>Peier et al. (2000) |
| | C57BL/6J | | Normal | | Van Dam et al. (2000) Olmos-Serrano et al. (2011) |
| **Spatial learning** | C57BL/6J; FVB/NJ C57BL/6J hybrid | Morris watermaze Reference task | Normal | Latency to platform; time on quadrant | Bakker et al. (1994)<br>Paradee et al. (1999) |
| | Hybrid FVB/NJ C57BL/6J | Barnes maze | Decreased | Time on the target hole | Yan et al. (2004) |
| **Working memory** | C57BL/6; FVB | Novel object recognition | Decreased | Exploring novel object | Ventura et al. (2004)<br>Busquets-Garcia et al. (2013) |
| | | | Normal | | Yan et al. (2004) |
| | FVB; C57BL/6J hybrid | Olfactory discrimination | Normal | Number of errors | Mineur et al. (2002)<br>Moon et al. (2008) |
| | C57BL/6 | Radial maze | Normal | | Yan et al. (2004)<br>Guo et al. (2012) |
| | C57BL/6; FVB | Y/T-maze | Decreased | Alternation | Bilousova et al. (2009)<br>Udagawa et al. (2013) |
| **Cognitive rigidity** | C57BL/6; FVB | Morris watermaze Reversal task | Normal | Time in target quadrant | Paradee et al. (1999)<br>Yan et al. (2004) |
| | | | Decreased | | Kooy et al. (1996)<br>D'Hooge et al. (1997) |
| | | Five-choice serial reaction time task | Decreased | Number of errors | Krueger et al. (2011) |
| **Social preference and novelty** | C57BL/6 | Social preference | Normal | Time spent with social stimulus | Mines et al. (2010)<br>Bhattacharya et al. (2012) |
| | | Social novelty/ interaction | Decreased | Time spent with novel mouse | Mines et al. (2010)<br>Bhattacharya et al. (2012) |

**(to continue)**

| Behavioural phenotype | Genetic background | Behavioural task | Observed phenotype | Parameter used | Reference examples |
|---|---|---|---|---|---|
| (continued) | | | | | |
| **Sensory gating** | FVB | PPI of acoustic startle | Decreased | Body flinches | de Vrij et al. (2008)<br>Baker et al. (2010) |
| | FVB/NJ<br>C57BL/6J hybrid | | Normal | | Peier et al. (2000)<br>Spencer et al. (2006) |
| | | | Increased | | Renoux et al. (2014) |
| **Seizures susceptibility** | FVB/NJ;<br>C57BJ/6J;<br>FVB/NJ<br>C57BL/6J hybrid | Audiogenic seizures | Increased | Seizures-like behaviour | Osterweil et al. (2013)<br>Veeraragavan et al. (2012)<br>Bakker et al. (1994) |
| **Communication deficits** | FVB/129 FVB | Ultrasonic vocalization | Decreased | Specific features of vocalizations | Rotschafer et al. (2012) |
| | | | Increased | Number of calls of pups | Lai et al. (2014) |
| **Circadian rhythms** | C57BJ/6J | Locomotor activity | Increased | Ambulatory counts | Baker et al. (2010) |
| | | | Decreased | Wheel running | Zhang et al. (2008) |

**Table 1.3 Behavioural abnormalities seen in Fmr1 KO mouse** (Modified from Santos et al., 2014 and Kazdoba et al., 2014).

Once more, genetic background differences seem to affect the behavioural output; social interaction performance appears to be dependent on the background strain into which the *Fmr1* mutants have been bred (Moy et al., 2009; Spencer et al., 2011). Despite several studies suggesting that individuals with FXS have social interaction deficits and social phobia, it has been proposed that these social deficits are due to hyperarousal and augmented social anxiety rather than a lack of interest in social interactions. A behaviour supporting this idea is known as the "Fragile X handshake"; affected individuals will shake the interviewer's hand and acknowledge their presence but will actively avoid eye contact until the interviewer looks away (Lozano, Rosero, & Hagerman, 2014); the behaviours of the mouse model described here, may differentially account for these factors.

Along with various other cognitive delays, children with FXS exhibit delays in all major developmental milestones, including language development (Abbeduto et al., 2008; Finestack, Richmond, & Abbeduto, 2009; J E Roberts, Mirrett, & Burchinal, 2001). Rodent pup ultrasonic vocalizations (USVs) are considered to be biologically meaningful (Fischer & Hammerschmidt, 2011), as they are emitted by young pups primarily during stressful situations (i.e. maternal separation) (Maria Luisa Scattoni, Crawley, & Ricceri, 2009) and elicit behavioural responses by the parents (mainly mother). It has been shown that the type and duration of USVs emitted by the *Fmr1* KO mouse pups (8 days old) were different from the control littermates, following maternal separation (Roy, Watkins, & Heck, 2012); however, there was no apparent changes in the total number of calls. Recently, Lai and colleagues reported an increased number of USVs in seven-day old mouse *Fmr1* KO pups (Lai et al., 2014a) suggesting an age-dependent function of FMRP on communication. Moreover, adult male mice and rats emit ultrasonic vocalizations during interaction with females and in response to scents from sexually receptive females (Holy & Guo, 2005). Yet again the behavioural phenotypes related to social communications are fairly contradictory; studies focusing on ultrasonic vocalizations of *Fmr1* KO mice have been inconsistent in their findings. While there are reports of increases (Spencer et al., 2011) or no differences in the number of calls between *Fmr1* mutant and wildtype mice (Pietropaolo et al., 2011), other research groups have reported a significant reduction in vocalizations from *Fmr1* KO mice (S. E. Rotschafer et al., 2012), including deficits in specific call-types (Roy et al., 2012); although no apparent difference in mating behaviour was observed. Taken together, these data suggest that social interaction and communication phenotype in *Fmr1* KO

mice is multifaceted. Mutant mice seem to exhibit some aspects of normal sociability, but they also display some abnormalities in social behaviour and communication.

### 1.5.6 Neurological symptoms of the *Fmr1* KO mouse

Approximately 10-20% of individuals with full *FMR1* mutation exhibit childhood seizures which gradually decrease in adulthood (Hagerman & Stafstrom, 2009; Musumeci et al., 1999). Seizures associated with FXS are irregular, often partial, and can typically be managed with appropriate medication (Hagerman & Stafstrom, 2009; Heard et al., 2014). *Fmr1* KO mice have not been reported to display spontaneous seizures, but display enhanced susceptibility to audiogenic seizures, induced by exposure to a 125 decibel, high-intensity siren (Dolan et al., 2013; Osterweil et al., 2013a; Pacey, Heximer, & Hampson, 2009; Veeraragavan et al., 2012). Susceptibility to audiogenic seizures has been the most consistent behavioural phenotype in the mouse model and it is therefore usually implemented to examine the efficacy of potential FXS treatments (Michalon et al., 2012; Osterweil et al., 2013; Pacey et al., 2009; Yan et al., 2005). Audiogenic seizure vulnerability in *Fmr1* KO mice could reflect the seizure susceptibly seen in individuals with FXS, although the severity of audiogenic seizure in *Fmr1* KO mice varies in degree depending on age and background strain (Santos, Kanellopoulos, & Bagni, 2014).

Individuals with FXS have been reported to exhibit hyperarousal and augmented sensitivity to sensory stimuli (Verkerk et al., 1991). For example, individuals with FXS have heightened and more frequent responses and reduced habituation to a variety of sensory stimulations (e.g., olfactory, auditory, visual, tactile, and vestibular stimuli) as measured by electrodermal responses (Miller et al., 1999). Electrophysiological recordings in the auditory cortex demonstrated enhanced responses to auditory tones in *Fmr1* KO mice, indicating that auditory neurons of *Fmr1* KO mice are hypersensitive to stimuli (Rotschafer & Razak, 2013). These sets of data are consistent with the increased responses to high intensity tones seen in individuals with FXS (Rojas et al., 2001; Van der Molen et al., 2012b). "Prepulse inhibition" (PPI) has been used to evaluate the ability of human and rodents to filter irrelevant information in their surroundings, called sensorimotor gating. It occurs when a weak pre-stimulus weakens the response to a strong stimulus (pulse) which follows within 100 milliseconds (Braff, Geyer, & Swerdlow, 2001; Swerdlow et al., 2001). Abnormal sensory inhibition may reflect a deficit in processing and prioritizing incoming information, a feature also seen in

schizophrenic patients (Braff et al., 2001; Rihs et al., 2013). Treatments with antipsychotic medication has been shown to alleviate those deficits in both rats and humans (Curzon, Kim, & Decker, 1994; Sánchez-Morla et al., 2009; Suryavanshi et al., 2014). It is known that patients with FXS are hyper-aroused in situations of excessive stimulation and habituate poorly to sensory stimuli (Elizabeth Berry-Kravis, 2014). Deficits in PPI have been reported in individuals with FXS, correlating with other clinical FXS features, such as reduced IQ and attention (Frankland et al., 2004; D Hessl et al., 2009; Yuhas et al., 2011). Therefore, *Fmr1* KO mice and control littermates were tested in PPI in an acoustic startle task to examine possible sensorimotor gating deficits. These studies in *Fmr1* KO mice have yielded contradicting results. The majority of reports indicate *Fmr1* KOs exhibit enhanced PPI and decreased startle (Ding et al., 2014; Paylor et al., 2008; Pietropaolo et al., 2011). This is a significant effect but unfortunately in the opposite direction to the human FXS phenotype. Moreover, it fits well with enhanced extinction in fear conditioning paradigms. In contrast, other research groups have reported deficits in PPI for *Fmr1* KO mice (de Vrij et al., 2008), enhanced startle responses to low intensity auditory stimuli (Nielsen et al., 2002), and minimal or no PPI differences between *Fmr1* KOs and wildtype controls (Nielsen et al., 2002; Veeraragavan et al., 2011; Yan et al., 2004). As has been previously discussed, *Fmr1* KO's behaviour phenotypes are heavily influenced by genetic background (Pietropaolo et al., 2011; Spencer et al., 2011). Explanations for the inconsistent findings on PPI as reported by different research laboratories could include the use of different murine genetic backgrounds and subtle differences in testing protocols (Swerdlow, Braff, & Geyer, 2000). Of greater concern have been the contrasting phenotypes between the majority of PPI studies in the *Fmr1* KO mouse model and FXS clinical studies. However, in one published study Frankland and colleagues (2004) showed that *Fmr1* KO mice display a heightened reaction to auditory stimuli, similar to the human phenotype (Renoux et al., 2014). Despite the inconsistences in published reports, it is clear that FMRP plays a role in sensorimotor gating. The absence of FMRP could be underlying the altered sensitivity to sensory stimulation seen in both human patients and mouse models.

Another aspect of behaviour which seem to be recapitulated by the mouse model of FXS is abnormal circadian rhythms. Sleep difficulties are a common feature of patients with FXS (Elizabeth Berry-Kravis, 2014); in the mouse model of the syndrome, FMRP has been shown to be involved in the regulation of circadian rhythmicity as measured by locomotor analysis. In complete darkness, FMRP was found to regulate the duration of

the circadian period (J. Zhang et al., 2008) as shorter activity periods of wheel running were recorded in the *Fmr1* KO mice compared to wildtype controls. Interestingly, FMRP absence affects circadian rhythmicity differently in females and males; recorded ambulatory activity during the light phase was elevated only in the female *Fmr1* KO mice (Baker et al., 2010)and no changes were reported in males. A caveat of these studies is that the affected circadian patterns seen in *Fmr1* KO mice cannot be directly compared to the sleep disturbances seen in human; simply because lack of activity (as measure by wheel running) does not mean sleep.

### 1.5.7 Lessons learned and lessons to be learned

Over the past 20 years, combined research by hundreds of laboratories working on FXS, has led to the identification of several hundreds of possible FMRP target mRNAs (Pasciuto & Bagni, 2014). A large number of these FMRP target mRNAs is known to have integral roles in synapse formation and function; a lot have also been shown to confer risk for autism. This fact might explain the very diverse and heterogeneous behavioural deficits seen in FXS; FMRP loss could be influencing multiple circuitries and cause alterations in various receptor pathways in different ways. Moreover, recent studies have shown that there is a convergence not only in behaviour but also key pathophysiological mechanisms between the mouse model of FXS and non-syndromic forms of intellectual disability (Barnes et al., 2015), and between *Fmr1* KO mice (as a syndromic form of autism) and neuroligin 3 (*Nlgn3)* KO mice (as a non-syndromic form of autism) (Baudouin et al., 2012). Even though it is very well established that multiple behavioural abnormalities seen in the mouse model of FXS are due to FMRP loss and subsequent alteration of the glutamatergic signalling (Bear, Huber, & Warren, 2004), many other molecular pathways such as BDNF, mTOR, ERK1/2, cAMP, and PKC cascades are also affected (Osterweil et al., 2010; Sharma et al., 2010; Uutela et al., 2012; Wang et al., 2008). In addition, multiple neuronal circuits, such as the GABAergic, cholinergic, dopaminergic, and serotonergic systems, have been shown to be modified by FMRP loss (Deng et al., 2013; Gatto, Pereira, & Broadie, 2014). What's clear from these sets of data is that while certain aspects of FXS are recapitulated in the *Fmr1* KO mouse, other clinical features of the syndrome cannot be reproduced. This could be due to limitations of the behavioural paradigms used or because of limitations of the mouse as a model of neuropsychiatric diseases. Later in this thesis (Chapter 3) I will try to compare the two

favourite rodent species in biomedical research, mouse and rat, and explain why newly created rat models have the potential to expand our knowledge of the pathophysiology of many devastating neuropsychiatric disorders.

Although it is well-known that the core genetic cause leading to FXS is the silencing of the *FMR1* gene, the wide spectrum of disabilities and diversity of physiological and cognitive features among patients with FXS lead us to believe that the cure of the disease could be difficult when targeting a single downstream affected pathway. The combination of pharmaceutical treatments targeting different molecules altered in FXS might be the key for the amelioration of FXS deficits. Having said that, the use of established and newly created animal models has been and will be of vital importance in advancing our understanding on the molecular basis of FXS, in order to be able to identify key affected molecular mechanism, which may lead to extended behavioural improvement when targeted. In the following introductory chapter, I will review recent advances in FXS treatment development and summarise FXS clinical trials which utilise symptom-based and mechanism-based treatments.

# 2. Therapeutic approaches in FXS

## 2.1 From Symptom-based to Mechanism-based therapeutic interventions

Drug discovery is by no means a novel concept. For millenia, we have identified the protective and therapeutic capacity of herbs and their crude extracts, like poppy seeds or willow tree bark. This premodern approach was pretty straighforward: focus exclusively on efficacy and safety without concern for mechanism of action (Enna & Williams, 2009). Remarkably, the Greek physician Hippocrates in the fourth century BCE described headache relief and fever reduction from the bitter powder of the willow tree in writings and was aware of the utility of willow bark in the treatment of inflammatory pain (Ugurlucan et al., 2012). Nearly two and a half millennia down the line, chemists working for Bayer isolated the active substance – salicylic acid – edited its formulation in order to reduce gastric side effects, and started selling it as aspirin in 1899. Similarly, the powerful analgesic morphine, was first isolated from opium poppy in 1804, industrially produced by Merck in Germany in the late 1820s, and later modified to be commercially sold as a cough suppressant in 1898, under the trade name Heroin. All of the above were done without any knowledge of the processes being targeted in the human body. The mechanism of action underlying the effects of these medications, and in the earliest example of salicylic acid, even the chemical compound itself, was completely unknown when these drugs were popularised. In this way, the empirical, symptom-based, approach led to the discovery of original CNS drugs.

Following the genomic revolution of the 1990s, the current high-throughput era has drastically reshaped our approach to the discovery of novel therapeutics. Today, research efforts focus on a hypothesis-driven, mechanism-based approach. Drug discovery emphasises target identification and the discovery of new chemical compounds which can act on a preselected molecular target site. This approach requires a very good understanding of the target physiology and a progressional integration of cellular and tissue in vitro studies, ultimately to animal models that can within reason predict the human responses (predicitve validity of a model).

This approach, also called "molecular medicine", begins with the identification of patients with similar or identical presentation of the disorder (Krueger & Bear, 2011). This can be very challenging when it comes to psychiatric and neurological disorders

which are highly heterogeneous in behavioural symptoms and genetic aetiology. Identifying multiple, more specific disorders from what originally appeared to be one syndrome with a diverse phenotype, will increase the likelihood of discovering the underlying mechanisms causing each disorder. A profound understanding of the genetic aetiology of the disease consequently allows for the generation of animal models with adequate construct validity. The application of basic neurobiology research in these models aims to characterise the molecular, cellular, and circuit processes being affected; these processes should be tightly associated with a measurable, behavioural manifestation. Molecular targets, such as receptors or proteins taking part in critical signal transduction cascades, may be amenable to pharmacological or other interventions. Genetic or small molecule approaches are validated first using in vitro assays and later in the animal model. It is critical to remember that irrespective of how much different disorders overlap, it is best to consider them independently at these first steps of the drug development process. Nevertheless a single therapeutic strategy could effectivelly alleviate symptoms in several or even all related disorders. Keeping this in mind, the current hope is that the identification of core molecular process contributing to FXS pathophysiology will lead to drug development which will be able to treat not only FXS but also autism spectrum disorders and intellectual disabilities more generally.

Over the last 20 years, the study of molecular, cellular, and behavioral alterations in existing animal models of FXS has significantly increased our understanding of FMRP function and the human disease pathophysiology, while providing us with potential molecular targets and candidate treatments in FXS, autism, and other associated neurodevelopmental disorders. Most of the current targeted treatments which are undergoing clinical trials have attempted to adjust the excitatory/inhibitory imbalance seen in FXS which is believed to contribute to its core pathophysiology. In the brain of individuals affected by FXS,there is likely to be excessive excitatory, mainly glutamatergic, signaling, coupled with deficits in inhibitory, mainly GABAergic, signaling. In the following paragraphs I will summarise existing symptomatic treatments followed by a summary of treatment undergoing clinical trials, categorised based on a hypothesised drug mechanism (Table 2.1).

## 2.2 Symptom-Based Interventions

To date, most of the drug treatment in FXS is symptomatic. The two most widely used medications are stimulants that help with attention and hyperactivity and selective serotonin reuptake inhibitors that can control aggression which individuals sometimes display due to elevated social anxiety (Levenga et al., 2010). In addition to the use of pharmacological agents, patients with FXS also seem to benefit from behavioural therapy addressing speech, general communication and emotional problems (Moskowitz, Carr, & Durand, 2011). The latter has also been demonstrated in the FXS mouse model; an enriched environment can positively affect behaviour, and thus this behavioural intervention might also be beneficial for human patients (Restivo et al., 2005). While both existing types of treatment, pharmacological and non-pharmacological, impact symptoms only and do not improve any core behavioural deficits associated with the disease, multi-drug therapies based upon individual phenotype are currently the most common method of helping affected individuals.

### 2.2.1 ADHD treatments

Individuals with FXS are often been diagnosed ADHD. The comorbidity rate is approximately 73%, as measured in an all-male cohort for subjects scoring 15 or higher on the Conner's abbreviated scale, which is indicative of ADHD (Baumgardner et al., 1995). Even individuals not diagnosed with ADHD, often display hyperactivity and difficulty focusing in a classroom setting. On top of the human observations, Ventura and colleagues (2004) have shown that treating *Fmr1* KO mice with amphetamine -known treatment for ADHD- can have beneficial effects in behaviour.

So far, there have been three clinical trials testing medications aimed at the treatment of ADHD symptoms in FXS. The first in FXS was a double-blinded placebo-controlled study of the stimulants methylphenidate (Ritalin) and dexamfetamine. Participants were treated for one week with each of methylphenidate, dexamfetamine, and placebo. Methylphenidate treatment was shown to improved patient behaviour, based on the Comprehensive Teacher Rating Scale, while no improvement was observed with dexamfetamine (Hagerman, Murphy, & Wittenberger, 1988). The second trial has looked at the ability of L-acetylcarnitine (LAC) to reduce the ADHD symptoms observed in boys with FXS. This double-blinded, placebo-controlled, clinical trial found significant

improvement in the Clinical Global Impressions (CGI) scale for both parent and teacher response scores, as well as in the Vineland Adaptive Behaviour Scale (VABS) and Adaptive Behaviour Composite (ABC) score (Torrioli et al., 2008). The most recent trials of the three focusing on ADHD symptomatology in FXS examined the effects of the antiepileptic drug valproic acid (VPA). In an open-label trial of VPA, the only significant change was in hyperactivity measured by the Conner's Parent Rating Scale (Torrioli et al., 2010). Taken together, data from clinical trials show that while compounds like LAC and VPA may have some beneficial effects against ADHD symptomatology observed in FXS patients, stimulants are more commonly used since they show more robust effects (Roberts et al., 2012).

### 2.2.2 Oxytocin

It is common for individuals with FXS to experience extreme social anxiety, which is often coupled with severe eye gaze avoidance and hyperarousal (Reiss & Hall, 2007). The neuropeptide oxytocin (OXT) has been shown to have profound anxiolytic effects, besides its many other prosocial and reproductive effects. Hence, there is increasing basic research and medical interest in its potential therapeutic use for the treatment of neuropsychiatric disorders, such as anxiety disorders, posttraumatic stress disorder, as well as autism and schizophrenia, among others (Neumann & Slattery, 2016). The effects of intranasal oxytocin on social anxiety were tested in a double-blind, placebo-controlled trial involving eight individuals with FXS. Subjects' heart rate, heart rate fluctuation, eye gaze incidents, and concentration of salivary cortisol in response to a social stressor were amongst the outcome measures. Treated individuals showed significant improvement on eye gaze and salivary cortisol measures (Hall et al., 2012). Despite the promising results of the study, a larger study is essential in order to adequately evaluate the benefits of oxytocin in FXS.

### 2.2.3 Aripiprazole

Aripiprazole, also known as Abilify, belongs to class of drugs called atypical antipsychotics. They are usually prescribed by clinicians to contain hyperarousal seen in individuals with FXS and autism (Ching & Pringsheim, 2012). Despite their widespread use, clinical trials of antipsychotics in FXS are very limited. Amongst the newer generation antipsychotics, the only reported clinical testing in FXS has been with

aripiprazole. In a 12-week, open-label trial, 10 subjects received aripiprazole treatment. As a result, individuals showed significantly improved performance on CGI scale, Social Responsiveness scale (SRS), and the Children's Yale-Brown Obsessive Compulsive Scale Modified for Pervasive Developmental Disorders (PDDs) (Erickson et al., 2010).

### 2.2.4 Melatonin

Sleep disturbances, and more specifically insomnia, is another symptom associated with FXS. Based on parent/caregiver reports, approximately 32% of boys with FXS experience some form of sleep irregularity. Irregular night sleep patterns and difficulties falling asleep were the most reported issues (Kronk et al., 2010). In a double-blind, placebo-controlled trial of melatonin, lasting four-weeks, individuals with FXS and ASD (12 subjects, 6 with FXS) were treated. Results demonstrated a significant increase in sleep duration, decreased latency to sleep, and caused earlier sleep time onset (the clock time when the child fell asleep) in individuals who got the treatment. Sleep awakenings were decreased but the difference between treated and untreated individuals was not significant (Wirojanan et al., 2009).

### 2.2.5 Selective Serotonin Reuptake Inhibitors

To date there are only a few clinical trials assessing the value of selective serotonin reuptake inhibitors (SSRIs) in FXS. Existing evidence suggests this class of antidepressant drugs may have beneficial effects. In a case study involving individuals with autism, a low-dose of sertraline, also known as Zoloft, led to improvements (eight of nine subjects) in irritability, anxiety, and behavioural decline induced by a transition in daily routine (Steingard et al., 1997). A retrospective chart review of studies in 45 children with FXS, showed that the 11 subjects who were treated with sertraline showed improved language development (Indah Winarni et al., 2012). Moreover, in a very recent study, 52 children with FXS aged 2 to 6 years old were treated with sertraline for 6 months. Although analysis of the primary outcomes showed no improvement, secondary exploratory analyses revealed significant improvement on motor and visual perceptual tests, as well as some cognitive measures (Greiss Hess et al., 2016). Finally, in a survey of individuals with FXS, it was found that fluoxetine, also known as Prozac, led to the greatest behavioural activation compared to any other drug in its class (Hagerman et al., 1994). It's clear from the above that, despite the small number of clinical trials in FXS, SSRIs are a commonly prescribed treatment for the management of anxiety and

depression related symptoms which could also have beneficial effects in cognitive impairments associated with FXS.

## 2.3 Modulating Excitatory Neurotransmission

Research in the mouse model of FXS has revealed extensive alteration in signalling and/or localization of several glutamatergic receptors (Bostrom et al., 2016). Several clinical trials have been focussing on testing compounds reducing excitatory neurotransmission by antagonism of group I metabotropic glutamate receptors (mGluRs) and more specifically mGluR5 (Fenobam, AFQ056, RO4917523). Alternative strategies include drugs which block the N-methyl-D-aspartate (NMDA) receptors (memantine) or the α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid receptors (AMPAR) (CX516) have also been tested in individuals with FXS.

The mGluR theory of FXS proposed by Bear and colleagues (Bear et al., 2004) suggests that excessive signalling through mGluRs is a significant contributor to the behavioural, electrophysiological, and molecular abnormalities associated with the loss of FMRP. The brainchild of Bear and his colleagues was one of the first hypotheses describing a link between the loss of FMRP in FXS and changes in molecular and cellular mechanisms of known importance for synaptic development and function in the normal brain. The mGluR theory was based on a few key observations: (1) FMRP was shown to acts as a translation regulator for several genes at the synapse (Brown et al., 2001), (2) mGluR signalling is known to be coupled with synaptic protein synthesis (Weiler et al., 2004), (3) direct evidence connecting FMRP loss to an increase of mGluR signalling downstream effects (Chuang et al., 2005; Huber, 2000), and finally (4) the loop of evidence closes with the fact that many of the aforementioned downstream effects rely on intact mRNA translation at the synapse (Huber, 2000; Karachot et al., 2001; Raymond et al., 2000; Zho et al., 2002). Over the last 10 years, experimental data from countless studies have supported the mGluR theory for FXS (Bostrom et al., 2016). One of the most direct pieces of evidence supporting the role of mGluR in FXS, came from Dolen and colleagues. They were the first to demonstrate that a reduction in the levels of mGluR5 in *Fmr1* KO mice, is enough to normalize elevated protein synthesis, dendritic spine morphology and some behavioural abnormalities (Dölen et al., 2007). However, a more recent study which included a more thorough behavioural assessment, revealed that genetically reducing mGluR5 in *Fmr1* KO mice has a limited beneficial effect in behavioural deficits. This suggests that mGlur5 signalling alterations may not lead to improvements as robust as

originally indicated (Thomas et al., 2012). Furthermore, several studies using the mouse model of FXS have shown that strong mGluR5 antagonists, like MPEP, can improve FXS related phenotypes, including receptor expression, behavioural deficits, and abnormal dendritic spine morphology (Michalon et al., 2012; Yan et al., 2005). I should also point out that the mGluR5 antagonists used in preclinical *Fmr1* KO studies so far, were also shown to maintain analgesic effects in mGluR5 KO mice. This data suggests that these molecules are not as eclectic as we would like and are likely to have additional molecular targets which could potentially contribute to the promising treatment effects previously discussed (Montana et al., 2009).

### 2.3.1 Fenobam

Fenobam is a non-benzodiazepine and potent negative allosteric modulator of mGluR5. It was synthesised in the late 1970s as a novel anxiolytic drug. It binds to the target in a non-competitive manner and has inverse agonist properties. This makes its mechanism of actions very similar to MPEP (a different selective mGluR5 antagonist developed by Novartis) (R. H. P. Porter et al., 2005). Studies in *Fmr1* KO mice have reported some beneficial effects of fenobam on molecular, cellular and behavioural levels but the results were not conclusive (Vinueza Veloz et al., 2012; Wang, Smith, & Mourrain, 2014). In order to evaluate, primarily, its safety and its pharmacokinetic properties, an open-label, single-dose study in adult males and females with FXS was conducted almost 8 years ago (Berry-Kravis et al., 2009). The effects on sensory gating, attention, and behavioural inhibition were also explored. The small experimental sample included six young males and six young female adults with FXS (18.7–30.7 years). There were no significant adverse events and the medication was well tolerated. That was an important outcome because previous reports in non-FXS individuals who received fenobam treatment in high doses (four times the daily highest dose in the FXS trial) for a period of 4 weeks, reported adverse CNS effects including insomnia, vertigo, paresthesia, and even hallucinations (Friedmann et al., 1980). The primary measured outcome of the study, pre-pulse inhibition (PPI) was tested at baseline and after treatment with a single dose of fenobam. Six out of twelve (50%) subjects met a performance criterion of at least 20% improvement compared to baseline. The limited beneficial outcomes and potentially severe adverse effects suggest that future studies on fenobam as a long-term treatment option in FXS are highly unlikely to be considered.

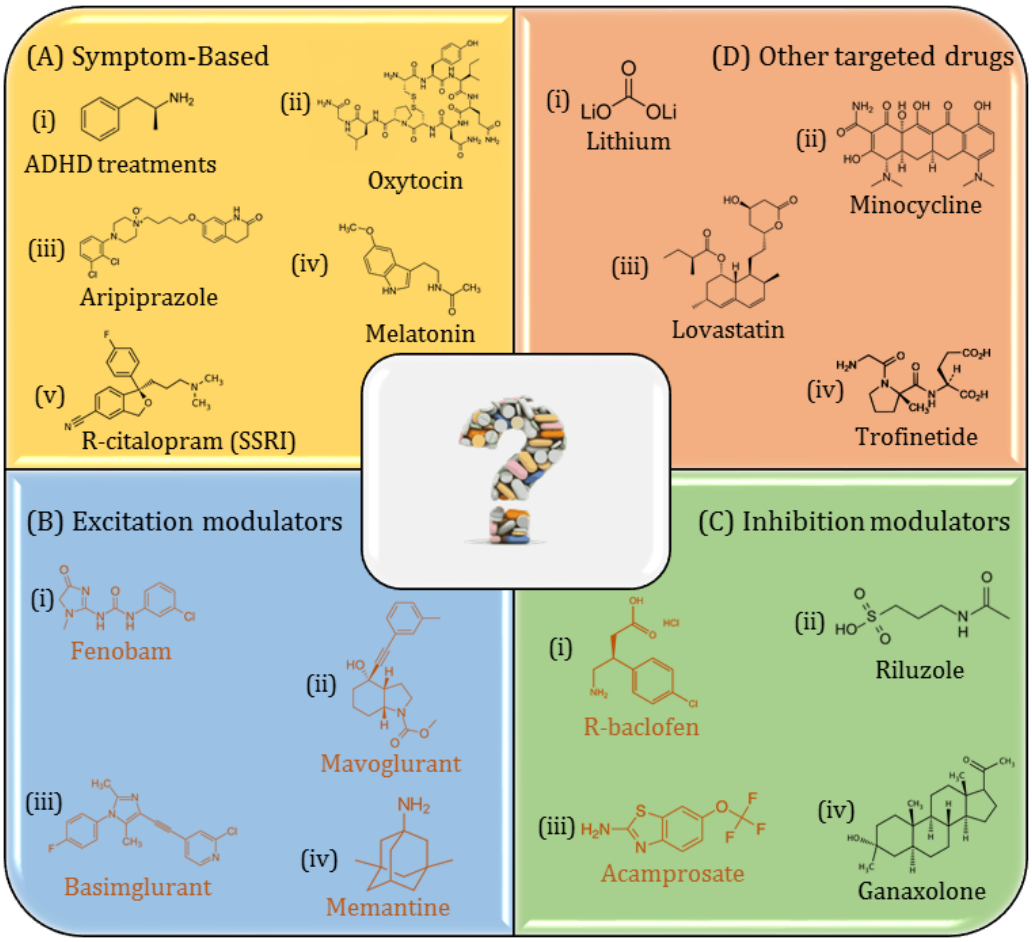



**Figure 2.1 Therapeutic strategies against FXS. (A)** Symptom based treatments are still in use but little is known about the mechanisms targeted in FXS patients. **(B)** Compounds targeting excitatory transmission have received a lot of attention due to promising pre-clinical data but failed to produce promising results in human clinical trials. **(C)** Molecules targeting inhibitory transmission have also received due to the well characterised inhibition/excitation ratio anomalies in the mouse model of FXS but so far have not produced any clear positive outcomes. Lastly, a number of other therapeutics which target core pathophysiology associated with FXS **(D)**. Their mechanism by which they exert their therapeutic effects is not fully understood so both pre-clinical and clinical studies are currently been conducted either with monotherapies or a combination of treatments. In red are compounds which were found to be inefficient in human clinical trials and their testing has been terminated.

### 2.3.2 Mavoglurant/AFQ056

Mavoglurant, also known by the product name AFQ056, is a non-competitive mGluR5 antagonist developed by Novartis Pharmaceuticals specifically as a candidate drug for the treatment of Fragile X syndrome. As in the case of fenobam, studies in mice showed that AFQ056 could reverse some behavioural deficits and spine morphology (Gantois et al., 2013; Pop et al., 2014). So far three clinical trials of mavoglurant in individuals with FXS have been completed. The first was a double-blind crossover study (subjects switched from placebo to treatment and vice versa during the trial), in which 30 subjects received 20 days of treatment. This study failed to find any positive effect of the treatment on any primary or secondary outcome measures, when the full study sample was analysed. Interestingly, when the researchers divided the participants according to methylation profiles, and limited their analysis to a small sub-set of seven individuals with complete *FMR1* promoter methylation, they found significant improvement across a wide range of outcome measures (Jacquemont et al., 2011). It is important to note that these seven participants showed minimal or no improvement while receiving the placebo treatment in one of the two stages of study, as measured by the Aberrant Behaviour Checklist (ABC). This finding may have contributed to the reported post-hoc effect found in that subgroup. The results of this initial study, led to two large international clinical trials in adults and adolescents: (1) a Phase IIb, double-blind, placebo-controlled, parallel group study in adult male and female participants (18–45 years) which lasted three months, and (2) a similarly controlled Phase III trial, in adolescents with FXS (12–17 years). Participants were assigned to two doses daily of 25 mg, 50 mg, 100 mg AFQ056, or placebo in order to evaluate the safety and efficacy of the three doses for treating the behavioural deficits associated with FXS. The ABC total score was used as a primary outcome measure, with the Clinical Global Impression-Improvement (CGI-I) scale and Repetitive Behaviour Scale-Revised (RBS-R) as secondary outcome measures. Unfortunately, neither of these studies found any significant improvements as a result of the treatment, on any primary or secondary outcome measures (Berry-Kravis et al., 2016). Following these negative results, the sponsor Novartis, terminated the open-label extension portion of the study in adolescents and discontinued the development program of AFQ056 for the treatment of FXS (ClinicalTrials.gov Identifiers: NCT01253629, NCT01357239) (Clapp, 2014).

### 2.3.3 Basimglurant/RO4917523

Another mGluR5 antagonist which was developed by Roche for the treatment of FXS and treatment resistant depression, is basimglurant (RO4917523). There is no published work testing basimglurant in *Fmr1* KO mice but a recent study showed that the orally bioavailable mGluR5-selective antagonist, CTEP, which is similar in structure, potency, and selectivity to basimglurant, can prevent cognitive impairments and prevent pathogenesis in an Alzheimer's disease mouse model (Hamilton et al., 2014). The effects of basimglurant as well as its safety and tolerability were studied in a Phase II clinical trial: 183 individuals with FXS, 14–50 years of age (ClinicalTrials.gov Identifier: NCT01517698) and in an additional study in 47 affected children and adolescents aged 5–13 years (ClinicalTrials.gov Identifiers: NCT01015430, NCT01750957). Although trial data remain unpublished at this time, treatment did not demonstrate efficacy based on the primary and secondary endpoints employed. Therefore, the sponsor of the trials, Roche, subsequently terminated its program for the development of basimglurant as a treatment for FXS (Santarelli, 2014).

Taken together, the disappointing results from both the mavoglurant and basimglurant trials, have led pharmaceutical companies to drastically move away from pursuing development of selective mGluR5 antagonists as potential targeted treatments for FXS. The fact that these clinical trials did not yield any promising results, does not conclusively answer the question of whether or not selective mGluR5 antagonists can alter FXS symptomatology and does not rule out the validity of the mGluR5 theory; however, these results do suggest that selective pharmacological decrease of mGlur5 signalling, alone, in humans with FXS, is not sufficient in order to ameliorate behavioural abnormalities when is used as a short-term treatment in the ages studied. Future trials of mGluR5 antagonists should target young subjects whose brains are dramatically more plastic compared to aged subjects. Targeting signalling imbalance during early postnatal brain development is possible to protect neural circuitry as it develops, potentially improving clinical outcomes, or even preventing the emergence of symptoms as a result. Finally, one of the current trends in field involves combined therapeutics, possibly including mGlur5 selective antagonists, with additional targeted drugs aiming at other key molecular and cellular mechanisms which might contribute to the FXS behavioural phenotype.

### 2.3.4 Memantine

Memantine (3,5-dimethyladamantan-1-amine) is a non-competitive antagonist acting on the N-methyl-D-aspartate (NMDA) receptor. There is evidence of NMDA receptor dysfunction in FXS, but the overall direction of the effect is unclear, appearing to depend on brain region and age. Memantine was the first medication targeting the glutamatergic system that the US Food and Drug Administration (FDA) approved for the management of Alzheimer's disease. A large body of work supports its effect in this devastating disorder; while it does not cure or reverse Alzheimer's, it does effectively treat a wide range of cognitive symptoms (Matsunaga, Kishi, & Iwata, 2015). Memantine treatment is also being explored in humans with other neurological disorders including Fragile X-associated tremor/ataxia syndrome (FXTAS) and Down Syndrome with mixed results so far (Hanney et al., 2012; Seritan et al., 2014; Yang et al., 2014). Only one pilot open-label study has been conducted related to FXS until now. Six participants who had previously been diagnosed with both FXS and PDD/NOS (diagnosed using criteria of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition) received an average of 34.7 weeks of memantine treatment, in order to test the tolerability of memantine and its effectiveness against a number of symptoms associated with FXS (Erickson, Mullett, & McDougle, 2009). While four subjects showed an improvement in general symptomatology, as measured by the CGI-I, there were no robust effects in any specific symptom domains. Furthermore, two of the subjects displayed increased irritability and had to discontinue memantine treatment.

## 2.4 Modulating Inhibitory Neurotransmission

Along with the increase in glutamatergic signalling, the signalling imbalance observed in FXS is, in part, due to deficits in inhibitory GABAergic function. The fragile X mouse models (*Fmr1* KO) display reduced GABA(A) subunit receptors, reduced synthesis of GABA coupled with augmented degradation of GABA and overall deficits in GABAergic input in multiple brain regions, including the hippocampus, striatum, amygdala, and somatosensory cortex (Idrissi et al., 2012; Lozano, Hare, & Hagerman, 2014; Olmos-Serrano et al., 2010). This reduced GABAergic input seen in FXS leads to various behavioural impairments including hypersensitivity to sensory stimuli, increased seizure susceptibility, and elevated anxiety. Moreover, deficits in the GABA receptor structure and function, in different brain regions, have been repeatedly associated with behavioural abnormalities and attentional processing deficits linked to anxiety disorders

and autistic spectrum disorders (Frye et al., 2016; Prager et al., 2016). Moreover, studies using *Fmr1* KO mice have already shown that drugs which act as positive modulators of $GABA_A$ receptors can improve behavioural and neurophysiological deficits (Braat & Kooy, 2015). All the above suggest that the GABAergic system represents a promising target for new treatments against core deficits of FXS.

### 2.4.1 R-baclofen/arbaclofen/STX209

The active right enantiomer of racemic baclofen, R-baclofen/arbaclofen, is a $GABA_B$ agonist. It is a GABA derivative and has been used as a skeletal muscle relaxant, primarily used to treat spasticity. Recent knowledge related to the contribution of GABA signalling in the excitation/inhibition imbalance, seen in FXS, led Seaside Therapeutics to reformulate arbaclofen under the code name STX209, and study it's efficacy in individuals with FXS and ASD. STX209 acts at presynaptic $GABA_B$ receptors. This led to the hypothesis that it inhibits glutamate release from presynaptic terminals, therefore reducing the neuronal hyperexcitability observed in FXS models and human patients. In the *Fmr1* KO mouse model, STX209 has been to shown to restore to normal a number of abnormal phenotypes; from reducing susceptibility to audiogenic seizures (Pacey et al., 2009), normalising excessive dendritic spine density and protein synthesis (Henderson et al., 2012), reducing repetitive behaviours and reversing social deficits (Silverman et al., 2015). A four-week, Phase II trial of STX209 recruited 63 individuals (55 male), carrying a full *FMR1* mutation, aged 6–40 years; it was completed in 2010 and showed that the drug was well tolerated (Berry-Kravis et al., 2012). The study was designed as a double-blind, placebo-controlled clinical trial with a two-period crossover conducted across 12 centres in the USA. STX209 did not show a significant difference over placebo on the primary endpoint, the Irritability Subscale of the Aberrant Behaviour Checklist (ABC-I). Nevertheless, post-hoc analyses did reveal significant improvements in parent-reported problem behaviours on the Visual Analog Scale (VAS) and on the Social Avoidance subscale of the ABC (ABC-SA); additional positive trends were also seen on multiple global measures. Interestingly, the more socially impaired subset of participants (based on the ABC-LSW at baseline), showed significant improvement on the Vineland II-Socialization raw score, on the ABC-Social Avoidance scale, and on all global measures. These very promising results lead to two phase III clinical trials in FXS individuals (Berry-Kravis et al., 2014). Unfortunately, no significant improvements were detected in the adolescent/adult Phase III trial. The Phase III trial in children found no

significant effect on any primary outcome measures, but did find an effect on the Fragile X Irritability subscale of the Aberrant Behaviour Checklist, a secondary outcome measure.

### 2.4.2 Acamprosate

Acamprosate, also known as Campral, is an FDA approved medication for the management of alcohol dependence and is currently being tested for efficacy in FXS. Its mechanism of action is still largely unknown and somewhat controversial; experiments done in Xenopus oocytes showed that acamprosate fails to alter activation of several tested GABA or glutamate receptor subtypes (Reilly et al., 2008). It is likely that acamprosate alters excitatory/inhibitory balance by modulating NMDA receptor transmission and may indirectly affect $GABA_A$ receptor transmission (Boismare et al., 1984; Kalk & Lingford-Hughes, 2014), probably in a pleiotropic manner (Mann, Kiefer, Spanagel, & Littleton, 2008). The first study looking at the effects of acamprosate in individuals with FXS was a small three-subject open-label trial in which all subjects showed improvements, as measured by the CGI-I (Erickson, Mullett, & McDougle, 2010). Acamprosate was then tested in a group of 12 children with FXS (6-17 years) for a period of 10 weeks, in a new open-label trial. Again acamprosate was shown to significantly improve the performance of participants on a number of outcome measures including various subscales of the ABC, CGI Severity, Social Responsiveness Scale (SRS), Attention Deficit Hyperactivity Disorder Rating Scale (ADHD-RS), and subscales of the Vineland Adaptive Behaviour Scale (VABS). On the CGI-I, the primary outcome measure, nine of the twelve subjects were either ‘‘very much improved’’ or ‘‘much improved’’ (Erickson et al., 2010). Moreover, in a separate published paper from the same study, acamprosate was shown to reduce plasma amyloid precursor protein (APP) and secreted APPα (sAPPα), and increase brain-derived neurotrophic factor (BDNF); although individual responses to treatment did not correlate with the extent of the change in BDNF plasma levels (Erickson et al., 2013; Erickson et al., 2014). Based on the results of these two open-label studies, acamprosate is currently undergoing a Phase II double-blind, placebo-controlled study in individuals with FXS (clinicaltrials.gov, NCT01911455).

| Compound | Preclinical testing in mice | | Open label Clinical trials | Placebo-control Clinical trials | |
|---|---|---|---|---|---|
| | Physiological effects | Behavioural effects | Outcome | Primary outcomes | Secondary outcomes/ post-hoc analysis |
| ADHD treatments | N.A | (+) Ventura et al., 2004 | (+) Torrioli et al., 2010 | (-) Hagerman et al., 1988 | (+) Torrioli et al., 2008 |
| Oxytocin | N.A | N.A | N.A | N.A | (+) Hall et al., 2012 |
| Aripiprazole | N.A | N.A | + Erickson et al., 2010 | N.A | N.A |
| Melatonin | N.A | N.A | N.A | N.A | (+) Wirojanan et al., 2009 |
| SSRIs | N.A | N.A | N.A | - Greiss Hess et al., 2016 | (+) Greiss Hess et al., 2016 Indah Winarni et al., 2012 |
| Fenobam | (+) Wang et al., 2014 | (+) Vinueza Veloz et al., 2012 | + (small) Berry-Kravis et al., 2009 | N.A | N.A |
| Mavoglurant | (+) Pop et al., 2014 | (+) Gantois et al., 2013 | N.A | (-) Berry-Kravis et al., 2016 | (-) Berry-Kravis et al., 2016 |
| Basimglurant | N.A | N.A | N.A | (-) Santarelli 2014 | N.A |
| Memantine | N.A | N.A | (-) Erickson et al., 2009 | N.A | N.A |
| R-baclofen | (+) Henderson et al., 2012 | (+) Silverman et al., 2015 | | (-) Berry-Kravis et al., 2012 | (-) Berry-Kravis et al., 2014 |
| Riluzole | N.A | N.A | (-) Erickson et al., 2011 | N.A | N.A |
| Acamprosate | N.A | N.A | (+) Erickson, Mullett, & McDougle, 2010 | N.A | N.A |
| Ganaxolone | (+) Braat et al., 2015 | (-) Heulens et al., 2012 | N.A | N.A | N.A |
| Lithium | (+) Choi et al., 2011 | (+) Liu et al., 2011 | (+) but (-) in primary Berry-Kravis et al., 2008 | N.A | N.A |
| Minocycline | (+) Bilousova et al., 2009 | (+) Rotschafer et al., 2012 | (+) Paribello et al., 2010 | (-) Leigh et al., 2013 | (+) Schneider et al., 2013 Leigh et al., 2013 |
| Lovastatin | (+) Osterweil et al., 2013 | (+) Osterweil et al., 2013 | (+) Çaku et al., 2014 | N.A | N.A |
| Trofinetide | (+) Deacon et al., 2015 | (+) Deacon et al., 2015 | N.A | N.A | N.A |

**Table 2.1 Summary of therapeutic approaches for FXS to date.** In orange symptom based treatments, blue excitation modulators, green inhibition modulators and red other mechanism based treatments. References are examples from existing literature. For more details see Sastre et al., 2015. IGF-1: insulin-like growth factor 1, SSRIs: selective serotonin reuptake inhibitors, (-): negative outcomes, (+): positive outcomes

### 2.4.3 Riluzole

Riluzole is a prescription drug approved by FDA for use by individuals with amyotrophic lateral sclerosis (ALS). It has been also shown to have antidepressant properties and act as an anxiolytic in obsessive-compulsive disorder (Grant et al., 2007; Zarate et al., 2004). Although the action of riluzole on glutamate receptors has not been elucidated fully yet, it is hypothesized to work by inhibiting glutamate release (Debono et al., 1993; Martin et al., 1993) and potentiating post-synaptic $GABA_A$ receptor activity (Jahn et al., 2008). Although there are no published studies in the mouse model of FXS, riluzole was one of the first drugs, hypothesised to act as a $GABA_A$ agonist, to be studied in FXS clinical trials. A six week open-label prospective pilot study (100 mg/day) took place 6 years ago (Erickson et al., 2011) with a primary outcome of repetitive, compulsive behaviour, which is a common comorbid disorder with FXS; the sample was six adults with FXS. The study showed that treatment with riluzole was associated with a clinical response only in one of six subjects. Peripheral extracellular signal-related kinase (ERK) activation, which is known to be altered in fragile X KO mouse models (Weiler et al., 2004), was significantly corrected in all subjects despite the lack of significant clinical improvement.

### 2.4.4 Ganaxolone

Ganaxolone (3a-hydroxy-3B-methyl analog of allopregnanolone) is a CNS-selective steroid and positive allosteric modulator of the $GABA_A$ receptors (Greenfield, 2013). Ganaxolone has been shown to act effectively as an anticonvulsant in diverse rodent models of seizure disorders, at tolerable doses and as potent sedative at higher doses (Carter et al., 1997). In *Fmr1* KO mice, ganaxolone has been shown to alleviate symptoms such as audiogenic seizures (Braat et al., 2015; Heulens et al., 2012). Furthermore, it is known to be well tolerated by human adults, children, and infants (Kerrigan et al., 2000; Monaghan et al., 1997). A Phase II trial in approximately 60 child carriers of the full mutation, aged 6–17 years, is currently taking place in California, US (ClinicalTrials.gov Identifier: NCT01725152). The study aims to determine the safety, tolerability, and efficacy of ganaxolone for the treatment of anxiety and attention deficits seen in FXS patients by using a randomized, double-blind, placebo-controlled, 6-week crossover design with a 2-week washout period between the two treatment stages.

## 2.5 Other Mechanism-based Treatments

### 2.5.1 Lithium

Lithium has been used for more than 60 years as a psychiatric medication, and especially for the treatment of major depressive disorder and bipolar disorder (Rybakowski, 2011). Long before it was the subject of preclinical and clinical testing, lithium had been used, off-label, to treat aggression and mood instability seen in individuals with FXS (Liu & Smith, 2014). Over the last 10 years, lithium has been shown to ameliorate a wide range of phenotypes associated with the loss of FMRP. *Fmr1* KO mice treated with lithium showed improvements in hyperactivity (Liu et al., 2011; Yuskaitis et al., 2010), social preference (Liu et al., 2011), cognition (King & Jope, 2013; Liu et al., 2011; Yuskaitis et al., 2010), aberrant dendritic spines (Liu et al., 2011), hippocampal plasticity (Choi et al., 2011), protein synthesis (Choi et al., 2011; Franklin et al., 2014; Liu et al., 2011) and seizure susceptibility (Min et al., 2009). Based on the extensive literature, lithium's mechanism of action seems to be the inhibition of glycogen synthase kinase-3 (GSK-3) (Jope, 2003), which has been shown to be altered in the *Fmr1* KO mouse (Jope & Roh, 2006; Portis et al., 2012). All the aforementioned preclinical data led to an open label treatment trial of lithium. Fifteen individuals with FXS, ages 6-23 were treated for two months, and showed improvement on a wide range of secondary measures such as ABC total scores, the CGI and the VAS. On the contrary, no effect was observed in the primary outcome measure, the Irritability Subscale of the ABC. While there were general behavioural improvements, the study also had a large number of adverse events, already known for lithium, including aggression, polyuria (increased urination), and polydipsia (increased thirst) (Berry-Kravis et al., 2008). The open-label design of this clinical trial makes it difficult to draw robust conclusions from the results. Although these findings are somewhat promising, the known side-effect profile of lithium probably limits its widespread use in patients with FXS.

### 2.5.2 Minocycline

Minocycline is a semi-synthetic broad-spectrum bacteriostatic antibiotic and it has an FDA approval for the treatment for acne (Strauss et al., 2007). Minocycline has been shown to inhibit matrix metalloproteinase 9 (MMP-9), which has been found to be elevated in the hippocampus of *Fmr1* KO mice (Bilousova et al., 2006; Dziembowska et al., 2013). Furthermore, *Fmr1* KO mice which received minocycline treatment displayed

reduced hyperactivity (Bilousova et al., 2009), reversal of communication deficits (Rotschafer et al., 2012), normal social recognition memory (Yau et al., 2016) and improvements in the immature dendritic spine phenotype (Bilousova et al., 2009; Siller & Broadie, 2012). There are two human clinical trials reported to date. In the first pilot study, 20 patients with FXS, 13-35 years, participated in an initial open-label trial. Minocycline led to significant improvement in CGI, ABC-C and the ABC irritability, hyperactivity, and inappropriate speech subscales after eight weeks of treatment (Paribello et al., 2010); minor side effects were observed. These promising results led to an additional study. This time minocycline efficacy was tested in randomized, double-blind, placebo-controlled clinical trials. Fifty-five children and adolescents aged 3.5–16 years with FXS underwent a minocycline treatment for 3 months. Subjects who received the drug demonstrated significant improvements in CGI-I but no significant improvement was found on any specific measure of the behavioural domains tested (Leigh et al., 2013). Even though no significant effects of minocycline on VAS scores were detected upon initial analysis, ad-hoc analysis did reveal significant improvements in anxiety and mood-related symptoms. Results for this study could have been affected by study design weaknesses; including unblinding of subjects when they completed the study, unblinding related to drug side effects, and the fact that investigators were aware of preliminary efficacy results. Twelve subjects (mean age 10.5 years) were taken from the same study and tested in a passive, auditory oddball paradigm. It was shown that minocycline treatment could normalise a specific electrocortical measure called event-related potentials (ERPs) (Schneider et al., 2013). This could be indicative of a normalisation in hypersensitivity to auditory stimulation which individuals with FXS exhibit.

### 2.5.3 Lovastatin

Lovastatin, also known as Mavacor, is a statin that has been FDA approved for the long-term management of familial hypercholesterolemia (Descamps et al., 2011). As a statin, lovastatin is an inhibitor of the enzyme HMG-CoA reductase, an enzyme that catalyses the conversion of HMG-CoA to mevalonate. This pathway is upstream to Ras signalling. Work by Cerezo-Guisado and colleagues, in cultured rat brain neuroblasts, revealed that lovastatin can indeed inhibit Ras signalling, an upstream effect that resulted in reduction in ERK1/2 activation (Cerezo-Guisado et al., 2007). This study supported previous findings by Xu and colleagues done in fibroblasts (Xu et al., 1996). The extracellular

signal-related kinase (ERK1/2) intracellular signalling pathway has been shown to be connected with the pathophysiology associated with FXS. Acting downstream of mGluRs, the ERK1/2 signalling pathway is integral for maintenance of normal synaptic plasticity and the regulation of activity-dependent protein synthesis (Gallagher et al., 2004; Osterweil et al., 2010). Elevated ERK activity under baseline conditions, has been shown except from the FXS mouse model in human post-mortem brain tissue as well (Wang et al., 2012). Reducing ERK1/2 activation indirectly, by inhibiting its activating kinase MEK with commercially available inhibitors U0126 and SL327, effectively reversed the audiogenic seizure phenotype in *Fmr1* KO mice (Wang et al., 2012) and reduced the increased basal level of protein synthesis seen in the hippocampus of *Fmr1* KO mice (Osterweil et al., 2010). Recently, Osterweil and colleagues confirmed that lovastatin indirectly inhibits Ras signalling and increases basal ERK1/2 activation. Moreover, *Fmr1* KO mice treated with lovatatin, showed normalised level of protein synthesis, hippocampal plasticity and audiogenic seizure susceptibility (Osterweil et al., 2013). Despite the known safety profile of lovastatin and the aforementioned very promising preclinical results, only one clinical trial has studied lovastatin's efficacy against FXS symptomatology so far. Çaku and colleagues (2014) reported an open-label trial in 15 patients (13 males, 6-31 years old). Treatment response was assessed before and after treatment using the ABC-C, total score (primary outcome), as well as the subdomains of the FXS validated version of the ABC-C, and CGI-I, and VABSII (secondary outcomes). Significant improvements were observed in the primary outcome, after 4 and 12 weeks of treatment. There was also moderate improvement on the CGI-I, but the open-label nature of the trial precludes any strong inferences of efficacy at this stage of development (Çaku et al., 2014). In addition to the behavioural evaluation of the treated individuals, blood samples were collected in order to analyse relevant biochemical markers. Quantitative Western blotting analysis in platelet samples, showed that ERK and Akt phosphorylation levels were normalised as well as ERK activity. Interestingly the changes in ERK phosphorylation correlated well with the clinical response to the treatment (Pellerin et al., 2016). One of the potential issues that arises with the possible use of lovastatin as a treatment for FXS is the lipid metabolism of the patients. Individuals with FXS have been reported to have lower levels of low- and high-density lipoproteins and total cholesterol. Therefore, participants should be placed on lipid monitoring during future lovastatin trials (Berry-Kravis et al., 2015).

### 2.5.4 Insulin Growth Factor 1/Trofinetide/ NNZ-2566

NNZ-2566, or trofinetide, is a synthetic analogue of a naturally occurring peptide, called glypromate or GPE, which is derived from insulin-like growth factor 1 (IGF-1). Trofinetide has been shown to have neuroprotective properties; in a rat model of traumatic brain injury, trofinetide leads to improving recovery, reducing apoptotic cell death, and reducing neuroinflamation (Cartagena et al., 2013; Wei et al., 2009). Trofinetide's efficacy has already been studied in recent Phase II clinical trial in individuals aged 16 to 45 years with Rett syndrome. Although the study has not been published yet, Neuren Pharmaceuticals has reported that the drug was well tolerated and met pre-specified criteria for improvement (Pharmaceuticals, 2016). Treatment with the entire IGF-1, which trofinetide mimics, has also been shown to improve symptomatology associated with Phelan-McDermid syndrome (PMDS) in the mouse model of the syndrome (Bozdagi, Tavassoli, & Buxbaum, 2013), cultured human neurons (Shcheglovitov et al., 2013), and a Phase I clinical trial in children (Kolevzon et al., 2014). PMDS, also called 22q13 deletion syndrome, is a rare developmental disorder caused by heterozygous deletion of the terminal of chromosome 22 (22q13.3) which includes the SHANK3 gene, or a loss of function mutation on the SHANK3 gene. SHANK3 gene loss of function mutations have been associated with ASDs (Boccuto et al., 2012; Phelan & McDermid, 2012). In the only publish study using the mouse model of FXS, trofinetide has been shown to reverse some learning and memory deficits, reduce hyperactivity, normalise abnormal dendritic spine morphology, and restore normal extracellular signal-related kinase (ERK) signalling and, interestingly, restore normal testicular size (Deacon et al., 2015). A double-blind, placebo-controlled early phase trial of trofinetide in patients with FXS, has recently been completed. This study was designed to investigate the safety and tolerability of a liquid oral formulation of trofinetide in adolescent and adult males with FXS (Treagus, 2015).

## 2.6 Treatment development for FXS: Quo Vadimus?

Over the last 10 years, drug development for FXS has received an increased amount of attention from both basic science researchers as well as large and small pharmaceutical companies. Basic, translational and clinical research breakthroughs continue to identify novel targets and develop new promising therapeutic treatments for patents. Based on its prevalence (1 in 5000), FXS is classified as a rare disease (EMA EU/3/15/1452). As a result, the Food and Drug Administration (FDA) as well as the European Medicines

Agency (EMA) have adjusted the criterion for demonstrating drug efficacy to a single Phase III clinical trial with positive primary and secondary outcomes; as opposed to two Phase III trials in disorders affecting larger populations (that's mainly due to the difficulties of recruiting more participants for a second large scale clinical trial). As in any other clinical study, the primary endpoint, which is preapproved by the FDA or EMA and specified before the clinical trial begins, must be significantly improved in the treatment group compared to participants who received placebo. Despite the less strict criteria for approval, there has not yet been a single treatment medication which is approved for the treatment of FXS.

Clinical trials so far have revealed a number of promising medications with positive behavioural responses, but have the final behavioural readouts been adequately objective? Thorough evaluation of drug efficacy requires a set of standardized, disease-specific, outcome measures; considerable effort is already being made in that direction. Primary endpoints of clinical trials in FXS have typically included the Aberrant Behaviour Checklist (and other subsection scores), the Clinical Global Impressions Improvement of Severity subscales (CGI-I or CGI-S), the Social Responsiveness Scale (SRS), or other care giver-, teacher-, or clinician-based rated scales. In research on developmental disorders it is possible to observe a significant improvement of subjects (20–30%) in response to placebo, when the aforementioned subjective evaluation methods are used; this effect is often tricky to avoid, even with a placebo lead-in phase during the trial (Waschbusch et al., 2009). It is clear from the above that the commonly used outcome measures listed, can be inadequate to track improvements in the FXS phenotype, resulting in a push to develop reliable, FXS-specific outcome measures (Berry-Kravis et al., 2013). The FXS-specific Fragile X Symptom Rating Scale is currently being validated in several clinical trials, including the Phase II trial of trofinetide (clinicaltrials.gov NCT01894958). Another scale being validated for use in FXS is the Pediatric Anxiety Rating Scale revised for FXS (Russo-Ponsaran et al., 2014). Progress in clinical trial endpoints and biomarker development are becoming top priorities in the field (Elizabeth Berry-Kravis et al., 2013).

Less subjective measures such as eye-tracking, pre-pulse inhibition, brain imaging, evoked related potentials (ERP), and blood biomarkers are slowly beginning to be included in early Phase II drug development as more objective measures of a treatment's efficacy and potential engagement with the underlying pathophysiology of the disorder (Pellerin et al., 2016). Molecular biomarkers, which are being explored as indicators of treatment response and signalling proficiency, include, but are not limited to, cyclic

adenosine 3′, 5′-monophosphate (Kelley et al., 2007), ERK (Weng et al., 2008), BDNF (Erickson et al., 2013), amyloid beta-protein precursor and cleavage proteins (Erickson et al., 2014), MMP9 (Dziembowska et al., 2013), and event-related potential (Schneider et al., 2013); it is likely that the latter may only be applicable to higher-functioning individuals, as many subjects with FXS find the procedure too stressful to tolerate. These molecular and physiological biomarkers, have not been used as 'primary' endpoints in any large placebo-controlled trials in FXS yet. Nevertheless, even the inclusion of these types of assessment is important for identifying changes, positively associated with the given treatment, keeping in mind the strong placebo effect on standard parent/ caregiver report rating scales. Ideally, additional research focusing on the correlations between behavioural symptomatology/severity and its physiological/molecular correlates, the use of objective biomarkers, will be more common in the future, providing a way to a clearer treatment evaluation.

The possibility of divergence in response treatment between various sub-populations defined by factors such as IQ, premutation status, or even gender, obviously complicates the problem of treatment evaluation even further. For the purpose of ad-hoc analyses, several studies used symptom severity: social withdrawal (Berry-Kravis et al., 2012) or methylation status (Jacquemont et al., 2011) to stratify participants. Unfortunately, in both instances, when the trials were repeated in larger sample sizes, the findings were not replicated. Although it is still considered controversial, the expression levels of FMRP and methylation status of the *FMR1* gene have been correlated with cognitive ability (positively and negatively respectively), whereas little work has been done on the relation between the extent of CGG trinucleotide repeat and cognition (Chudley et al., 1983; Steyaert et al., 1996). Surprisingly, it was recently shown that the number of CGG repeats can differ across different tissue types in the same affected individual (Lokanga et al., 2013). This suggests that the number of repeats based on blood mononuclear cells (the cell type often used for FXS diagnostic and stratification purposes) may not always directly translate to that in the brain. If this finding is correct, it is obvious that inaccurate trinucleotide repeat characterisation can severely complicate efforts to use blood biomarkers for clinical trial inclusion/exclusion criteria.

One major question which need to be addressed in future FXS research, and in neuroscience as a whole, is whether correcting lifelong abnormal synaptic morphology and function can or will lead to improved behavioural symptomatology. More specifically, in FXS treatment development, the main question is whether modulation of

synaptic imbalance in the adult brain, is adequate to restore normal cognitive function in FXS. Excitation/inhibition imbalances, which show a distinct developmental profile (Gatto & Broadie, 2010), can cause the brain circuitry to develop improperly with potentially permanent consequences in behaviour. We should keep in mind that FMRP is expressed ubiquitously across the brain starting from early stages of embryonic development.

On top of the complexity of the human disease and the difficulties in assessing the outcomes of clinical trials objectively, the reasons for our failure to develop a successful treatment for FXS so far, arises from the fact that the mouse model of FXS has some limitations. It is important to recognize the inherited limitations of the data produce and of the mouse model as a system. Clear interpretation of results, replication of the studies, and further studies on initial results need to receive more attention from the research community. Furthermore, it is a common problem in research that negative molecular, electrophysiological and especially behavioural responses are very rarely being reported; this can drastically skew the attitude of the research community, and the pharmaceutical industry towards the efficacy of a treatment. As discussed earlier, behaviour in *Fmr1* KO mice is known to be very unreliable; it is critical that authors are aware of potential confounding factors in their behavioural tasks. For example, many tests in rodents, can be heavily influenced by changes in locomotor activity. If, by any chance, a proposed treatment reduces basal levels of locomotor activity in the *Fmr1* KOs, this could affect a number of tests' readouts; reduce marble burying (reduced repetitive behaviours) and time spent in the open arm of the elevated zero or plus maze (perceived as reduced stress). When reporting and interpreting the efficacy of a pharmacological treatment, it is also really important to mention whether there was a robust baseline deficit between the vehicle-treated *Fmr1* KO mice and wildtype littermate controls. The differences between mouse and human physiology are obvious and translating results between species is challenging. Without thorough assessment, careful interpretation, and full disclosure of all experimental procedures in preclinical models, it is extremely difficult to get a clear picture of the effectiveness of a treatment. Nevertheless, this does not mean that a robust improvement in a single domain of the mouse behavioural (observed in several different tasks) is not a significant finding. Individuals with FXS exhibit a very multifaceted behavioural phenotype so that even if a drug only improves reliably one of the affected behavioural domains, it would be incredibly beneficial.

Even if we examine treatments where behavioural (limited in many cases), electrophysiological and molecular improvement/normalization were shown in the mouse model and moderate improvements were also shown in small scale clinical trials, expansion into larger sample sizes was unsuccessful in most cases. A reason for that could also be that the human equivalent of the majority of the behavioural and physiological readouts or symptom domains examined in the mouse model studies, are either not being assessed in the human clinical trials (i.e., audiogenic seizure susceptibility, object memory, PPI), or the equivalent human symptom domain is not heavily affected or easily translated into a relevant mouse behavioural task (social preference, inhibitory control) (Kramvis et al., 2013). Therefore, the efficacy of a treatment cannot be adequately examined for a number of behaviours across mouse model and human patients. For example, one of the most commonly used FXS preclinical mouse behaviour tests is the audiogenic seizure paradigm, partly because increased seizure susceptibility is one of the most common behavioural deficits seen in *Fmr1* KO mice. Several mGlur5 antagonists and many other treatments, especially those targeting excitatory/inhibitory imbalance, were effective at reducing the number or the severity of audiogenic seizures in *Fmr1* KO mice (Michalon et al., 2012). These results could suggest that the examined treatments effectively reduce hyperexcitability seen in the mouse brain; for obvious ethical reasons, seizure susceptibility is not something that is assessed in human clinical trials and the direct effect of a possible hyperexcitability attenuation, does not have a clear correlation to other symptom domains, such as anxiety or inattentiveness. Moreover, treatment with mavoglurant was shown to improve deficits in startle response in a PPI paradigm in the mouse model, but PPI could not be assessed in the clinical trial because several participants with FXS could tolerate this procedure easily (Levenga et al., 2010).

Of course mice are still relevant as a model for FXS and there is a lot more to learn from studies on them. For example, robust genetic manipulations are still only possible in mice. Genetic rescue in the *Fmr1* KO mouse by creating double mutants that harbour a mutated/deficient allele such as mGlur5 (Dölen et al., 2007), muscarinic M4 (Veeraragavan et al., 2012), p70 ribosomal S6 kinase (Bhattacharya et al., 2012), or amyloid β-protein precursor (APP) (Westmark et al., 2011) can be really powerful experiments. However, we have to keep in mind a major limitation of genetic rescue studies. This rescue approach is significantly different from administering a drug, which will proceed to modify the function of a receptor or any other signalling molecule, to an

infant, an adolescent, or even an adult. Double-mutant mice which are generated by crossing two single-mutant mice, will have "received the treatment" exerted by second genetic manipulation, from conception, every day, throughout the day, rather than the typically short-duration treatment used in a FXS clinical trial. Furthermore, in the case of a pharmacological approach, the optimal dose is usually unknown (since we are dealing with novel therapeutic strategies) and the highest tolerable dose is usually used. Nevertheless, both genetic and pharmacologic approaches can reveal potential core mechanisms which have been affected by the loss of FMRP, and can give greater insight into the likelihood of success of a treatment's for FXS.

It looks like mouse models often take the blame for one of the most inconvenient truths in translational research: Even after mouse studies suggest that a medication will be safe and effective, more than 8 out of 10 potential therapeutics fail when tested in large scale clinical trials (Arrowsmith, 2011; Ledford, 2011). Mouse models of various conditions, especially neuropsychiatric, are regularly thought to be poor predictors of an experimental drug's efficacy (poor predictive validity). As we previously discussed, the real reason for the observed poor predictive validity is often that the preclinical experiments are not rigorously designed; but in the case of neuropsychiatric disorders, mice might have certain inherited limitations as a species. In the following introductory chapter, I will be discussing how the emergence of new rat KO models can be a potential "game changer", for translational research.

# 3. From Mouse to Rat

## 3.1 Rats are not oversized mice

The outward similarity of mice and rats can convey the false impression that mice are essentially smaller, faster breeding rats. The evolutionary distance between mice and rats, however, is bigger than one might imagine; the rodent lineage which gave rise to these two species split between 18 and 40 million years ago; hence the genetic material shared between a rat and a mouse has had more evolutionary time to diverge than one might expect (Gibbs et al., 2004; S Kumar & Hedges, 1998). Homo sapiens has common ancestry with non-human primates about 6 to 7 million years ago, therefore mice and rats on average are much more different from each other than we are from chimps.

The Norway rat, *Rattus norvegicus,* was the first mammalian species to be domesticated for scientific purposes; the first recorded rat dissection dates back to 1621 when Theophilus Müller and Johannes Faber of Italy's Accademia dei Lincei in Rome perform a dissection of a pregnant wild specimen; work on rat physiology dates back to the early 19th century, when experimenters concentrated on the effects of food and oxygen deprivation (Abbott, 2004). The rat has been a very important model in biomedical research ever since, due to its well-characterized physiology and convenient size and, more importantly, the rat is a species with its own biological particularities which make it, in several circumstances, an advantageous model compared to a mouse. For example, rat arthritis and hypertension models, including transgenic animals, have long been used because they recapitulate, better than mice, human clinical aspects such as gender differences (Paul et al., 1994; Taurog et al., 1999).

Behavioural neuroscientists have historically preferred the larger and less temperamental rat. Mice are difficult to work with because they are generally hyperactive and more impulsive than rats; mice are also slower and less flexible learners compared to rats (Ellenbroek & Youn, 2016). For instance, a widely used experimental set-up for researchers interested in addiction, involves training rats to press a lever in order to get a small quantity of a certain drug such as cocaine (drug self-administration studies). Rats appear to learn faster and it is clear that they deliberately press the lever. On the other hand, mice look much more impulsive and they do not seem to have a certain plan; they rush and press the lever randomly (Chistyakov & Tsibulsky, 2006; Thomsen & Caine, 2005).

Even though rats have been used in both basic and applied biomedical research for many years now, the mouse is currently the king. Since 1990, the number of yearly published scientific reports indexed by PubMed in which mouse models have been used increased threefold, overtaking the number of papers devoted to the rat in 2002. The mouse genome has been extensively studied and genetic manipulation techniques have been far more advanced than in any other mammalian model. But rat genetics have begun to catch up after the report of the first cloned rat in 2003 and sequencing of the rat genome in 2004. Technologies such as zinc-finger nuclease technology have now been used to create dozens of KO rat lines (Dolgin, 2010). Moreover, recent advances in gene editing, like the CRISPR/Cas9 system provide outstanding possibilities for targeted modification of the genome, which are often extremely efficient. New KO rat models are being developed every month, which reveals the excitement in biomedical research about re-establishing the rat as the main animal model (Bao et al., 2015; Li et al., 2013). In the following few pages I will discuss why rats have the potential of being an invaluable tool in biomedical research and more specifically research focussed on neurological and neuropsychiatric disorders.

## 3.2 Rat in translational research

The development of technology allowing for targeted genetic manipulation of mouse embryonic stem cells 25 years ago, led to an explosion in biomedical research. Not surprisingly, the mouse has overtaken the rat and has become the most widely used model organism (Mayford et al., 1997). Yet the rat has traditionally been the preferred model organism in biomedical research, so that even until 2001 the number of published research reports in which rats were used was larger than that of mouse publications. In fact, in 1989, the year in which the creation of the first KO mouse was reported (Thompson et al., 1989; Zijlstra et al., 1989), there were 70% more publications on rats than mice. That bias toward rats in biomedical research, has given us today an enormous archive of historical physiological data on the rat (Gill et al., 1989). Furthermore, most in vivo assays, particularly those in behavioural and cardiovascular research, were initially developed and validated on the rat and only in the recent past have been adapted to be used for mice. Understanding of rat genetics is the single field where rats have been lagging behind mice. However, this has changed with the sequencing of the rat genome (Gibbs et al., 2004; Mullins & Mullins, 2004) and the development of tools such as the rat genome database (Shimoyama et al., 2015). Finally, the advent of new genomic editing

tools, such as zinc finger nucleases (ZFNs) and CRISPR/cas9 gene editing system, has now enabled precise genetic manipulation of the rat genome and germ-line transmission, setting the stage for the resurgence of the rat as the model organism of choice.

### 3.2.1 Larger sample volumes

By weight, a rat is more than 10 times the size of a mouse. The larger size means larger tissues and samples (Kleiber, 1947; Medigreceanu, 1910). This can mean a reduction in the number of animals required for a study, or enable the study of molecules too low in concentration to be measured reliably in the mouse. There are also more opportunities to measure multiple biomarkers/metabolites from the same sample, further reducing animal requirements (Parasuraman, Raveendran, & Kesavan, 2010). Small sample volumes require highly sensitive assays and are prone to high variability and false readout, while the larger sample volumes afforded by rat models reduce these technical limitations. Moreover, with blood volume of approximately 25mL, it is a lot easier to get multiple blood samples from rats than mice (1.5-2.5mL), enabling time-course sampling (for both animals a maximum of 1% of the circulating blood volume can be removed every 24 hours) (Parasuraman et al., 2010; Teilmann et al., 2014).

### 3.2.2 Easier surgery

The larger size of the rat compared to mice, allows for surgery that is much easier to perform. Experimenter's training times are reduced when rats are the models of choice, reducing simultaneously the time needed for reliable data acquisition and eventually publication. More easily performed surgery also means fewer experimental errors, leading to increased data collection efficiency, reduction in experimental costs and in the number of animals used (Ellenbroek & Youn, 2016). Small substructures can be more easily studied and targeted, for example microinjections or cannulations into small brain nuclei such as the arcuate nucleus of the hypothalamus is far easier to perform in the rat. For example, disconnection studies (multiple brain areas in both hemispheres being deactivated in order to study the importance of functional connections between different brain areas) can be performed much more efficiently in rat models, even in neonates (Lipska et al., 2002; Zeeb & Winstanley, 2013). Furthermore, implantation of probes in order to monitor brain activity (something relevant to research in neurodevelopmental disorders) is possible in rat pups starting from the 14$^{th}$ day of their lives (Langston et al., 2010).

### 3.2.3 Higher resolution imaging

Imaging technologies are advancing rapidly with the emergence of techniques such as two-photon functional imaging of neuronal activity using calcium-sensitive (Svoboda & Yasuda, 2006) and voltage-sensitive (Ferezou, Bolea, & Petersen, 2006) dyes and protein markers, quantum dots (Marshall & Schnitzer, 2013), diffusion tensor imaging (Alexander et al., 2007), and fMRI among many others. Even though some of them require certain genetic tools already available in mice (like voltage and calcium dyes for two-photon imaging), the translational nature of the rat as a model makes it an ideal organism for imaging aimed at uncovering structural and functional irregularities related to disorders. Perhaps the biggest advantage of the rat over the mouse, which has already been discussed, is the increase in spatial resolution due to the rat's larger size; spatial resolution in PET imaging has been estimated to be up to 10 fold greater in the rat than the mouse) (Zheng et al., 2015).

An example of an imaging technique with potential translational value is resting state functional Magnetic Resonance Imaging (rsfMRI). Interest in rsfMRI, an imaging method commonly used to study functional connectivity in the brain, has recently increased and has opened interesting and flourishing lines of investigation. The idea of measuring the brain's resting state became popular among human researchers and various resting state networks have been identified over the recent years. These observations led to a number of intriguing studies which investigated functional connectivity correlates in both neurologic and psychiatric disorders (van den Heuvel & Hulshoff Pol, 2010), depression (Greicius et al., 2007), dementia (Zhang et al., 2013) and schizophrenia (Kantrowitz et al., 2015). Consequently, rsfMRI became a very attractive candidate for identifying (early) disease signatures as it is a non-invasive technique which is undemanding for the patient due to its limited scanning time. rsfMRI experiments on animals are up to this date scarce, and are mainly on rats and monkeys (Kannurpatti et al., 2008; Moeller et al., 2009; van Meer et al., 2010; Vincent et al., 2007), with a few exceptions on mice (Grandjean, Schroeter, Batata, & Rudin, 2014). In a study published 5 years ago, Jonckers and colleagues (2011) compared functional connectivity between mice and rats using rsfMRI. They found that rats produced less variable data than mice, the imaging resolution was higher (i.e. 4 separate components for the entorhinal cortex compared to 2 for mice), but the overall signal to noise ratio was the same between the two rodent species. The use of rsfMRI in rat models clearly has the potential to give us more insight

into, and understanding of, the potential of this technique as a clinical diagnostic tool. Rat models make it possible for us to experimentally alter functional connectivity using drugs and/or genetic lesions. Additionally, rsfMRI could be used to evaluate the efficacy of potential treatments in rat models (a preclinical stage), and examine how that translates to human patients who received the same treatment, (clinical trial stage), bridging the gap between the two stages of treatment development.

### 3.2.4 Physiology closer to humans

Over the years, several anatomical and physiological differences have been highlighted between mice and rats (Logan et al., 1988; Stepanichev et al., 2016; Witte et al., 2010). Additionally, rats are consistently more representative of human physiology than mice, but still not as close as larger animals (i.e. swine, dogs, macaque) (Lelovas et al., 2008; Radermacher & Haouzi, 2013). The heart rate of a mouse is ~600 beats per minute (310-840bpm), while the rat is less than half of that (300-450bpm) and therefore closer to the human average of 70 bpm. Adding to the previous the fact that rats have larger heart and blood vessel size, it is obvious why they are preferred in cardiovascular research over mice (a quick PubMed search shows that there are 6 times more published reports on rat than mouse). Furthermore, during the past decades, researchers working on drug discovery have preferred to screen the efficacy of a drug in genetically modified mice, models of disorders, and then switch models and assess safety and toxicity in rats, mainly due to the large volume of historical safety data in the rat and the greater physiological similarities between rats and humans compared to mice and humans. This methodology relies heavily on extrapolations of used mouse dosing to rat, and the assumption that this dose would have similar efficacy in the rat. The uncertainty in this approach is clearly far from ideal, as drug efficacy has been observed to be highly variable in different mouse strains, let alone in different species (Fattore et al., 2002; Paterson et al., 2003). Recently developed genetically modified rat models can now address this problem, enabling researchers to conduct both drug efficacy and safety studies not only in the same species but even in the same background strain.

### 3.2.5 Rich behavioural profile

I have already mentioned that the rat has been the work horse of experimental psychology. Rats' performance in behavioural tasks is much more reliable and robust

than that of mice; due to their increased variability, mouse behavioural assays typically require cohort sizes of up to 50% larger than those needed for rats (Ellenbroek & Yun 2016). Generally, rats perform better than mice on behavioural assays addressing learning and memory mechanisms and addiction; I mentioned earlier that rats are the preferred species for drug self-administration studies. The ability to classify subtly different behavioural responses is what can make the rat a powerful model of neuropsychiatric disorders. An example of that was reported by Steiner and Redish (2014). They showed that when rats realised they made a mistake, in a decision making task, they displayed signs of "regret". Regret is different from disappointment, which is simply when things are not working out. Regret is the recognition of one's mistake and the realisation that if one had done something differently, things would have been better. Steiner and Redish trained rats to run around a circular track past a series of four arms, each leading to a different food. As the rat came to the entrance of each arm, a tone indicated how long the rat would have to wait to receive that food. The rat could choose whether to stay or go, depending on how much it liked that food and how long it would have to wait. Whenever a rat refused a "good deal" only to realise that the next arm was offering a worse deal, it would stop and look back at the previous arm it had skipped. The rats showed three behaviours consistent with regret: (1) they only looked backwards in the "regret" arm, and not in the "disappointment" arm, (2) they were more likely to take a "bad deal" after their mistake, and (3) instead of taking their time to eat and groom themselves after they finished eating, the rats in the regret conditions ate quickly and rushed to the next arm. Of course, some differences in the cognitive performance between mice and rats could be due to the fact that most of the widely used cognitive tasks were first developed in rats and then just "transferred" with minor modifications to mice, without taking into account ethological difference between rodent species (Jaramillo & Zador, 2014).

In assays assessing pain, rats are less likely to be susceptible to anxiety-induced analgesia and, as previously discussed, perform much more reliably; in fact, pain research is one field where mice never surpassed rats in the number of publications. Interestingly, a recent study showed that the gender of experimenter heavily affects the response to painful stimuli in mice (Sorge et al., 2014). Moreover, rats are highly social compared to mice, which are more territorial and aggressive. An example is juvenile play; young rats will display playful wrestle behaviour with cagemates, much like kids do, while mice do not (Wöhr & Scattoni, 2013). Due to aggression and social rigidity in mice,

cages of male mice (the sex that is most frequently used in biomedical research) are a mixture of dominant and subordinate animals that vary greatly in terms of behaviour, physiology and immune function (Berry, 1970; Brain, 1971; C. A. Hendrie, Weiss, & Eilam, 1996) and this is, at least, a major source of variance in all studies using this rodent species. Cognitive and social behaviour differences will be discussed in more detail in the following paragraphs of this section, as these domains are two of the main affected behavioural domains in FXS and autism.

### 3.2.6 More translational

Taking together all of the previously discussed advantages of rats over mice, everything points to the idea that rat models are more translational than mouse models. The recent creation of KO rat models may be the best possible way to bridge the gap between basic preclinical research and clinical research, which can hopefully lead to the development of new treatments. Rats have been used in the pharmaceutical industry for years to predict how human patients will metabolize medication and to identify and study potential side effects. The results of these studies are essential before Phase I trials, addressing tolerance, can begin in humans. In the previous chapter, I discussed recent drug development efforts against FXS. Several translation failures so far have led to uncertainty in the field. As a result, the mouse model of FXS has recently come under scrutiny. Two examples that highlight the differences between the two rodents, once more, come from two comparative pharmacology studies. Rats and mice were treated with two previously discussed drugs (R-baclofen and MPEP) in an attempt to examine their efficacy against nicotine addiction. In both studies, differences between species was reported; mice also showed an inflexibility in dose-response curves and the dose of self-administered nicotine (Fattore et al., 2002; Paterson et al., 2003). The newly created rat models are now aiming to reclaim their position as the animal model of choice because of all the reasons mentioned above.

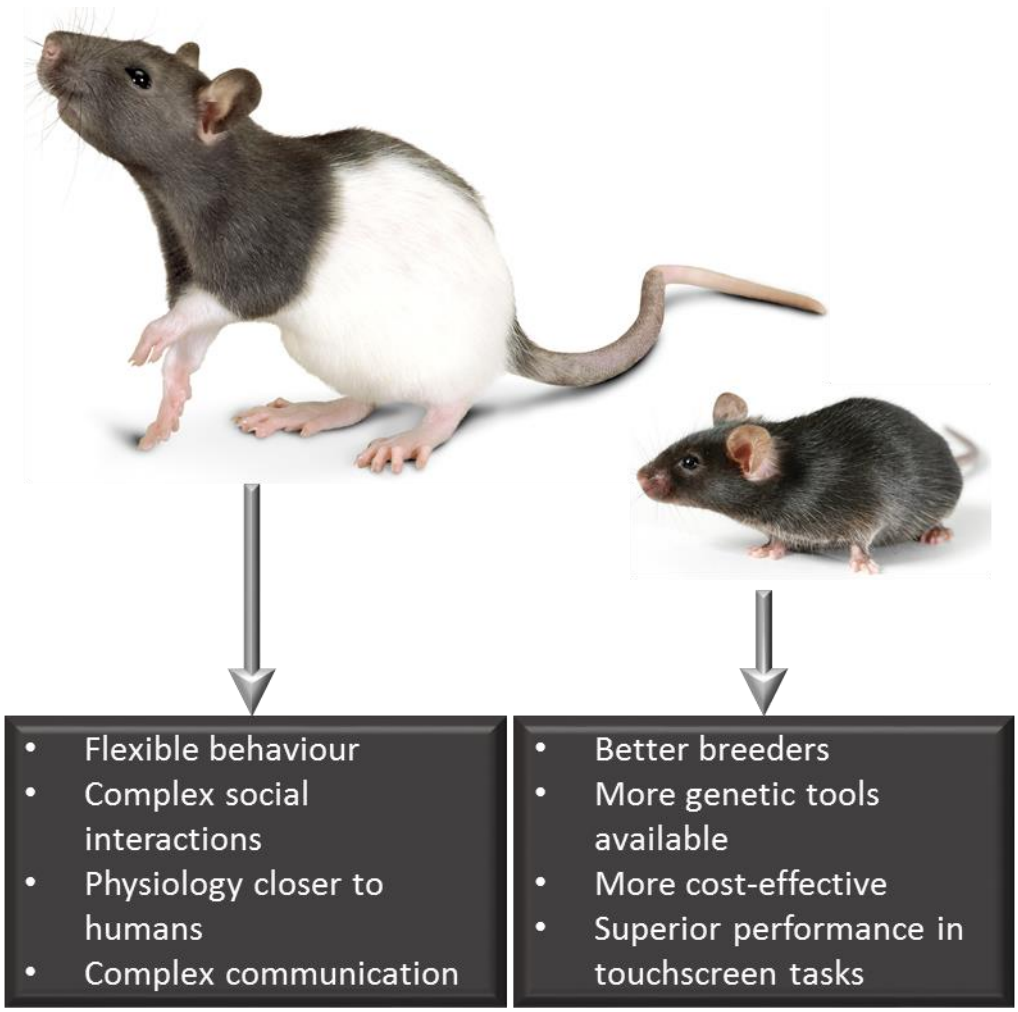



**Figure 3.1 Rats are not oversized mice.** The outward similarity of mice and rats can give somebody the false impression that mice are essentially smaller, faster breeding rats but there are core differences between the species.

## 3.3 Rat as a model organism of ASD and FXS

Validity is a key concept when assessing the utility and reliability of animal models. As originally stipulated by McKinney and Bunney (1969), the ideal animal model of a disorder should mimic the disorder's aetiology, pathophysiology/symptomatology, and response to treatment. These criteria proposed by McKinney and Bunney laid the foundation for the concept of construct, face, and predictive validity of animal models respectively (D. C. Blanchard, Summers, & Blanchard, 2013; Willner, 1984). Specifically, in model organisms of neuropsychiatric disorders, construct validity expresses the conceptual framework of the model – that is, the commonality between the underlying neurobiological mechanisms in the model system/organism and those underlying the behaviour in the pathological state being observed in human patients. Face validity expresses the similarities between the symptoms displayed by the affected individuals and the behaviours exhibited by the model organisms. Lastly, the criterion of predictive validity relates to the capability of the model organism to predict the progression of the disorder modelled, as well as the response to therapeutic interventions; whether or not a medication will be successful when tested in the clinical trials (Willner, 1984), also including sensitivity of the animal model in question, to pharmacological manipulations affecting the disease in humans, either in a positive or in a negative direction (D. C. Blanchard et al., 2013). In the following pages I will discuss the potential advantage of rat models over mice in modelling FXS and ASD(Cenci, Whishaw, & Schallert, 2002). I will focus on the three core behavioural domains being affected in FXS and ASD, namely anxiety/hyperactivity, social interactions and cognitive deficits, and explain why the rat is a more suitable model organism.

### 3.3.1 Differences in anxiety-hyperactivity

Anxiety and hyperactivity are common behavioural problems associated with FXS and ASD (Leitner, 2014; Wheeler et al., 2014). The neurobiological mechanisms connecting anxiety/hyperactivity phenotypes and FXS/ASD remain elusive but there are available symptom-based treatments like SSRIs and ADHD medications (methamphetamines) that can partially alleviate the behaviours in affected individuals. Over the last 20 years behavioural phenotyping in the mouse model of FXS has yielded some contradictory results. The three main behavioural paradigms used to access anxiety-like and hyperactivity-like behaviours in rodents and specifically in *Fmr1* KO mice are the open field test, the elevated plus or zero maze and the light-dark box task (Bouwknecht &

Paylor, 2008). Although, rats do not seem to have an obvious advantage over mice when modelling anxiety related behaviours, differences between the species' natural response to stressors lead to different readouts in these tasks. An obvious difference in that respect is that mice are primarily herbivores while rats are opportunistic (herbivores and carnivores depending on their habitat's natural sources). This key difference in natural behaviour means that rats are bolder when exploring new environments which are potential unfriendly; a behaviour which is essentially assessed in all three aforementioned tests.

Rats and mice are used extensively in the field of depression and anxiety research because of the wealth of genetic and physiology data available. Nevertheless, summarising the findings from studies made using rats, mice or other species, as 'rodent models' does not take into consideration the potential importance of these species differences. This is also a very high risk strategy that assumes depression and anxiety to be general mammalian features that can be modelled in any animal of this class (Hendrie et al., 2013). In relation to innate fear and depression related tasks, there are important differences between rats and mice and their significance cannot be ignored.

Even though there are not many comparative studies specifically in behaviour, there are a large number of studies looking at differences in the neurotransmission systems between rats and mice, relative to mood disorders and specifically in depression, anxiety and innate fear. For example, galanin and galanin receptor 1 (GalR1) have been connected to the serotonergic system (Misane et al., 1998) and have been shown to be implicated in cellular processes related to neurological disorders such as anxiety and depression (Bellido et al., 2002). A comparative study showed that galanin and GalR1 have different expression profiles in the central nervous systems of mice and rats; there is a lack of galanin and GlR1 in the mouse dorsal raphé nucleus, a part of the serotonergic system which has long been implicated in mood disorders (Larm, Shen, & Gundlach, 2003). Consistent with this finding, two studies have demonstrated that drugs acting on the serotonergic system exert anxiolytic-like and panicolytic-like effects only on rats but not in mice (Blanchard et al., 1997; Griebel et al., 1997). The differences in neurotransmission between rats and mice seem to be even more widespread; it has been reported that exposure to restrain stress leads to different responses in the monoaminergic neurotransmission between rats and mice (Konstandi et al., 2000).

Differences have also been observed in the effect of corticotropin releasing factor (CRF), a molecule associated with depression and anxiety disorders (Binder & Nemeroff, 2010). It has been shown that intra-cerebroventricular administration CRF leads to a reduction of depression like behaviours in rats but not in mice (Dunn & Swiergiel, 2008). Moreover, Radulovic and colleagues (1998) showed that there are key differences in the distribution of Corticotropin-Releasing Ractor Feceptor type1 (CRFR1) between rat and mouse central nervous systems. The main species differences were observed in cortex and brainstem. Mice were found to express less CRFR1 in the neocortical areas and more CRFR1 in the brainstem than rats. Keeping in mind that the cortical CRF receptors have been implicated in stereotyped motor behaviours (Crawley et al., 1985) and cognitive function (Van'T Veer et al., 2012), while brainstem CRF receptors are mainly involved in fear and anxiety, one could speculate that besides the differences seen in stress response between the species, differential CRFR1 expression profiles could account, at least in part, for differences between the rats and the mice in studies of learning and memory (Whishaw, 1995).

### 3.3.2 Differences in social interactions

Perhaps the most important reason to consider rats as a superior animal model for the autism spectrum and other related neurodevelopmental disorders is that they express much more complex social behaviours than mice. Social interaction deficits are one of the three affected core behavioural domains in FXS and ASD. To date, many behavioural paradigms have been developed in order to assess related deficits in existing mouse models (Silverman et al., 2010; Wöhr & Scattoni, 2013). Nevertheless, the question of whether these human behavioural traits have strong endophenotype similarities to rodents still remains unanswered (Servadio, Vanderschuren, & Trezza, 2015).

Differences between rats and mice can be observed even while handling them (see 1.5.3). Anyone who has worked with both rodent species will easily see that mice are much more aggressive than rats. This may be due to the species' different social structures in the wild; mice normally live in small groups (depending on the availability of natural resources) where one extremely aggressive alpha male dominates and monopolizes all females. They are strongly territorial and show high levels of intra-male aggression whenever they are group housed. Rats, on the other hand, which are organised in bigger highly social colonies, have a more loose and dynamic hierarchy, with widespread

promiscuity and low levels of aggression (Barnett, 1976). Rats live together relatively peaceably under laboratory conditions, within which significant levels of full-blooded aggression are seen under only the most unusual of conditions (Barnett, 1976; Rodgers & Hendrie, 1982).

Social learning is another behavioural skill relevant to FXS and ASD affected traits. Social transmission of knowledge and emotions have been documented in both mice and rats (Galef, 1984; Knapska et al., 2010; Munger et al., 2010). The only type of social learning that has been documented in mice is socially transmitted food preference, in addition to the identification of one of the molecular pathways involved. It seems that a specific receptor expressed in olfactory sensory neurons which are part of a specially designed circuit in the olfactory system is required for the acquisition of socially transmitted food preferences (STFPs) in mice (Munger et al., 2010). On the other hand, rats seem to be able to share information related not only to food preference (Strupp & Levitsky, 1984) but also to emotions (Knapska et al., 2010) and pain sensitivity (Fanselow, 1985). Knapska and colleagues (2010) showed that a brief social interaction of a rat with a cage mate that has undergone an aversive learning experience, was enough to promote aversive learning in the otherwise naive animal. Another related study by Fanselow (1985), showed that odours released by stressed rats can produce analgesia in unstressed naive conspecifics. These two socially evoked responses may belong to group of evolutionary conserved behaviours that promote defensive responses to novel, potentially harmful, situations in their environment. Collectively, these observations of social influence on food preference and fear in rats, point to the fact that the neuroanatomical circuits, mediating the complexities of social interactions seen in rats, should be much more intricate in comparison to mice, suggesting that rats can be a more suitable model for disorders affecting social interactions like FXS and ASD. Furthermore, such studies provide a useful model system with which we can explore the many ways in which social interactions are affected in these disorders.

One other way of communication in rodents, which is extensively studied, involves the use of ultrasonic vocalisations (USVs). Rodents have developed an elaborate communication system which is associated with emotionally negative and positive states. Aversive low pitch vocalisations, termed 22kHz type USVs, are emitted in dangerous and life threatening situations, or situations causing discomfort, frustration, and significant stress and anxiety. On the other hand, high pitch vocalisations (50kHz

type USVs), include brief calls emitted in a variety of social non-aversive, appetitive, and pleasing situations (Burgdorf et al., 2013). As mentioned before, rats display a richer social behavioural repertoire compared to mice, exhibiting more "human-like" behaviours; thus it is not surprising that rats and mice seem to have differences in vocalisations as well. It has been shown that juvenile rats display 'rough-and-tumble' play (largely composed of non-serious chasing, fighting and rolling around together); mice on the other hand exhibit a very low percentage of time spent in contact social play (Siviy & Panksepp, 2011). Furthermore, it has been found that juvenile rats emitted a high number of high frequency USVs also in anticipation of play when they were placed alone in a chamber where they had played with a partner during the previous days (Knutson, Burgdorf, & Panksepp, 1998). Play is thought to have an important role in brain development and well-being of humans as well as other animals. Taking this into account, several studies show that rats selectively bred for low rates of play-related 50-kHz pro-social USVs can be used to model social deficit symptoms of autism (Burgdorf et al., 2013). Another indication of the rich communication repertoire of rats was shown by Blanchard and colleagues (Blanchard et al., 1990). They showed that rats' production of USVs, in response to a predator depends on the presence of conspecifics, meaning that they produce these alarm cries deliberately, reflecting in that way social facilitation (Crawford, 1939). Blanchard and colleagues hypothesized therefore, that rat USVs emitted in aversive situations serve as alarm calls to warn conspecifics (Blanchard et al., 1991). In support of this hypothesis, Kim and colleagues (2010) showed that the main vehicle for social transmission of fear in rats is potentially the emission of USVs. These observations have yet to be replicated in mice using the same apparatus (Visible Burrow System) and the same paradigm. The latest example suggests that mice lack the intense social bonding observed in rats. Conclusively, mice and rats have the capability to emit USVs mainly in a social context. The more colonial life style of the rat compared to the mouse is accompanied by the occurrence of ultrasonic vocalisations in a much wider variety of contexts. This species difference in the capability for, and evolutionary value of, communication of affective states could be linked to the more complex social structure of rats in comparison with mice.

Apart from the USVs, differences in the patterns of defensive behaviour in rats and mice were observed in the previously described studies (Blanchard et al., 1991; Blanchard et al., 1990). Upon the initial presentation of a predator, mice normally retreat from the open surface and return later engaging in risk assessment behaviour. This behaviour

consists of returning to the main hall area of the apparatus and peeking through an opening. If an obstacle obscures the view of the cat, the mice would change their vantage point. After a period of about 5 to 10 min of risk assessment, the mice retreat to the depths of the burrows and remain there for some hours. In contrast, the rats immediately retreat to the depths of the burrows and engage in prolonged freezing behaviour. The main reason why the rats do not engage in initial risk assessment appears to be related to their use of vocal communication. The first rat to observe the cat emits an alarm signal to alert other members of the colony about the potential danger; therefore, not all members of the colony have to see the predator in order to avoid it. One could hypothesise that the greater complexity of social organization of rats seems to have resulted in their use of different defensive strategies to that used by mice. This observed use of vocal communication in rats may suggest the use of a more complex behaviour than simple predatory inspection, as vocal communication is widely thought to involve relatively complex signalling (Marler, Evans, & Hauser, 1992).

Another difference between rats and mice which is possibly more relevant to ASD than FXS relates to prosocial behaviours like empathy. Behavioural tasks which assess empathy related behaviours have been developed for both rats and mice (Atsak et al., 2011; Bartal et al., 2011; Langford et al., 2006). Langford and colleagues (2006), working with mice, showed that the pain sensitivity of mice which observe cage mates in pain, is increased. In an attempt to determine the transmitting sensory modality of this behavioural response, they blocked sensory inputs individually, by placing physical barriers to sight and/or touch or by rendering mice anosmic or deaf. They found that visual blockade completely abolished the observed hyperalgesia, although based on the techniques used, pheromonal communication could not be ruled out. On a similar experiment in rats (Atsak et al., 2011) researchers showed that if a rat witnesses a conspecific receiving a mild electric foot shock, two things can happen; the witnesses reacts with a typical distress behaviour to the distress of the demonstrator rat, displaying empathetic freezing behaviour and the demonstrator's behaviour was in turn modulated by the behaviour of the witness, as in, demonstrators froze more following foot shocks if their witness froze more. The latter shows a reciprocal social communication. In a pair of more sophisticated paradigms, only possible in rats so far, Bartal et al. (2011) showed that under certain circumstances the rat can exhibit empathic behaviours. In their experiment, a rat was restrained in a small cylindrical cage in the middle of larger rectangular arena. A second, cage mate, rat was free to move into the arena, and

gradually learned how to open the door of the cage and free its cage mate. Opening this door takes some effort, and it took the rats a while to find out how to open it. In contrast to the misconception that rats would be selfish animals, the free rats were seen putting considerable effort into finding ways to open the door and free their captive conspecific. In an additional experiment, they gave the free rat the choice between getting a chocolate reward placed inside an identical cage, and freeing the constrained rat. Surprisingly the majority of the test subjects decided to free the caged rat first and then share the food reward. All female rats in the study displayed this behaviour, while 30% of the males did not, showing that females seem to exhibit more empathy related behaviours. One could suggest, that this behaviour was seen not because rats really display empathic behaviours but because they crave companionship, but recently Sato and colleagues (2015) reported a similar behaviour, putting those doubts to rest. In order to test rats' altruistic behaviour, they divided a Plexiglas box into two compartments using a transparent partition. On one side of the box, a rat was forced to swim in water (the water level was rising slowly). The only way the rat could escape was if a second rat, sitting safe in the dry compartment of the testing box, open a small escape hatch connecting the two sides. Interestingly, the rats in the dry side did not open the hatch when their conspecific was still dry, confirming that they were helping in response to others' distress, rather than because they just wanted company. Rats that had previously been in the wet compartment, learned how to save their cage mates much faster than those who had never been soaked previously, suggesting that it is empathy that drove their response (Sato et al., 2015).

### 3.3.3 Differences in cognition

There are many studies to date showing that rats are indeed much better at acquiring new information than mice. This difference could also be due to the higher impulsivity of mice. Operant tasks that require suppression of spontaneous behaviours are more difficult for mice to perform well. A wide range of popular behavioural tasks either are best performed in rat models or have been validated and optimized especially well in rats, including tasks related to reward (De Vries et al., 1998), sensory systems (Znamenskiy & Zador, 2013), working memory (Deacon & Rawlins, 2006), declarative memory (Dusek & Eichenbaum, 1997) and decision making (Steiner & Redish, 2014).

One of the most widely used behavioural tasks to assess learning memory in rodents is the Morris watermaze. It was initially designed for rats as natural swimmers, but it has been adjusted for mice ever since. The water maze has several advantages over conventional dry mazes. For example, there are no local cues such as scent traces from other rats and there is no fixed escape strategy. Experimental animals make good progress in the trials because they want to escape. Several studies have shown that even rat pups outperform the mice, especially in retention memory (Frick et al., 2000; Podhorna & Didriksen, 2005; Stranahan, 2011; Whishaw & Tomie, 1997; Whishaw, 1995). Both rats and mice are able to locate the hidden and visible platform, even when tested in an intense one-day training protocol. However, the two rodent species appear to use different strategies for locating the hidden platform; rats demonstrate a robust spatial strategy, whereas mice appear to utilize alternative non-spatial strategies which are neither consistent nor reliable. Moreover, early studies suggested that laboratory mice seemed to have a tendency to float, behaviour perhaps related to their perceived weakness in the water maze. Therefore, it was suggested that mice did not actually aim to find the platform, but simply waited until the experimenter rescued them, though water mazes have now been utilized extensively in thousands of published experiments with transgenic and knock-out mice. The key when using mice is to use procedures that minimize stress and improve performance in this task, like gentle handling for a few minutes every day before the start of training. The fact that rats seem to have greater visual acuity than mice and are natural swimmers gives them an obvious advantage in water maze tasks, of course, so we cannot claim that rats exhibit higher cognitive flexibility than mice based only on water maze results (Ellenbroek & Youn, 2016).

When mice and rats are compared in a spatial navigation task in dry mazes their performance is comparable (Cressant et al., 2007; Whishaw & Tomie, 1997). These results confirm that mice and rats can learn dry-land spatial tasks equally well but are likely to rely on different strategies even when confronted with the same paradigm. Cressant and colleagues (2007) confirmed what was previously observed (Frick et al., 2000); that the strategies of mice are much less robust and flexible than the ones used by rats, not allowing a rapid adaptation to a dynamic environment when a switch onto another sensory modality is ineffective. On this last note, several behavioural tasks have been used to assess cognitive flexibility, mainly in rats. These behaviours, such as attention shifting (Birrell & Brown, 2000), delayed alternation (Horst & Laubach, 2012) and delayed (non)matching-to sample tasks (Porter, Burk, & Mair, 2000) rely heavily on

prefrontal brain areas in mammals (Birrell & Brown, 2000). Interestingly, FXS patients exhibit difficulties in prefrontal cortex dependent tasks such as the Wisconsin Card Sorting (Van der Molen et al., 2012a). Rat and mouse equivalent of the tasks have already been developed (Tait, Chase, & Brown, 2013); once again the rat equivalent is more complex and rats perform much better in all aspects of testing. These differences in performance are important in order to be able to detect subtle differences after a potential treatment, so it is obvious that rat models can have a real advantage over mouse models when testing these complex behaviours.

Another type of a widely used set of behavioural paradigms is the spontaneous object exploration task. Again rats appear to perform better than mice either in short or long retention intervals (Bevins & Besheer, 2006) in novel object recognition, which is the simplest version of these tasks. In contrast a study by Stranahan (Stranahan, 2011) showed no difference between species but rats show increased exploratory behaviour; even so high exploration times are a very important element of behaviour in these types of tasks in order to get interpretable results. To date there is not a direct comparison between the species in other similar tasks like object in place or object in context but a quick look at the literature is enough to prove that rats outperform mice in both retention of memory, and performance index (Langston & Wood, 2010; Spanswick & Dyck, 2012).

Looking at physiology studies related to brain areas important for cognition can also give us an idea of the differences between mice and rats. An important comparative study showed that the adult neurogenesis in dentate gyrus (a process believed to be important for learning and memory amongst other behaviours) in rats, processes much faster and newly differentiated neurons have much higher chances of being incorporated into a functional circuit (Snyder et al., 2009). This suggests that new neurons may make a greater contribution to behaviour in rats than in mice. One other major difference between the rat and mouse dentate gyrus is that the peak of granule cell development is postnatal in the rat but prenatal in the mouse (Angevine, 1965; Schlessinger, Cowan, & Gottlieb, 1975), despite the almost identical gestation time in the two species. The delayed development of the dentate gyrus in the rat may result in a structure that is more versatile to changes in response to environmental stimuli and more plastic during adulthood. Behaviourally, this could result in rats showing a much more complex behavioural repertoire than mice and being able to acquire behavioural strategies that

demand significant plasticity (Whishaw et al., 2001) and/or that strongly rely on refined hippocampal function (Gerlai & Clayton, 1999). It is not yet known which of these two species shows rates of neurogenesis comparable to humans. However, the extended development and more intricate and flexible behavioural repertoire of the rat compared with the mouse (Whishaw et al., 2001) suggest that the rat's hippocampus may be the better model for that of the human.

Concluding, the mouse can perform similarly to the rat, in many behavioural tasks, but is almost always less sophisticated and with less capacity for modifying its initial behavioural response. Given that there is roughly a fourfold difference in the weight of rat and mouse brains and that the mouse cortex is only about 60% as thick as the rat's brain, it is reasonable to assume that rats have more synapses per equivalent volume of cortical tissue, resulting to a more complex behavioural repertoire (Whishaw et al., 2001). Therefore, the mouse seems to be relatively inefficient for neurobehavioral research as it is a species functioning at a lower level of complexity, relative to the rat, focussing primarily on just those behaviours directly needed for successful survival and reproduction. When used in learning and memory tasks, mice often seem to lack a solid plan and a robust strategy (Cressant et al., 2007; Frick et al., 2000); manipulations of neural processes are thus relatively limited in their ability to alter behavioural output. This may not be a drawback for the genetic analysis of behaviour and can aid in gaining insights into the genetic basis of more basic behaviours. However, this may be a stumbling block for those neurobiologists who aim to assess behavioural plasticity and social behaviour as a primary aim of investigation. In particular, research related to behavioural deficits of neurodevelopmental disorders, like autism spectrum disorders, requires animal models which can exhibit complex behaviours. Lastly, using mouse models may also may be a drawback in comparing the results produced in different laboratories, in which the focus of the analysis is on behaviours that might be a less pronounced part of an animal's natural behaviour. Crabbe, Wahlsten and Dudek (1999) have remarked on the widely divergent results that can be obtained from behaviour of mice tested in different laboratories even when the same tests using the same apparatus are applied. This could be one of the ways to explain the inconsistency of the behavioural phenotype of *Fmr1* KO mice.

**Figure 3.2 Rodent models are accelerating therapeutic development for neurodevelopmental disorders**. Translational neuroscience has made great effort in developing treatments based on understanding the causal genetic mechanisms underlying neurodevelopmental diseases. Today we stand at a therapeutic front line, with much more to learn and the recent generation of a rat models creates exciting new directions. (photo from Jeste and Geschwind 2016)

## 3.4 The age of KO rats

A number of technological problems have made it very difficult to specifically target genes in rats. On the other hand, scientists have been effectively manipulating genes in mice ever since researchers first discovered mouse embryonic stem (ES) cells in the 1980s (Evans & Kaufman, 1981). Recent advances in genome sequencing in rats (Jacob & Kwitek, 2002) and the development of methods to produce pluripotent stem cells from rats (Buehr et al., 2008; P. Li et al., 2008) paved the way for the development of novel genetic manipulation techniques possible in rats and this has led to the creation of KO rats. Before the emergence of ZFN technology (Geurts et al., 2009), there had not been a reliable technique for creating KO rat models. Chemical mutagenesis using ethylnitrosourea (ENU) and random insertion mutagenesis using gene-trap Sleeping Beauty transposons have been used to generate loss-of-function, or 'KO', mutations in important disease genes including rat models of cancer, eye development and immunodeficiency (Amos-Landgraf et al., 2007; Homberg et al., 2007; Zan et al., 2003). Recently, the CRISPR/Cas9 system has emerged as a highly efficient and advantageous alternative to the previously mentioned genetic manipulation approaches. The CRISPR/Cas9 system has been used to generate genetically modified rat strains which carry single or multiple mutations in genes relevant to selected disorders (Bao et al., 2015; Shao et al., 2014). While existing mouse models were created by cloning or using embryonic stem cells, CRISPR/Cas9 technology bypasses these techniques by targeting genes in vivo, creating knock out or genetically modified animals in a shorter amount of time and enabling KO in a wider range of mammalian species; for example, by direct injection of RNAs into one-cell embryos. Now that KO technology is available for rats and mice, scientists will be able to make choices based only on the question they want to answer. Thus, it is widely believed that the rats' natural advantages as experimental animals, combined with the wealth of new genetic information and gene manipulation techniques should lead to a surge of interest among biomedical researchers (Dolgin, 2010) (Iannaccone & Jacob, 2009; Wöhr & Scattoni, 2013; Zalocusky & Deisseroth, 2013).

It is also the case that more complex brains are not always preferable. As Vermaercke and colleagues showed, when rats and humans were trained in rule-based and information-integration category-learning tasks with visual stimuli, their performance was equal in the rule-based categorization, but rats outperformed humans on generalization in the information-integration task (Vermaercke et al., 2014).

Furthermore, and contrary to what I have discussed already, a recent test showed that even though rats learned faster than mice in a challenging rule-based auditory task, that tested perceptual ability as well as cognitive flexibility, their final performance was very similar. (Jaramillo & Zador, 2014). According to the authors, the performance differences researchers detect between mice and rats, could be due to the training protocols used in a wide range of behavioural tasks. The rat has been the workhorse of experimental psychology for many years, so most of the tasks were developed and optimized specifically for them. Since all of the genetic tools available in mice are still ages ahead of rats, careful adjustment of behavioural protocols could be an alternative way to study the neural mechanisms of normal and diseased brain states.

In closing, it is important to keep in mind that these recent innovations in genetic manipulation tools for rats do not mean the end of the lab mouse (Fig. 3.2). The hope is that by acknowledging and understanding the differences between species, the resources used in the past decades in studies, using mice as model organisms, will not be wasted and that both rodent species, rats and mice, will continue to be used in parallel, with a rational conceptual frame of reference based on objective features of comparative pathophysiology (rather than physiology). Only then could mouse and rat models take their real place in neuropsychiatric research along with other experimental approaches and in vitro (i.e., primary cultures, iPSC from affected individuals) studies before commencement of any human clinical trials.

# 4. Behavioural characterisation of a new rat model of Fragile X syndrome

## 4.1 Introduction

Over the last two decades, much has been learned about the pathophysiology associated to the loss of FMRP from mice and other model organisms of FXS (Chapter 1). Recent advances in techniques for manipulating genomes have allowed the generation of transgenic mammals other than mice. The recent generation of a rat model of FXS opens the door, not only to validate phenotypes across mammalian species, but also to address behavioural deficits (especially cognitive) using paradigms that are more challenging to address in mice. This cross-mammalian comparison, especially related to behavioural manifestations can be quite challenging if we take into account ethological differences between species (Chapter 3). The approach most likely to be used, to test the validity of new rat models is going to be the same as for any other previous model organism. Many candidate gene mutations, thought to be important for disorders, will be introduced in homologous rat genes and each mutant rat line will be evaluated for phenotypes analogous to the symptomatology of the human condition. Successful rat models should incorporate face validity (strong analogies to the pathophysiology of the human condition), construct validity (common biological underpinnings to the human disease, such as a genetic lesion or anatomical abnormality) and predictive validity (analogous system reaction to treatments which prevent or reverse symptoms in human patients) which is vital when testing the efficacy of new therapeutics (Silverman et al., 2010).

Choosing appropriate behavioural tests that are relevant to human neuropsychiatric disorders is not a trivial task. Certain symptoms may manifest only in humans or are inherently variable in severity. The same problem exists in the case of new rat models. In the case of *Fmr1* knockout rats, it is still not clear if common cellular and circuit pathophysiology with the mouse model leads to the same behavioural abnormalities. Therefore, this new model of FXS will enable us to directly examine whether common cellular dysfunction or behavioural outcomes of a genetic mutation are conserved across species.

## 4.2 The *Fmr1* KO rat

The first rat model for FXS has been made available almost 5 years ago. Therefore, literature is still very limited; there are only seven published studies (including a study describing part of the work described in this thesis) looking into cellular, circuit and behavioural abnormalities in *Fmr1* knockout rats.

The first report by Hamilton and colleagues (2014), focussed on the behavioural characterisation of *Fmr1* KO rats. No significant differences were found between groups *Fmr1* KO and WT rats, in a wide range of behavioural tasks but some behavioural abnormalities with relevance to autism were reported. Juvenile *Fmr1* knockout rats displayed reduced play behaviours during interaction with same genotype animals. This is interesting because contrary to mice, juvenile rats display a large repertoire of social and play behaviours (Thor & Holloway, 1984). No differences were observed in the three-chamber test, as all juvenile rats showed a strong preference for the "social" compartment, containing an unfamiliar stimulus rat. Moreover, a phenotype emulating repetitive behaviours was observed. Mutant rats chewed a wood block more. No cognitive or sensory processing abnormalities were reported except from small trends for enhanced PPI, indicating minor dysfunctions in sensory gating. The results in this first report are limited to only some subtle differences related to social and repetitive behaviour. *Fmr1* knockout rats do not seem to express a variety of behavioural deficits seen in the mouse model, but the rich behavioural of rats, like juvenile play (Wöhr & Scattoni, 2013), could reveal behavioural differences which indicate that this rat model will complement existing mouse models.

Two more studies examined deficits related to sensory processing, and specifically auditory stimuli (Engineer et al., 2014; Ruby, Falvey, & Kulesza, 2015). Engineer and colleagues reported that evoked potentials and spiking activity were significantly degraded in primary auditory cortex, anterior auditory field and the ventral auditory field in response to auditory stimuli. Further analysis revealed that activity in these brain areas contains significantly less information about sound identity in *Fmr1* knockout rats compared to wildtype littermates. Specifically, ventral auditory field which has been related to emotional regulation (Kimura, Imbe, & Donishi, 2010), showed the biggest differences. The second study examined morphology and neurochemistry in the auditory stem (Ruby et al., 2015). They reported that in absence of FMRP, specific neuronal types

have abnormal somatic and spine morphology, pointing to the fact that auditory dysfunction in FXS derive, at least in part, from malfunctioning brainstem circuits.

Another recently published study focussed on the postnatally developing visual cortex (Berzhanskaya et al., 2016a). They showed that the visual cortex of *Fmr1* null rats exhibits long periods of hyperactivity compared to wildtype littermates. Moreover, this hyperactivity was connected with reduced synchronisation of circuits in visual cortex, suggesting disrupted inhibitory function. As this thesis was being composed, another report from the same research group (Berzhanskaya et al., 2016b), showed that instead of the hyper-excitability previously observed, visual responses before eye-opening have reduced spike rates and an absence of early gamma oscillations, which is a marker for normal thalamic function at this age (Hartung et al., 2016). Surprisingly, despite this finding early in life, the developmental trajectory of visual responses in *Fmr1* null rats was found to be identical to wildtype littermates. Taken together these two studies suggest that early circuit deficits in this rat model of FXS have consequences on circuit function and are opposite those found in adults.

Lastly, another recently published study reported that FMRP loss leads to a dysregulation in reward processing in rats (Kenkel et al., 2016). Further behavioural analysis revealed that transgenic rats failed to discriminate a rewarding odour (almond) which is shown to elicit innate reward response in wildtype rats and a subsequent increased preference. These results provide support to evidence pointing to the reward system as a contributor to social deficits seen in individuals with FXS. The importance of this study apart from the results showing deficits in reward processing, is that it highlighted one of the advantages of rats as a translational model. Combining fMRI in awake rats with relevant behavioural assays in genetic rat models, represents an effective experimental approach that allows the identification of the effect of single gene mutations on neural circuits regulating emotion and cognition.

Taken together these studies suggest indicate that transgenic rats will complement existing mouse models, providing valuable insights into the pathophysiology associated with FMRP loss. Despite the diverse finding, it is obvious that cognitive deficits have been quite challenging to be identified so far in this new rat FXS model. This study is an attempt to characterise this rat model is the most thorough way possible within the limits of a PhD thesis. Behaviour was assessed on all three mainly affected trait groups in FXS: (1) anxiety/hyperactivity (2) social interactions/communication and (3)

cognition. For each behavioural trait, more than one tests were used in an attempt to allow a more detailed characterisation of the model. While the majority of work presented in this chapter is focussed on the commercially available model of FXS on an albino Sprague-Dawley background strain, in the last section. I present work done on a custom made rat model with the same genetic lesion on *Fmr1* gene, but on a Long-Evans hooded background strain. Since the mouse model has shown background strain influences behavioural phenotype heavily (Corinne M. Spencer et al., 2011), we wanted to examine whether the cognitive deficits Sprague-Dawley rats display, persist across different strains.

## 4.3 Methods

### 4.3.1 Animals

Sprague-Dawley *Fmr1* KO rat founders obtained from Sigma Advanced Genetic Engineering (SAGE) Labs (St. Louis, MO, USA), now part of Horizon Discovery, bred in-house and kept in a 12h/12h light dark cycle. Female *Fmr1* heterozygotes were crossed to WT SD males (Charles River labs) to produce *Fmr1* KO and WT littermate controls. Offspring were genotyped using primers for *Fmr1* lines, Fwd: 5'-TGGCATAGACCTTCAGTAGCC-3', Rev: 5'-TATTTGCTTCTCTGAGGGGG-3'. WT rats produced a 400bp PCR product while *Fmr1* KO a 278bp (Figure 4.1A). DNA samples were obtained after alkaline lysis of tissue samples (~2mm ear clips) using 600μL NaOH 50mM per biopsy and incubating in 96 degrees for 40 minutes. 60μL of Tris 1M pH 8, were used to neutralise the pH of the solution. 1μL of this sample was used for each PCR reaction.

Long Evans Hooded *Fmr1* KO rats, were obtained from Sigma Advanced Genetic Engineering (SAGE) Labs (St. Louis, MO, USA), now part of Horizon Discovery, bred in-house and kept in a 12h/12h light dark cycle. Female *Fmr1* heterozygotes were crossed to WT LEH males to produce *Fmr1* KO and WT littermate controls. Offspring were genotyped using primers targeting the eGFP cassette in exon 1 of *Fmr1* gene Fwd: 5'-ACGTAAACGGCCACAAGTTC-3', Rev: 5'- ATGCCGTTCTTCTGCTTGTC-3'. WT rats produced no PCR product while *Fmr1* KO a 421bp. Primers targeting the genomic sequence either way of the eGFP cassette insertion site were used as positive control in a separate reaction; WT rats produced a 400bp PCR product while *Fmr1* KO nothing; the two reactions were run separately and the products from the same DNA sample were loaded on the same column (different well) on agarose gel (Figure 4.1B). All

experimental subjects were male and group housed (2–5 animals/cage) to avoid effects of isolation. Ad libitum standard laboratory chow was provided throughout experimental procedures. All experiments were done blind to genotype. Testing was always performed in during the light phase of the cycle. Prior to the start of the study, all experimental procedures were approved by the University of Edinburgh centenary services and abided by the Animal Care (Scientific Procedures) Act 1986.

### 4.3.2 Experimental Design

Six different cohort of animals were used for the experiments described in this chapter. Cohort 1 (9 WT and 9 *Fmr1* KO SD) was tested in USV analysis and a Reference and reversal task in watermaze. Cohort 2 (8 WT and 8 *Fmr1* KO SD) was tested in marble burying test. Cohort 3 (12 WT and 12 *Fmr1* KO SD) was tested in a Delayed Matching to Place task in watermaze. Cohort 4 (10 WT and 10 *Fmr1* KO SD) were tested in open field test and light/dark box. Cohort 5 (16 WT and 16 *Fmr1* KO SD) was tested in spontaneous exploration tasks and three chamber social interaction test (for the social interaction test only 14 from 16 animals of each group were used). Finally, cohort 6 (16 WT and 16 *Fmr1* KO LEH) was tested in spontaneous exploration tasks. Statistical analysis was done using IBM SPSS Statistics 22.0 and GraphPad Prism 6. All graphs were produced in GraphPad Prism 6.

### 4.3.3 Behavioural Assays

#### Open field test

Testing was carried out in wooden square arena (1x1 m) painted grey, under dimmed light. The walls were not vertical, but on a 45-degree angle in order to prevent shadow creation. No handling was carried out before testing, as it could have masked any anxiety phenotype. Rats' behaviour was recorded using an overhead camera, for 30min. The open field was cleaned with 70% ethanol between rats. Anymaze software was used to analyse subjects' behaviour.

#### Light/dark box

A wooden box with an enclosed (dark) and an open (light) compartment was used (light 40x50x30 cm; dark 40x30x30 cm). The two compartments were connected through an opening (8x8 cm). Rats were placed in the light compartment and let to explore the box

for 5min. Rats' behaviour was recorded using an overhead camera. A transition between compartments was recorded only when all 4 paws crossed the opening. The apparatus was cleaned with 70% ethanol between rats. Anymaze software was used to analyse subjects' behaviour.

**Marble burying test**

Testing was carried out as previously described (Deacon, 2006a). Transparent plastic cage (Tecniplast) (48x26.5x21 cm) was filled approximately 5 cm deep with wood chip bedding, lightly tamped down to make a flat, even surface. 15 black glass marbles were place in a regular pattern evenly spaced, each about 4 cm apart. Animals were placed in the cage for 2 h and the number of marbles buried (to 2/3 their depth) with bedding were recorder after 30 min and at the end of the experiment. Due to technical limitations rat behaviour was not monitored.

**USV analysis**

Dirty bedding was collected from cages containing males and transferred daily for seven days prior to the experiment, to cages containing stimulus females. This was done to ensure that the 14 females used would be receptive to male courtship (Moncho-Bogani et al., 2002). Oestrus in females was determined based on lordosis behaviour (Dulac & Torello, 2003; Kow & Pfaff, 1998). Every female was used maximum twice as stimulus to avoid decline in interest in males after repeated exposure. Apparatus was a transparent plastic cage (Tecniplast) (480x265x210mm). A microphone able to record in the ultrasonic frequency range (Avisoft-Bioacoustics) was placed approx. 10 cm above experimental cage on holding hand and connected to a computer running the appropriate acquisition software (Avisoft-RECORDER). Camera was placed next to experimental cage in order to record courtship behaviour. Male subjects were independently housed for 1 hour before the experiments, in cages outside the experimental room. Females were in a separate room from males. Each male was placed in the experimental cage approx. 15sec before female. USVs were recorded during a 3min interaction session between a male and female and for additional 3min after the stimulus female was removed from the cage. Males were weighed before the experiment as vocalisations' frequencies are heavily influenced by weight. Due to the special nature of the dataset, data analysis was primarily carried out by Dr Caterina Michetti Prof Maria Louisa Scattoni using Avisoft-SASLab Pro (Avisoft-Bioacoustics). Probability of

vocalizations within each strain was calculated as number of calls in each category for each subject/total number of calls analysed in each subject and standardized by angular transformation (M. L. Scattoni, Ricceri, & Crawley, 2011).

**Social interaction task**

The backbone of this protocol is based on previously described experiments (McKibben, Reynolds, & Jenkins, 2014). The apparatus was rectangular box divided into three equally sized chambers (each chamber 21×63×45 cm; total size 63 ×63×45 cm). An overhead camera was used to record animal behaviour. The central chamber was connected to the left and right cambers with doors. Right and left chambers contained a wire mesh cylindrical enclosure (18cm diameter). Juvenile stimulus SD rats (n=8) (Charles River Laboratories, UK), complete strangers to the experimental subjects, were be habituated for 20 min to placement in a wire cage 24 h before testing. These rats were counterbalanced across genotypes and their location in the left vs right side chamber were counterbalanced between trials and tested rats. Each of them was not used for more than 2 rats in a row, as their loss of interest in the experiment animal would introduce bias. The experiment is divided in to three stages: Stage 1-Habituation; The test rat was placed in the middle chamber and allowed to explore only this chamber for 10 min. Barriers were place in the two doorways leading to the right and left chamber. Stage 2-Social interaction: following habituation, a stimulus rat (stranger 1), that had no prior contact with the experimental rat, was placed in one of the side chambers enclosed in the wire mesh cylindrical enclosure that will allow nose contact. An empty, but otherwise identical wire cage was placed in the opposite chamber. The experimental rat was placed in the middle chamber and was allowed to explore the entire arena for a period of 5 min. This step was repeated two more times to test social habituation. Stage 3-Social novelty preference; Following stage 2, the experimental rat will return to the centre chamber. With stranger1 (now familiar) retained in the arena, a second, unfamiliar rat (stranger2) was placed in the empty wire cage in the opposite chamber. The subjects were allowed to explore the entire arena again for a period of 5 min. Between the stages, rats were transferred in to a holding bucket for 3 min and the maze was cleaned with 70% ethanol. Time spent sniffing the wire cages was recorded online using in-house developed scoring software (multitimer). For each session a Discrimination Index DI [(time exploring social chamber—time exploring empty or familiar rat chamber)/(time exploring both chambers)] was calculated.

**Spatial reference memory and reversal in watermaze**

Subjects were trained in three stages in a 2 m diameter water maze containing a 12 cm escape platform (Fig 4.2). First, rats were trained for 3 days on the visible platform version of the water maze (4 trials/day, 15 min ITI, extra-maze cues obscured, platform location moved each trial). In the second stage, extra-maze cues were visible and rats received one daily hidden-platform training session for seven consecutive days; each session began with a reinforced probe trial, followed by three training trials separated by a 15 min ITI. For probe trials, an Atlantis platform (Spooner et al., 1984) was raised to 1.5 cm below the water surface 1 min into the trial; for standard trials the platform was raised throughout. Each trial lasted a maximum of 2 min; rats failing to escape were guided to the platform. All rats remained on the platform for 30 s before removal from the pool. The third (reversal) stage was identical to the second, but the platform was relocated to the opposite side of the pool. Path length performance is plotted in meters (m) was compared to account for differences in swim speed. For probe trials, target crossings during the first 60 s were quantified.

**Delayed matching to place in watermaze**

Subjects were trained on a modified version of a DMP task in the water maze (Steele & Morris, 1999). The protocol for both pre-training and delay phases were the same; the platform was hidden in a novel location on trial 1 of each day and then remained in this place for trials 2–4, on which rats could use rapidly encoded place memory to reach the escape platform efficiently. The different platform locations were located on an inner ring (0.8-m diameter) or outer ring (1.4 m) concentric with the pool. Each trial lasted a maximum of 2 min; rats failing to escape were guided to the platform. All rats remained on the platform for 30 s before removal from the pool. All four start positions were used daily in an arbitrary sequence, to discourage egocentric strategies. During the first phase, rats received two 4-day blocks of pre-training (4 trials/day, 15 s ITI, extra-maze cues visible, platform location moved each day). In the second phase, rats received 15 days of delay training during which three different ITIs (15 s, 15 min or 2 h) were introduced between trials 1 and 2 (5 days of each ITI); for one of the 5 days at each delay, trial 2 of the day was run as a probe trial with an Atlantis platform (Spooner et al., 1984) raised to 1.5 cm below the water surface 1 min into the trial; for standard trials the platform was raised throughout. Probe trial performance was calculated as the percent time spent in a 20 cm diameter zone around the centre of the platform location during the first 60 s.

Perseveration index indicates the difference between the percent time spent in the previous day's target zone and the current day's target zone during the first 60 s of the probe trial.

**Spontaneous exploration tasks**

Subjects underwent object recognition (OR), object place (OP), object context (OC) and object place context (OPC) tasks as previously described (Langston & Wood, 2010). Animals were tested in a rectangular box (76 × 45 × 60 cm tall) that could be configured as either of two contexts (by changing floor/wall inserts). An overhead black and white camera was used to monitor the movement of the rat around the testing arena. The video signal was fed into a TV monitor on the desk of the experimenter. A computer ran an in-house timing program (National Instruments, LabView) whereby depression of a key on the computer keyboard would activate a timer. This was performed manually by the experimenter who observed the behaviour of the rat via the TV monitor and recorded the amount of time the rat was engaged in exploration (Fig. 4.3 A&B). To confirm that no bias was introduced, since the experimenter was not blind to object novelty identity, a second independent scorer, blind to genotype, object identity and task rescored portion of trials (96 trials) and scoring was compared yielding a very high correlation (Pearson $r = 0.91$, $p < 0.001$) (Fig 4.3 C). Training included a 5-day habituation period during which rats familiarize themselves with the apparatus, the two different contextual configurations of the testing arena, the type of objects that would be placed in the arena during testing and the locations in which these objects would be placed. Each day, rats were brought into the testing room in their home cage, which was placed on a bench near to the testing apparatus. Following the habituation, rats were tested on object recognition tasks (associative and non-associative) in the morning. On each trial, rats to be tested were removed from its home cage and placed in the holding bucket on a stool next to the testing apparatus. Appropriate objects were cleaned with 70% ethanol solution and attached at the appropriate locations in the testing box configured as either context 1 or context 2 (counterbalanced across rats). Rats were placed into the box facing the wall from the side opposite to the object positions and were let to explore the objects. Exploration was defined as the rat being within 2 cm of an object, directing its nose at the object and being involved in active exploration such as sniffing or whisking. Sitting on or next to an object without any signs of active exploration was not included. After 3 min subjects were removed from the box at the same point from which they entered.

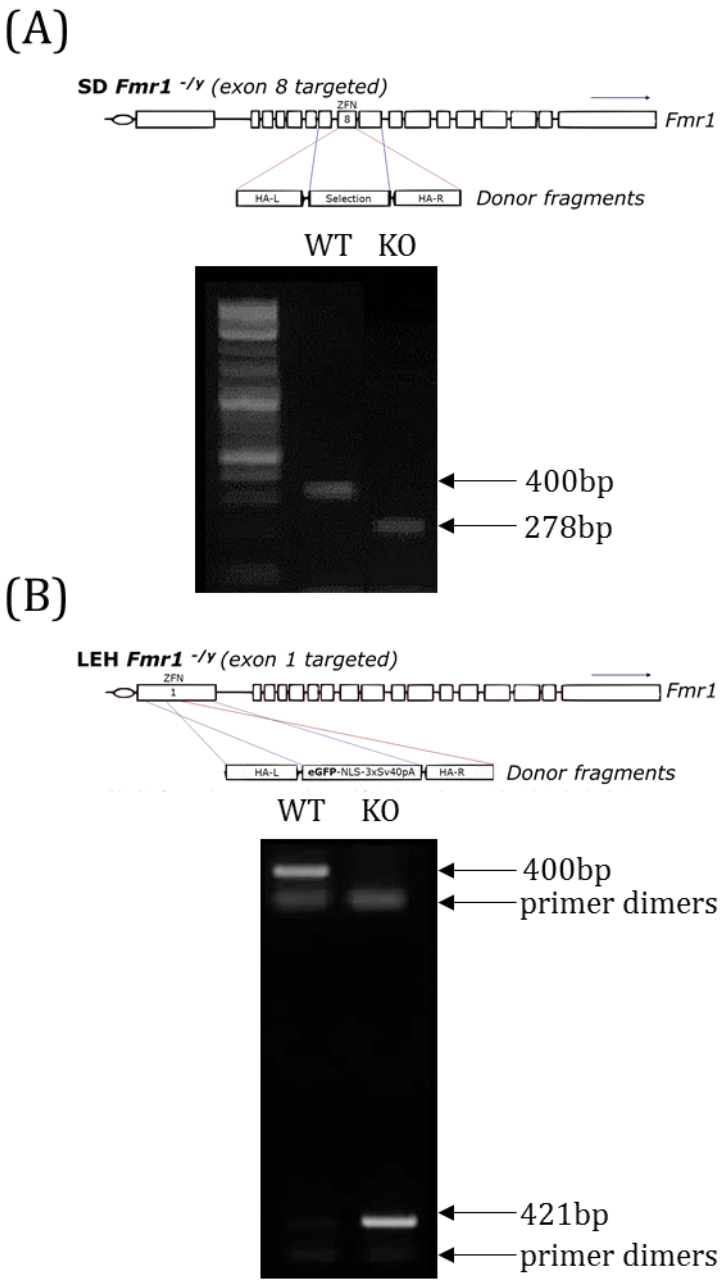



**Figure 4.1 Injection of targeted Zinc finger nuclease (ZFN) mRNAs resulted in the respective loss of FMRP in Sprague-Dawley (SD) and Long-Evans Hooded (LEH) rats.** Diagram of ZFN left and right homology arms (HA-L, HA-R) cleavage sites (selection) for the **(A)** commercially available Fmr1 KO SD rats and **(B)** custom made Fmr1 KO LEH rats. Note that the open reading frame of *Fmr1* gene has been interrupted in exon 1 by a eGFP gene cassette in the case of LEH rats and in exon 8 by a 122 bp deletion in SD rats. Using primers flanking the deletion region, the wild type and deletion alleles yield readily distinguishable PCR amplicons in SD rats**(A)**. In the case of LEH **(B)**, two reactions with two different primer sets were used for each DNA sample. One primer set flanking the insertion region gives a product of 400bp only for WT rats and another primer set targeting the eGFP gene was used to confirm the KO rats. The agarose gell used in this case had two combs in different positions; the two PCR products for a given rat were loaded in the upper and lower sections of gel (In the example upper for primers targeting *Fmr1*, lower for primers targeting eGFP) Furthermore primer dimers seem to be to be two close to the approx. 400bp products because if the short duration of electrophoresis.

(A)

(B)

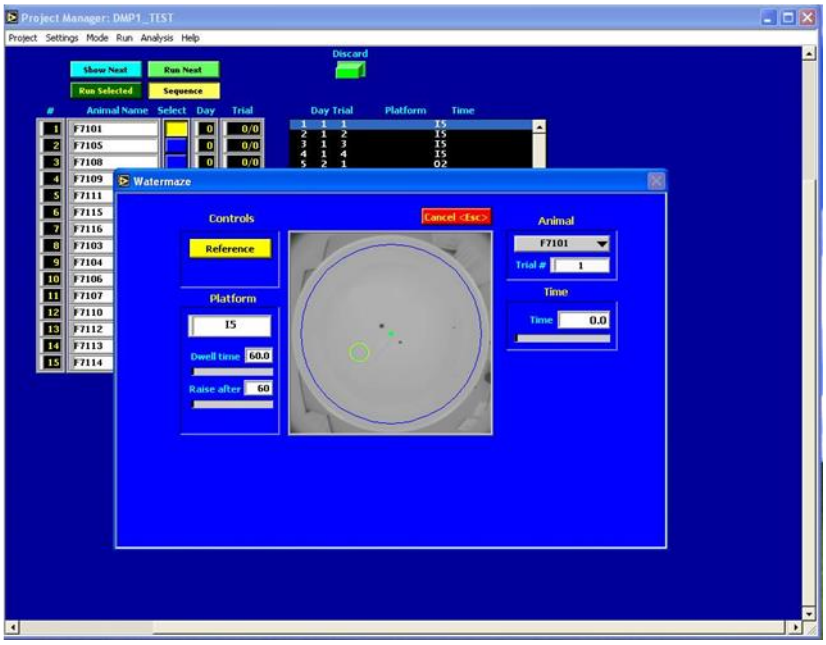



**Figure 4.2** Experimental setup **(A)** and acquisition software **(B)** used in watermaze experiments.

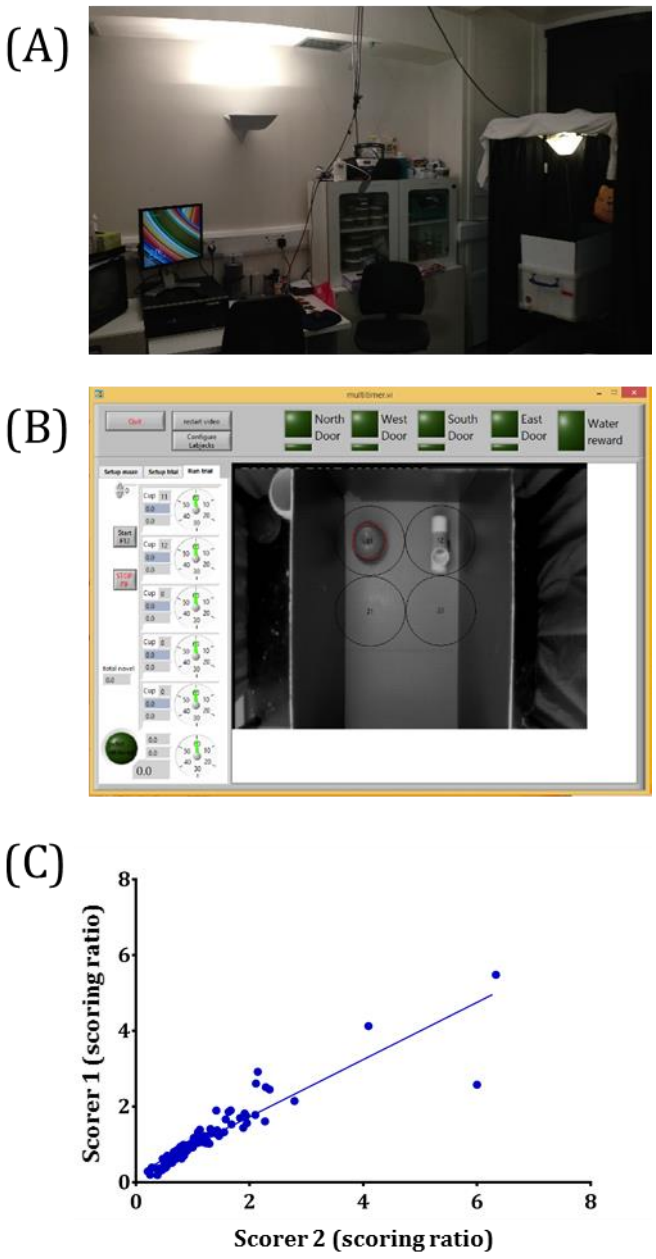



**Figure 4.3** Experimental setup **(A)** and acquisition software **(B)** used in spontaneous exploration tasks. Correlation of scoring between the main scorer (scorer 1) and an independent scorer (scorer 2). The values on both axis are ratios of scored exploration times between left and right object **(C)**.

During the retention interval of 2 min rat were returned to the holding bucket while the experimenter prepared the box for the next part of the trial. The floor and walls of the box were removed, cleaned and replaced in the appropriate context configuration. For OC and OPC recognition a second sample in the opposite context with appropriate objects followed. New objects were cleaned and placed in the box. For the test phase one object was a third copy of the two objects seen in the sample phase while the other object was completely novel. The test phase was carried out using exactly the same procedures as the sample phase. After the test phase, the rat was returned to its home cage. Rats received 2 trials (one/day) on each of the four tasks (order OR, OP, OC, OPC, OPC, OC, OP, OR), with 3 min sample phases, a 2 min retention interval and a 3 min test phase. For each test phase, a Discrimination Index DI [(time exploring novel object—time exploring familiar object)/(time exploring both objects)] was calculated. Trials in which a subject did not reach at least 10 sec of exploration for each object in the sample phase and 15 sec of total exploration in the test phase were excluded from the analysis.

For the second group of spontaneous exploration tasks (Object displacement {OD} and Object recognition, short and long term memory tasks) a different square arena was used (60x60x50 cm). Subjects were habituated for 5 days to the new apparatus. Rats received again 2 trials (one/day) on each of the four tasks [order OR (2min ITI), OD (2min ITI), OD (2min ITI), OR (2min ITI), OD (24h ITI), OR (24h ITI), OR (24h ITI), OD (24h ITI)], with 3 min sample phases, a 2 min retention interval and a 3 min test phase for the short term memory tasks, and 5 min sample phases, a 24 h retention interval and a 3 min test phase for long term memory tasks. Object displacement assesses spatial memory requiring allocentric representation of space therefore on all sessions, the rats will enter the testing box pseudo-randomly from one of the corners of the box.

## 4.4 Results

### 4.4.1 Fmr1 KO rats exhibit normal activity and anxiety levels

To determine whether Fmr1 KO rats experience the elevated activity and anxiety levels seen by many researchers in the mouse model of FXS (Santos, Kanellopoulos, & Bagni, 2014), we used three widely utilised tasks, open field test, light/dark box test and marble burying test. No differences were found between genotypes in any of these tests.

**Open field test**

We measured the total distance travelled and found no difference between groups (WT:160.8 ± 11.6cm; KO:172.6 ± 8.8cm; $t18 = 0.81$, $p = 0.43$ Fig. 4.4A). In attempt to examine whether there are different patterns in the exploratory behaviour between groups we analysed the distance travelled in 5 min epochs across the total 30 min of open field testing. We found no statistically significant main effect of genotype indicating that, overall, Fmr1 KO rats did not have abnormal activity compared with control rats (time epoch $F(5,90) = 96.86$, $p < 0.001$; genotype $F(1,18) = 0.649$, $p = 0.43$; genotype × time epoch $F(5,90) = 0.87$, $p = 0.50$; Fig. 4.4B). The significant effect of time and the absence of interaction with genotype, indicates that both Fmr1 KO and control littermates displayed a burst of activity while exploring a novel environment, which steady decreased as a response to habituation to the environment as testing progressed. Another measure assessing activity levels is mean speed (Walsh & Cummins, 1976). The mean speed was found to be almost identical between the two groups (WT:0.089 ± 0.006cm/sec; KO:0.096 ± 0.005cm/sec; $t18 = 0.81$, $p = 0.43$ Fig. 4.4E). We also found no statistically significant effect of genotype when we examined mean speed in different epochs (time epoch $F(5,90) = 96.75$, $P < 0.001$; genotype $F(1,18) = 0.658$, $p = 0.43$; genotype × time epoch $F(5,90) = 0.87$, $p = 0.50$; Fig. 4.4F). Max speed did not reveal any differences between groups either (data not shown). In order to assess possible anxiety phenotypes in open field we analysed the movement of animals into different area zones (Bailey & Crawley, 2009). An outer zone 30cm wide was set in the analysis software (Anymaze). Again no differences were seen in the total distance spend in the outer area (WT:158.8 ± 11.5cm; KO:170.1 ± 8.7cm; $t18 = 0.79$, $p = 0.44$ Fig. 4.4C) and when we looked the profile over time (time epoch $F(5,90) = 101.1$, $p < 0.001$; genotype $F(1,18) = 0.623$, $p = 0.44$; genotype × time epoch $F(5,90) = 0.87$, $p = 0.50$; Fig. 4.4D). Analysis of other areas (i.e. corners) or number of transitions between them did not yield any differences between groups either (data not shown).

**Light/dark box test**

The light/dark box test utilises the innate aversion of rodents to intensely illuminated spaces and on their spontaneous exploratory behaviour in response to mild stressors, like novel environment (Hascoët & Bourin, 2009; Hölter et al., 2015). Analysis of time spent in the two compartments revealed a main effect of compartment but no significant effect of group and no interaction indicating that both groups behaved similarly (compartment $F(1,36) = 10.96$, $p = 0.002$; genotype $F(1,18) = 0.0$, $p > 0.99$; genotype × compartment $F(1,36) = 0.033$, $p = 0.86$; Fig. 4.5A). The number of transitions between the two compartments was also identical between the groups (compartment $F(1,36) = 0.47$, $p = 0.499$; genotype $F(1,18) = 0.0$, $p > 0.99$; genotype × compartment $F(1,36) = 0.298$, $p = 0.59$; Fig. 4.5B).

**Marble burying test**

The marble burying test is thought to be assessing repetitive and perseverative behaviours but it also heavily influenced by novelty induced anxiety (Deacon, 2006; Thomas et al., 2009). In response to an aversive stimulus rodents are likely to engage into a burying behaviour, commonly referred to as "defensive burying" (Poling, Cleary, & Monaghan, 1981). Taking into account previous report showing repetitive behaviours in Fmr1 KO rats (Hamilton et al., 2014) we wanted to examine if marble burying could reveal similar phenotypes. Analysis of number of marbles left uncovered by sawdust in two time points revealed a significant main effect of time but no significant effect of genotype (time $F(1,14) = 29.34$, $p < 0.001$; genotype $F(1,14) = 2.01$, $p = 0.178$; genotype × compartment $F(1,14) = 0.059$, $p = 0.82$; Fig. 4.5C).

### 4.4.2 Fmr1 KO rats display minor communication and social interaction deficits

The second main group of behaviour traits we assessed was social communications and social interactions. The three chamber social interaction task and analysis of ultrasonic vocalisations (USVs) have been used extensively to explore social deficits in models of neurodevelopmental disorders (Wöhr & Scattoni, 2013). Both tasks revealed mild deficits in social interactions which are consistent with previous findings in the mouse model and in recently published work in the rat model of FXS.

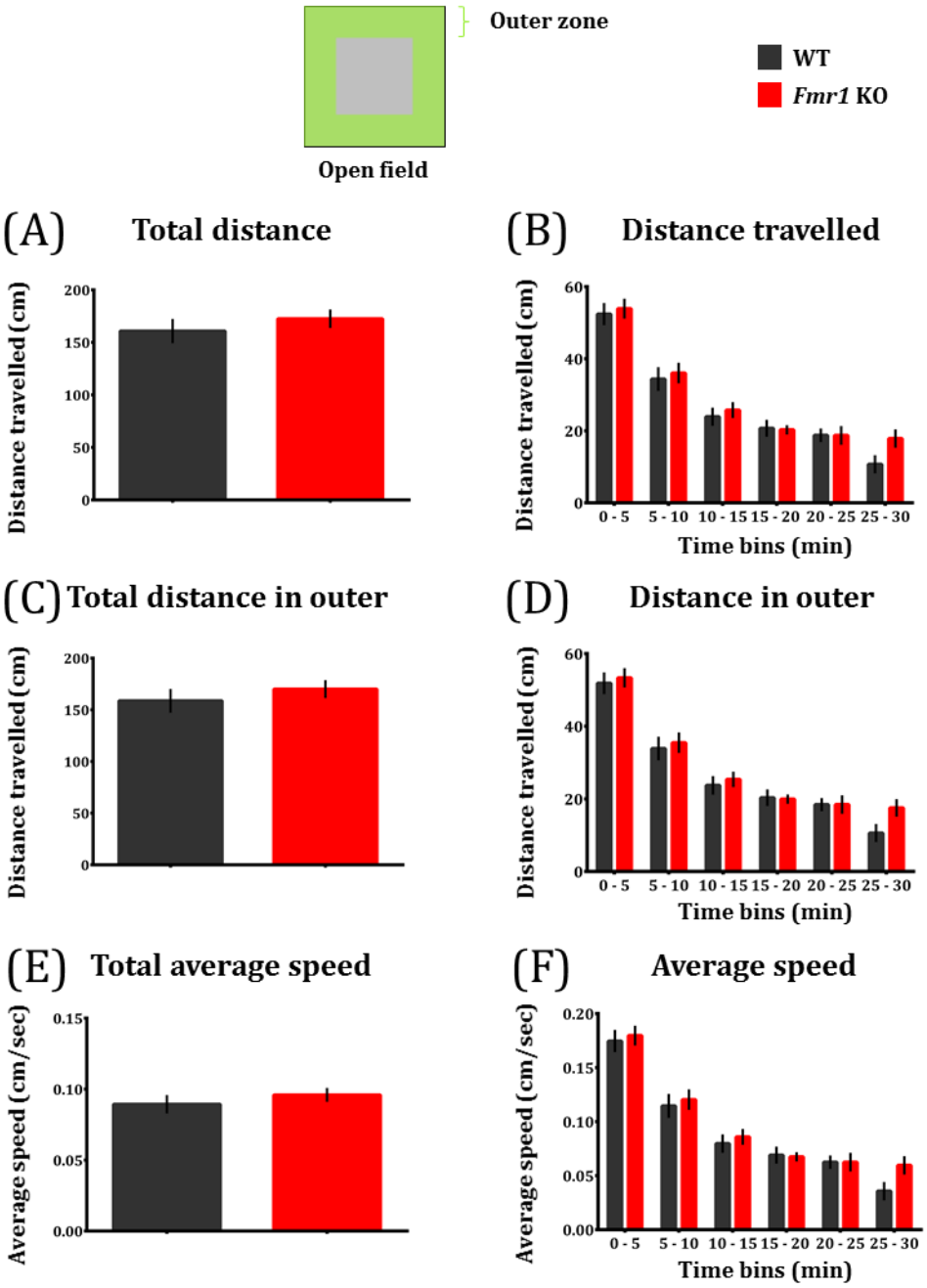



**Figure 4.4 Fmr1 KO rats exhibit normal activity and anxiety levels in open field test. (A)** Total ambulatory distance as well as distance travelled in 5 min epochs across the total 30 min of open field testing **(B)** revealed no differences between groups. Distance travelled in the periphery of the testing apparatus **(C)** as well as its profile across the testing **(D)** confirmed that Fmr1 KO rats experience normal anxiety levels. Hyperactivity was further assessed by analysis of the total average speed and speed profile across the total 30 min of open field testing confirming similar activity level between groups.

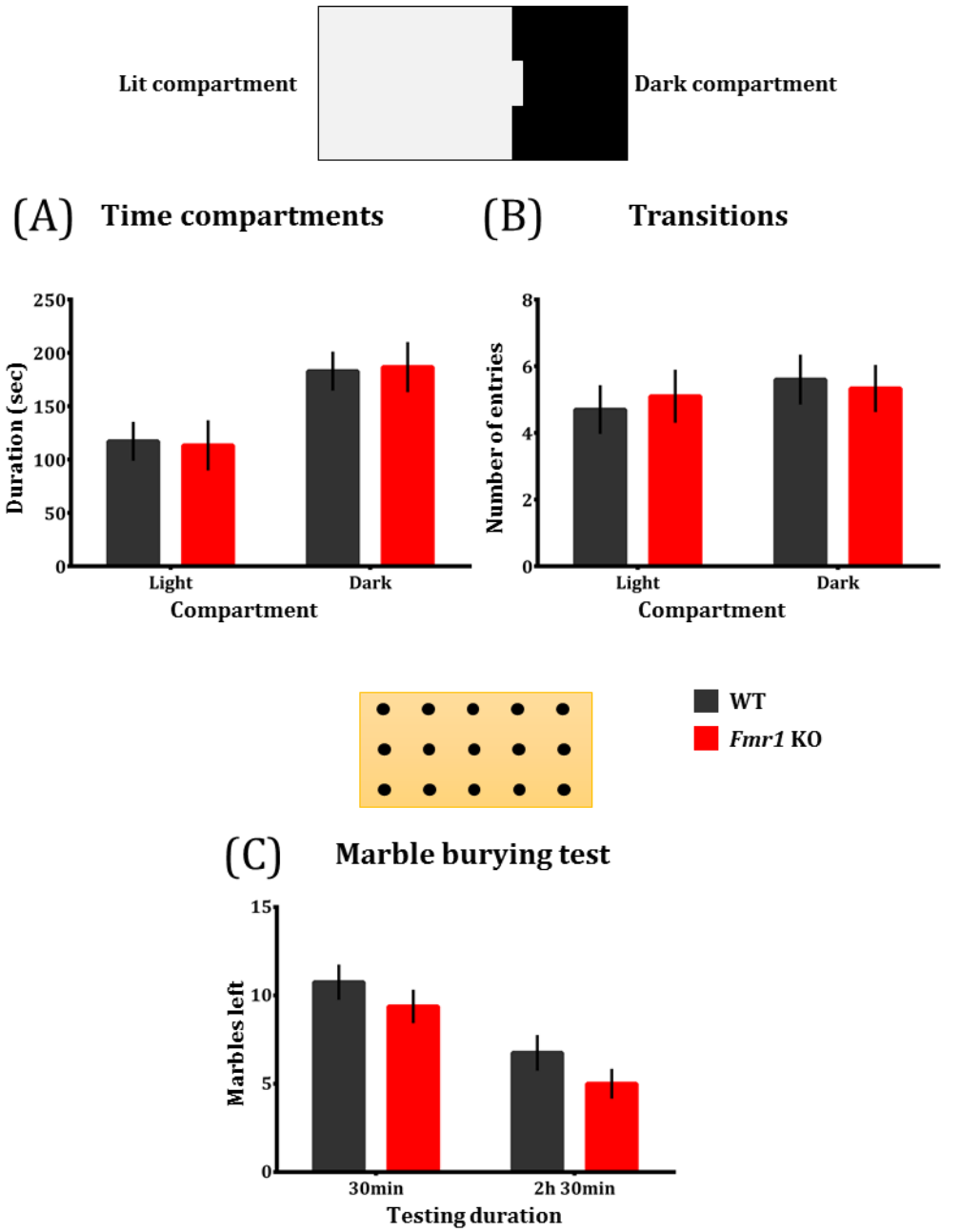



**Figure 4.5 Fmr1 KO rats experience normal anxiety level and show no repetitive behaviours.** Light/dark box testing revealed no differences in time spent between the two apparatus compartments **(A)** and equal number of transitions **(B)** between Fmr1 KO and wildtype rats. **(C)** Marble burying test confirmed that novelty-induced anxiety and repetitive behaviours are not augmented in Fmr1 KO rats.

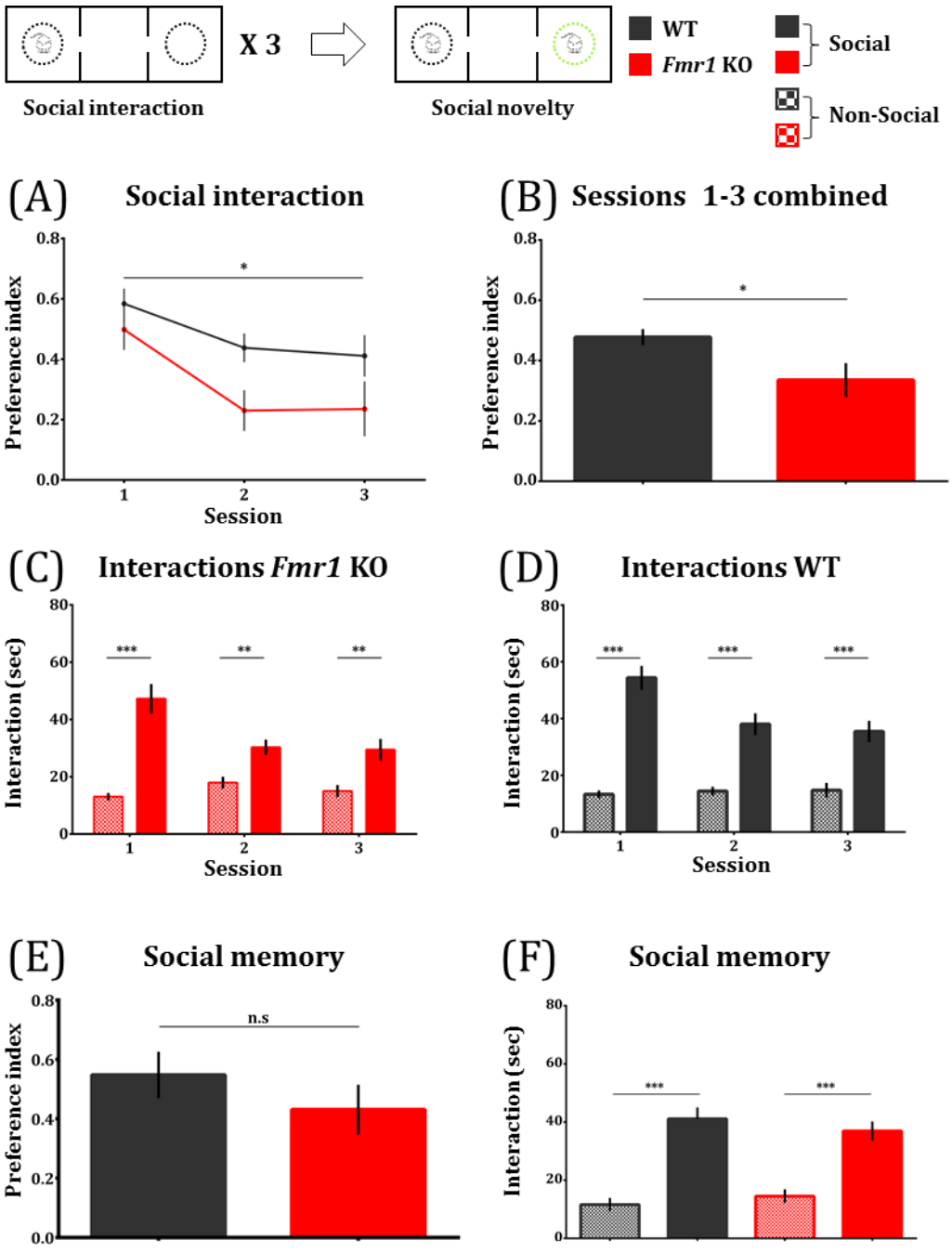



**Figure 4.6 Fmr1 KO rats display subtle social interaction deficits. (A)** Fmr1 KO rats tend to explore less a stimulus rat over 3 repeated sessions, showing a steeper decline in their interest in the last two sessions. **(B)** Averaging exploration between all three sessions reveals a genotype difference in social preference. Both Fmr1 KO **(C)** and WT rats **(D)** show preference to the social stimulus. Both groups show good memory for a social stimulus (E) and both preferentially explore a novel conspecific rather than a familiar juvenile rat (F). *p<0.05

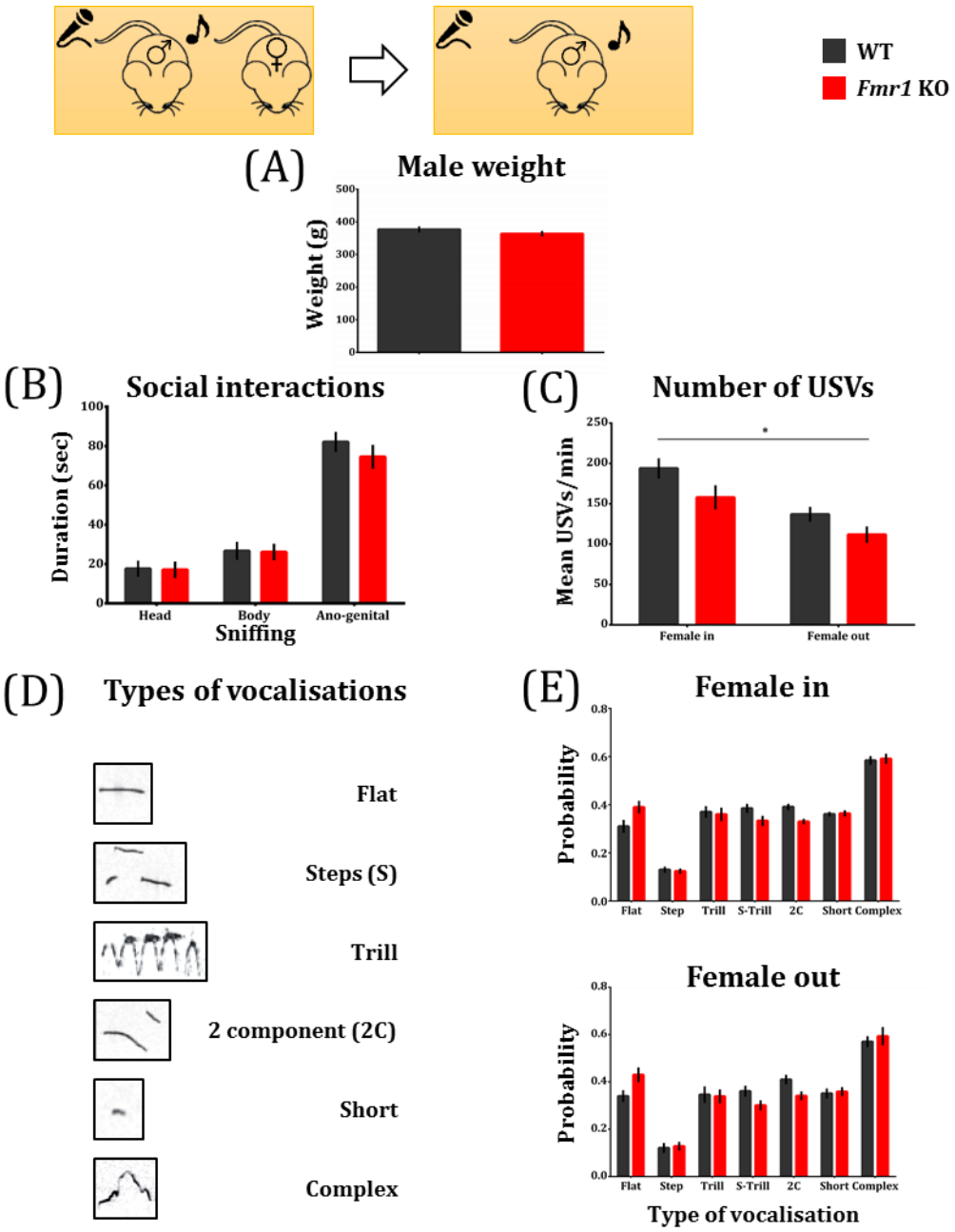



**Figure 4.7 Fmr1 KO rats display minor communication deficits in social interaction task.** Subjects used have similar body weight **(A)**. Social investigation of female in oestrous was almost identical between groups **(B)**. Total number of vocalisations over the two phases of testing revealed that Fmr1 KO rats vocalise less compared to their wildtype littermates **(C)**. Comparison of different categories of calls **(D)** produced by the two groups revealed no differences in the probability of each type of call irrespectively of the female presence **(E)**. *p<0.05

### Three chamber social interaction task

Previous work by Hamilton and colleagues (Hamilton et al., 2014) showed that Fmr1 KO rats exhibit reduced play behaviours during adolescence but the show a strong preference, equal to wildtype littermates, to a social stimulus over an object in three chamber social task. We wanted to see if repeated exposure to the same social stimulus would lead to any differences in social habituation. For that reason, instead of a single exposure subjects were presented with the same stimulus juvenile rat three times (Fig 4.6). Analysis of the preference index revealed that Fmr1 KO rats overall have a lower interest in the social stimulus than their wildtype littermates (session $F(2,52) = 7.8$, $p = 0.0011$; genotype $F(1,26) = 6.52$, $p = 0.017$; genotype × session $F(1,14) = 0.519$, $p = 0.598$; Fig. 4.6A). When values for each animal were averaged across the three sessions KO animals clearly showed a diminished interest (WT: 0.48 ±0.027; KO: 0.34 ±0.056; $t26 = 2.29$, $p = 0.031$; Fig. 4.6B). Nevertheless, both groups showed a strong preference for the social stimulus, over the three sessions (all multiple t-tests $p < 0.01$ Bonferroni corrected; Fig 4.6 C, D). In order to explore possible social memory deficits, we used a fourth session; an unfamiliar rat was introduced to the chamber and the subjects were tested in their ability to recognise a novel over a familiar rat. Both groups of rats showed strong preference for the novel social stimulus (WT $t26 = 6.47$, $p < 0.001$; KO $t26 = 5.52$, $p < 0.001$; Fig. 4.6 F) and there were no differences between groups in their preference for the unfamiliar social stimulus (WT: 0.55 ±0.078; KO: 0.43 ±0.084; $t26 = 1.02$, $p = 0.32$ Fig. 4.6E).

### USV analysis

Rats have a very rich repertoire of vocalisations ranging from fear-induced vocalisations to vocalisations specific to mating behaviour. As a result, it is thought that analysis of these vocalisations during different social paradigms could reveal deficits in rodent models of human conditions which include communication deficits (Wöhr & Schwarting, 2013). We used the male courtship paradigm which has been previously used in rats (McGinnis & Vakulenko, 2003) and in the mouse model of FXS (Rotschafer et al., 2012). The paradigm consists of two phases; first a stimulus female on oestrous interacts with a male subject and then the male is recorded while being alone in the testing apparatus. The weight of animals is known to affect vocalisation frequency (Wöhr & Schwarting, 2013), so subject were weighed before the experiment and no differences were found between genotypes (WT: 377.1 ±9.49; KO: 363.8 ±8.39; $t20 = 1.05$, $p = 0.31$ Fig. 4.7A).

The interactions between the female stimulus and the subjects were analysed and classified. No differences were seen in any of the types of interactions examined (type of interaction $F(2,48) = 95.12$, $p < 0.001$; genotype $F(1,16) = 0.55$, $p = 0.46$; genotype × type of interaction $F(2,48) = 0.36$, $p = 0.697$; Fig. 4.7B). Analysis of the mean number of vocalisations, during both phases of the paradigm, revealed a significant effect of genotype (female presence $F(1,32) = 18.82$, $p < 0.001$; genotype $F(1,16) = 6.61$, $p = 0.042$; genotype × female presence $F(1, 32) = 0.21$, $p = 0.650$; Fig. 4.7C). Although there was an overall genotype effect, the total number of calls was no significantly different between genotypes ($p = 0.075$, data not shown). For that reason, a detailed analysis of seven different categories of calls was carried out (Fig. 4.7D, E) in order to examine whether loss of FMRP in rats causes limited and call-type specific deficits in ultrasonic vocalization (Roy, Watkins, & Heck, 2012). No differences were observed in any of the different categories of calls with either female present (call type $F(6,112) = 95.21$, $p < 0.001$; genotype $F(1,16) = 0.292$, $p = 0.9712$; genotype × call type $F(6,112) = 2.87$, $p = 0.224$; Fig. 4.7E), or male subjects alone (call type $F(6,112) = 54.98$, $p < 0.001$; genotype $F(1,16) = 0.007$, $p = 0.566$; genotype × call type $F(6,112) = 2.21$, $p = 0.067$; Fig. 4.7E).

### 4.4.3 Watermaze tasks reveal normal spatial memory in Fmr1 KO rats

To start addressing whether the loss of FMRP leads to impaired cognitive function in rats, we employed two tasks in the widely used watermaze apparatus (Morris, Garrud, Rawlins, & O'Keefe, 1982). The first is a spatial reference memory and reversal task which has been used to assess memory and cognitive flexibility in the mouse model of FXS (Santos et al., 2014). The second is delayed matching-to-place (DMP) task, which is a rather unusual version of the watermaze protocols in which rats (or mice) learn to escape to the hidden platform which is moved to a new location daily and performance is recorded across many days or weeks (da Silva, Bast, & Morris, 2014). Neither of these hippocampus dependent tasks revealed any differences in cognitive performance between groups (Till et al., 2015).

#### Spatial reference memory and reversal task

This task assays the ability of subjects to learn to navigate a circular pool using distal cues to locate a hidden, submerged escape platform. During the first phase of the task, both Fmr1 KO and wildtype rats showed a progressively decreased path length needed to reach the platform (training day $F(6,96) = 21.89$, $p < 0.001$; genotype $F(1,16) = 1.66$, p

=0.22; genotype × training day F(6,96) =0.56, p =0.76; Fig. 4.8A). Moreover, the time spent in a zone around the platform location during probe trials across days increased (training day F(6,96) =10.30, P <0.001; genotype F(1,16) =0.04, p =0.85; genotype × training day F(6,96) =0.66, p =0.68; Fig. 4.8C). This data indicate that spatial learning and memory is equivalent between genotypes and that Fmr1 KO rats have intact spatial navigation capacity. To assess cognitive flexibility, rats then underwent a reversal learning task during which the platform was moved to the opposite side of the pool; the decrease in overall path length (training day F(6,96) =27.58, p <0.001; genotype F(1,16) =0.48, p =0.50; genotype × training day F(6,96) = 1.68, p = 0.13; Fig. 4.8B) and the increase in time spent in the new platform location zone (training day F(6,96) =14.8, P <0.001; genotype F(1,16) <0.001, P =1; genotype × training F (6,96) =1.18, p =0.32; Fig. 4.8D) reveal a comparable learning of the new platform position between genotypes. Although analysis of cognitive parameters revealed no difference in learning in either of the two the phases of the task, swimming speed was significantly increased in Fmr1 KO during the reference memory phase (training day F(6,96) =4.54, P <0.001; genotype F(1,16) =6.52, p =0.02; genotype × training day F(6,96) =1.43, p = 0.21; Fig. 4.8E) but not during reversal (training day F(6,96) =6.67, p <0.001; genotype F(1,16) =2.93, p =0.11; genotype × training day F(6,96) =0.61, P =0.72; Fig. 4.8F).

**Delayed Matching to Place (DMP) task**

To explore further the effects of FMRP loss in behavioural flexibility, we used a DMP task which is similar to the spatial reference memory task of water maze except that the location of the hidden platform location is updated daily (da Silva et al., 2014) (Fig. 4.9). This assay assesses the ability of an animal to learn a new location of a hidden platform in a single trial as measured by its performance in the following three trials. During the eight days of the pre-training phase, both Fmr1 KO and wildtype rats showed similar decreases in path lengths taken to escape over trials 2–4 compared with the first trial of the day (trial FD1-4(3,66) =21.19, p <0.001; genotype FD1-4(1,22) =0.21, p =0.65; genotype × trial FD1-4(3,66) =0.86, p =0.47. trial FD5-8(3, 66) =19.10, p <0.001; genotype FD5-8(1,22) =0.23, p =0.64; genotype × trial FD5-8(3,66) =0.18, p = 0.91 Fig. 4.9A). During the second phase, variable time delays were introduced between the first and second trials of each day [15sec, 15min or 2h inter-trial intervals (ITI)]. Although the task was made substantially more demanding, both Fmr1 KO and control rats performed similarly at each ITI as measured by their path lengths to escape (trial F15sec(3,66) =115.2, p <0.001; genotype F15sec(1,22) =0.45, p =0.51; trial × genotype F15sec(3,66)

=1.14, P =0.34. trial F15min(3,66) =91.6, p <0.001; genotype F15min(1,22) =2.04, P =0.17; trial × genotype F15min(3,66) =0.56, P = 0.64. trial F2hr(3,66) =85.93, p <0.001; genotype F2hr(1,22) =0.17, p =0.69; trial × genotype F2hr(3,66) =1.23, p = 0.30; Fig. 4.9B). A reduction in path length between the first and second trial of each day known as 'savings' reflect the learning from a single trial. The savings from trials one to two again did not reveal any differences between groups (delay F(2,44) =4.77, p =0.01; genotype F(1,22) =2.58, p =0.12; delay × genotype F(2,44) =0.14, p =0.87; Fig. 4.9C). Probe trials, during the second trial, measuring time spent searching in the target zone of each day further confirmed analogous one-trial spatial learning between groups across all used ITIs (delay F(2,44) =4.27, p =0.02; genotype F(1,22) =0.34, p =0.56; delay × genotype F(2,44) =1.26, p =0.29; Fig. 4.9D). Analysis of the time spent in a zone around the platform location on the previous day (expressed in negative values) suggests no difference in cognitive flexibility and no signs of perseveration across genotypes (delay F(2,44) =2.07, p =0.14; genotype F(1,22) =0.61, p =0.44; delay × genotype F(2,44) =0.54, p =0.59; Fig. 4.9E).

### 4.4.4 FMRP loss leads to hippocampus-dependent, spatial and episodic-like memory impairments

To investigate further the effect of FMRP loss on cognitive function, rats were tested on a battery of spontaneous recognition memory tasks testing non-associative memory in object-recognition (NOR), spatial memory in object displacement (OD) tasks and associative memory in object-context (OC), object-place (OP) and object-place-context (OPC) tasks (Fig. 4.10 and 4.11). Interestingly, while no impairments were observed in most of the tasks, a very robust deficit was detected in the most complex OPC task which is hippocampus-dependent and involves the associative recognition of objects, their spatial locations and the local context (Eacott & Norman, 2004; Langston & Wood, 2010).

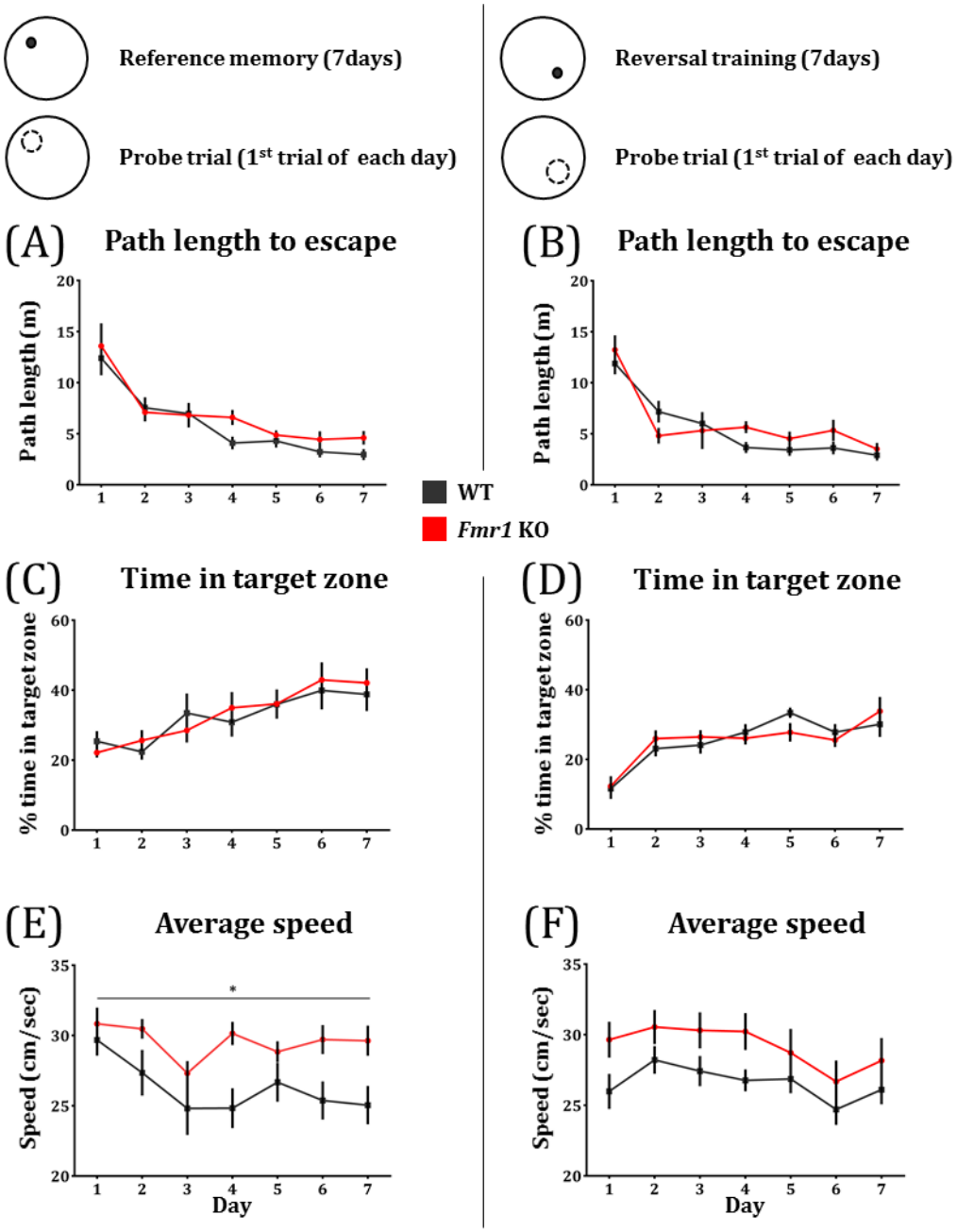



**Figure 4.8 *Fmr1* KO rats have normal spatial reference memory acquisition and reversal learning.** **(A)** *Fmr1* KO rats learn the hidden-platform version of the water maze similarly to WT littermates as measured by a decrease over days in the path taken to escape **(B)** and the time spent in a zone around the platform during daily probe trials **(C)**. Performance during reversal learning was comparable between genotypes as measured by path to escape (B) and the time spent in a zone around the new platform location, during daily probe trials **(D)**. **(E)** *Fmr1* KO rats swim faster than WT littermates over reference memory training but not over reversal **(F)**. *p<0.05

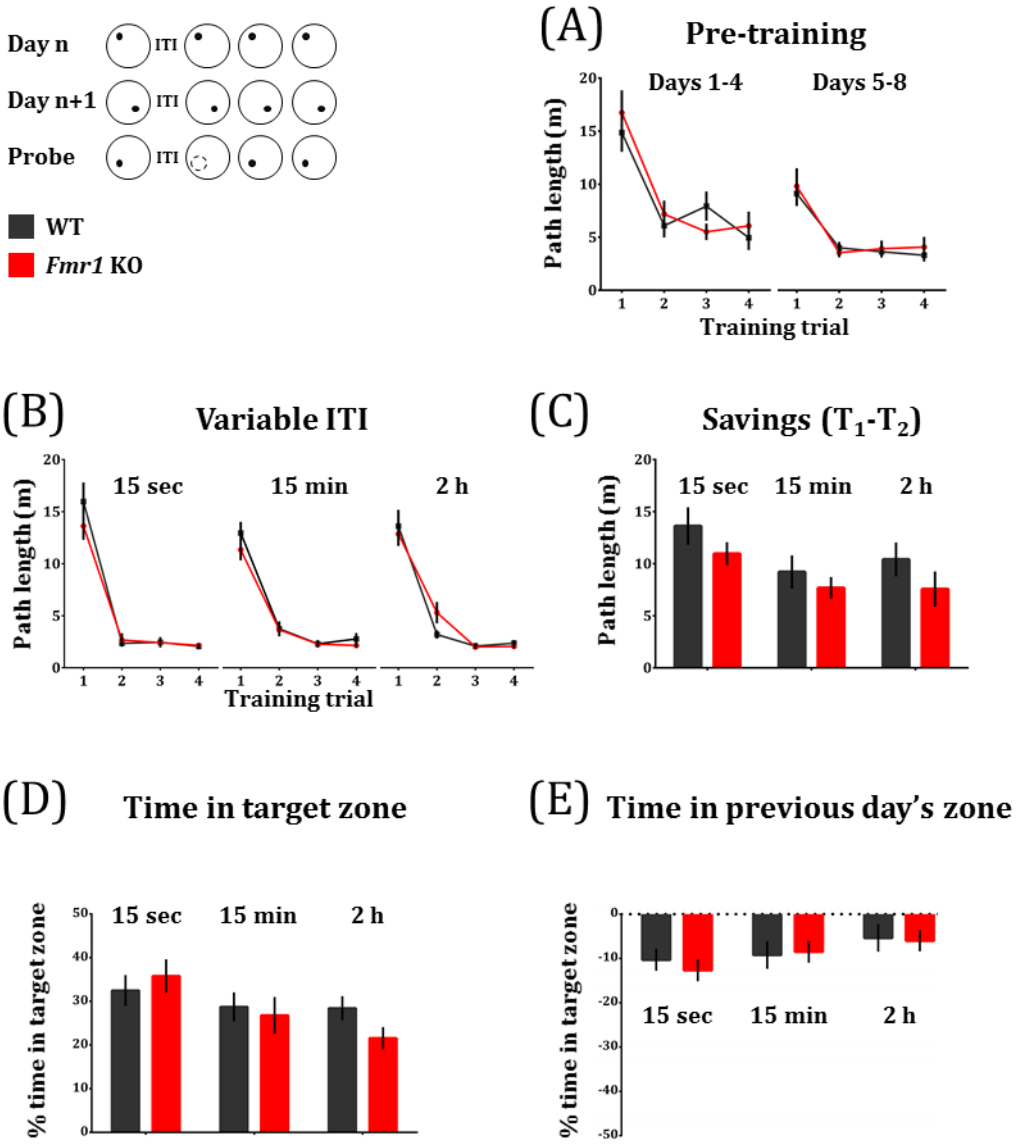



**Figure 4.9 One-trial spatial learning is intact in *Fmr1* KO rats. (A)** *Fmr1* KO rats learn the DMP task similarly to WT littermates as measured by a decrease in the path length taken to escape over trials within a day. Introducing a variable time delay between the first and second trials of the day does not affect performance of *Fmr1* KO rats compared with WT as measured by path length to escape **(B)**, savings **(C),** time spent searching in the target zone on probe trials **(D)** or time spent around the location of the target on the previous day on probe trials **(E).**

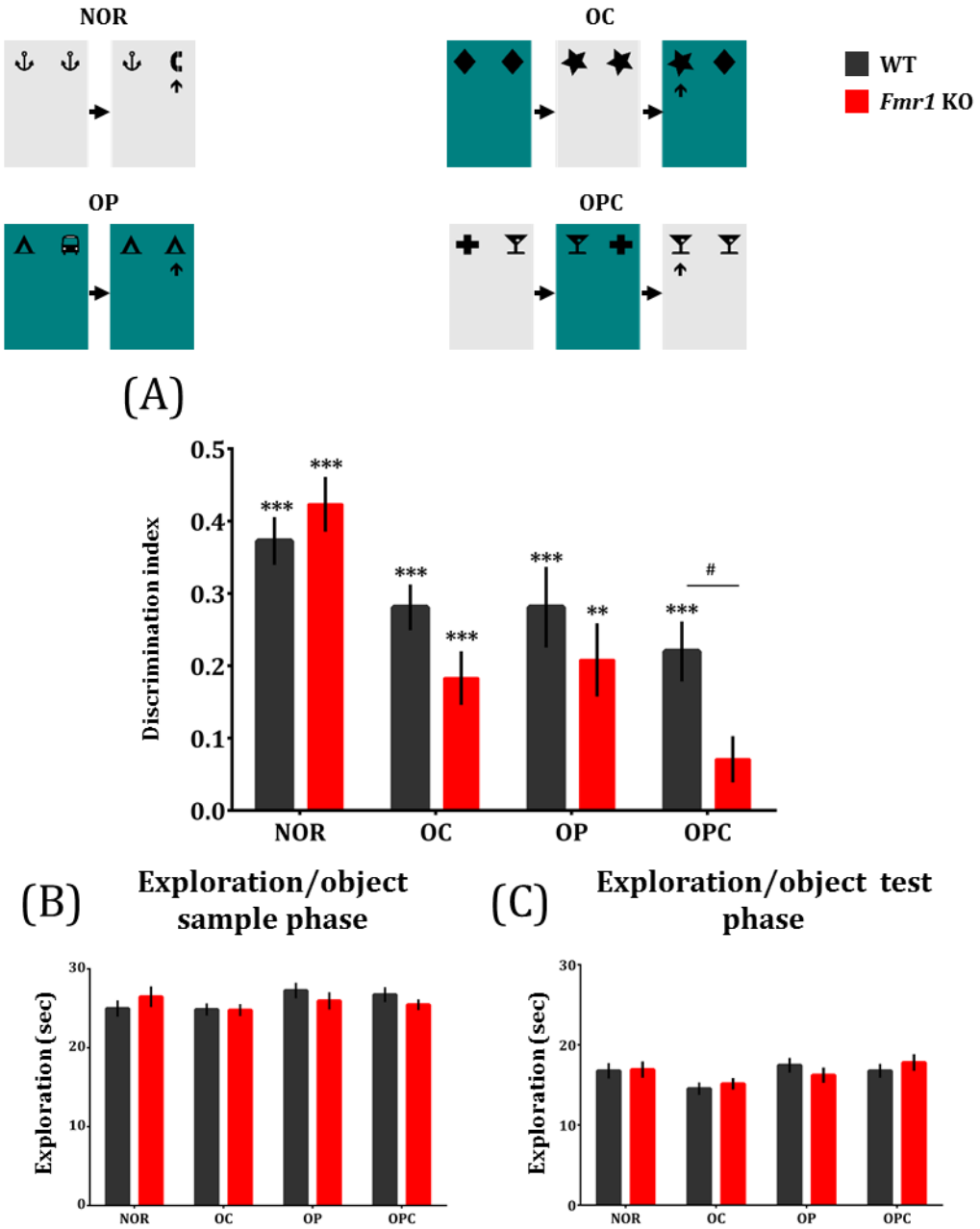



**Figure 4.10 Loss of FMRP results in impaired performance on spontaneous object exploration task assessing episodic-like memory.** On top: a schematic of the spontaneous exploration tasks for novelty preference. **(A)** WT rats exhibit memory for all four tasks as measured by above chance performance. In contrast, Fmr1 KO rats do not perform above chance levels in an OPC task that requires the ability to form associations between objects, their locations and the context, but do exhibit memory for the individual components as measured by above chance performance in object recognition, object-place and object-context tasks. Object exploration in sample **(B)** and test phase **(C)** of the tasks is similar between the two genotype groups. * $p<0.05$ difference from chance (DI =0) # $p<0.05$ difference between genotypes

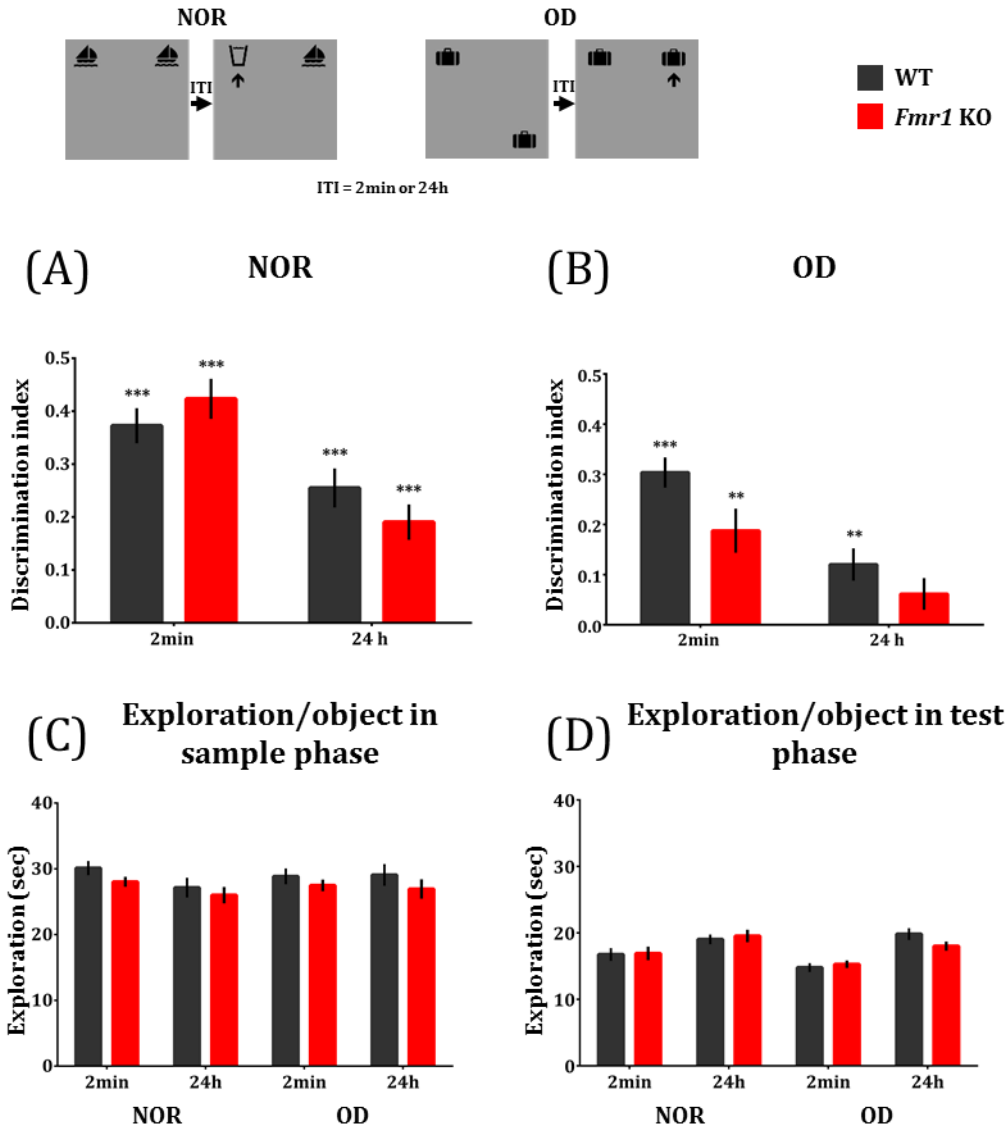



**Figure 4.11 Fmr1 KO rats show impairment in long-term spatial object memory.** On top: a schematic of the spontaneous exploration tasks for novel object and object displacement tasks. **(A)** Both groups have intact short and long-term object memory. WT rats exhibit memory for spatial object memory at both short and long delays while Fmr1 KO rats show only short term spatial memory **(B)**. Object exploration in sample **(C)** and test phase **(D)** of the tasks is similar between the two genotype groups. * $p<0.05$ difference from chance (DI =0)

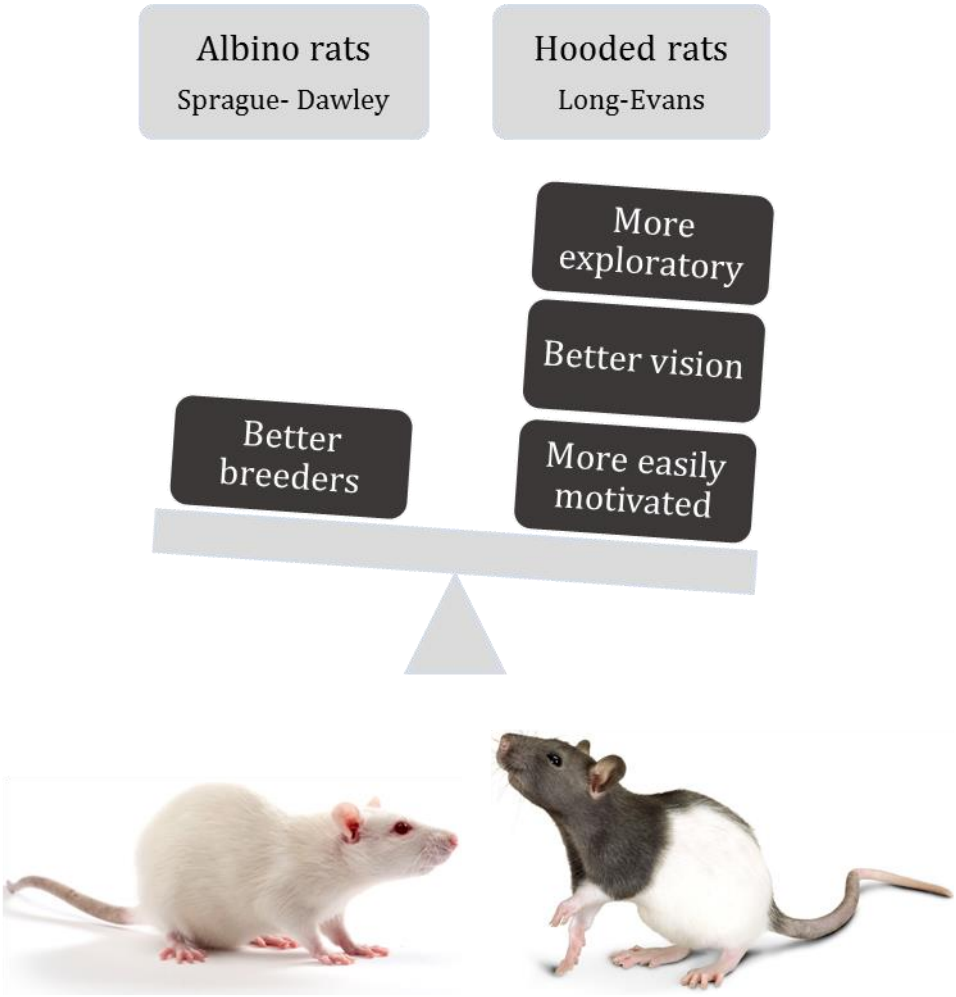



**Figure 4.12** Hooded rats are considered more suitable for behavioural testing due to their increased innate curiosity for novelty.

**Short term non-associative and associative spontaneous exploration tasks**

These four tasks assessed the subjects' ability to discriminate novel from familiar objects, and novel from familiar object-context, object-place and object-place-context associations over a short (2 min) delay. Both groups showed significant memory in NOR, OC and OP tasks but only wildtype rats performed above chance in OPC task (WTNOR: 0.37 ±0.03, t15 = 11.26, p <0.001; WTOC: 0.28 ±0.03, t15 =8.88, p <0.001; WTOP: 0.28 ±0.06, t15 =5.03, p <0.001; WTOPC: 0.22 ±0.04, t15 =5.32, p <0.001; KONOR: 0.42 ±0.04, t15 =11.20, p <0.001; KOOC: 0.18 ±0.04, t15 =4.93, p <0.001; KOOP: 0.21 ±0.05, t15 =4.1, p =0.004; KOOPC: 0.07 ±0.03, t15 =2.21, p =0.17; all values Bonferroni corrected Fig. 4.10A). Fmr1 KO rats showed a decreased preference for novelty in OC and OP tasks compared to their wildtype littermates, but only in OPC there was a significant difference between groups (NOR: t120 =0.88, p =0.85; OC: t120 =1.69, p =0.32; OP: t120 =1.26, p =0.61; OPC: t120 =1.26, p =0.04; all values Bonferroni corrected). In order to confirm that this finding was true memory deficit rather than an effect of impaired encoding we analysed the exploration time during the sampling phase of the tasks. We saw that both groups interacted with object on a very similar level indicating that the observed deficit is not due to poor encoding (task F(3,120) =1.2, p =0.32; genotype F(1,30) =0.18, p =0.67; task × genotype F(3,120) =0.92, p =0.43; Fig. 4.10B). We further analysed the exploration time during the test phase of the tasks in order to see if the observed impairment in OPC task is due to decreased interest in the objects. Although we found a main effect of task, no differences between groups were detected (task F(3,120) =1.87, p =0.039; genotype F(1,30) =0.05, p =0.83; task × genotype F(3,120) =0.58, p =0.63; Fig. 4.10C).

**Short and long term spatial and non-spatial object memory**

Taking into account the OPC deficit and the fact that no differences between genotypes were observed in the hippocampus dependent watermaze tasks, we want to assess spatial memory at both a short (2min) and along (24h) delay using object-displacement (OD) task. For OD task we used a different square testing apparatus, so in order to be sure that animals can perform in this new environment we used NOR as a positive control (Fig 4.11). Both Fmr1 KO rats their wildtype littermates performed significantly above chance in both delays in NOR task (WTNOR2min: 0.37 ±0.03, t15 = 11.26, p <0.001; WTNOR24h: 0.26 ±0.04, t15 =6.92 p <0.001; KONOR2min: 0.42 ±0.04, t15 =11.20, p <0.001; KONOR24h: 0.19 ±0.03, t15 =6.92, p <0.001; all values Bonferroni corrected; Fig. 4.11A) confirming the intact object memory after FMRP loss. Although both groups

performed similarly in OD task at short delay (WTOD2min: 0.30 ±0.03, t15 = 10.07, p <0.001; KOOD2min: 0.19 ±0.04, t15 =4.27 p =0.002; all values Bonferroni corrected; Fig 4.11B), only wildtype rats performed above chance at a 24h delay (WTOD24h: 0.12 ±0.03, t15 = 3.73, p =0.008; KOOD24h: 0.06 ±0.03, t15 =1.95 p =0.28; all values Bonferroni corrected; Fig 4.11B). Despite this finding, no statistically significant differences were found in any of the two delays in either NOR or OD task (NOR2min: t30 =1.01, p =0.32; NOR24h: t30 =1.3, p =0.20; OD2min: t30 =2.17, p =0.16; OD24h: t30 =1.30, p =0.20; all values Bonferroni corrected). As previously, we also analysed raw exploration times during sample (task F(3,120) =1.4, p =0.26; genotype F(1,30) =3.59, p =0.061; task × genotype F(3,120) =0.08, p =0.97; Fig. 4.11C) and test phase (task F(3,120) =11.57, p <0.001; genotype F(1,30) =0.08, P =0.77; task × genotype F(3,120) =0.92, p =0.43; Fig. 4.11D) and found no differences in the exploratory behaviour between groups.

### 4.4.5 Spatial and episodic-like memory impairments persist across different rat strains

Following our findings that Fmr1 KO rats, on a Sprague-Dawley background, exhibit deficits in the OPC task, we explored whether the newly created Fmr1 KO Long-Evans-Hooded (LEH) rats exhibit the same deficit. The main goal was to determine whether the behavioural phenotype described previously persists across strains, since the mouse model of FXS has been shown to have many strain-specific behavioural phenotypes (Chapter 1). Previous research has shown that hooded rat strains are more suitable for behavioural testing than albino (Birch & Jacobs, 1979; Broersen & Uylings, 1999; Clemens et al., 2014) (Fig. 4.12); thus we were very pleased to discover in line with our previous findings in SD rats, LEH Fmr1 KO rats exhibit episodic-like (OPC) memory and spatial (OD) memory deficits.

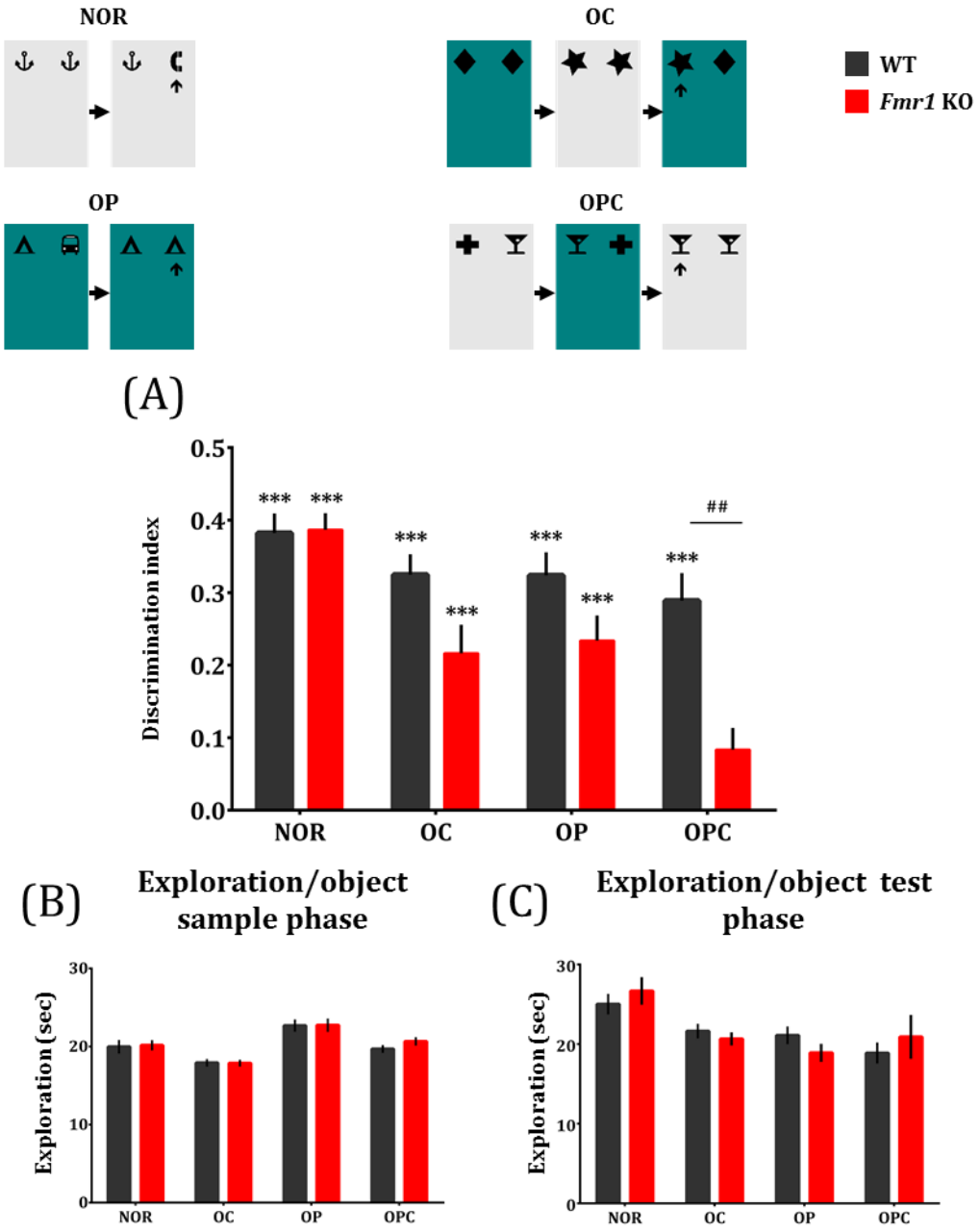



**Figure 4.13 LEH *Fmr1* KO rats are unable to remember object-place-context associations.** On top: a schematic of the spontaneous object exploration. **(A)** WT rats exhibit memory for all four tasks as measured by above chance performance. In contrast, *Fmr1* KO rats do not perform above chance levels in an OPC task that requires the ability to form associations between objects, their locations and the context, but do exhibit memory for the individual components as measured by above chance performance in object recognition, object-place and object-context tasks. Object exploration in sample **(B)** and test phase **(C)** of the tasks is similar between the two genotype groups. * $p<0.05$ difference from chance (DI =0) # $p<0.05$ difference between genotypes.

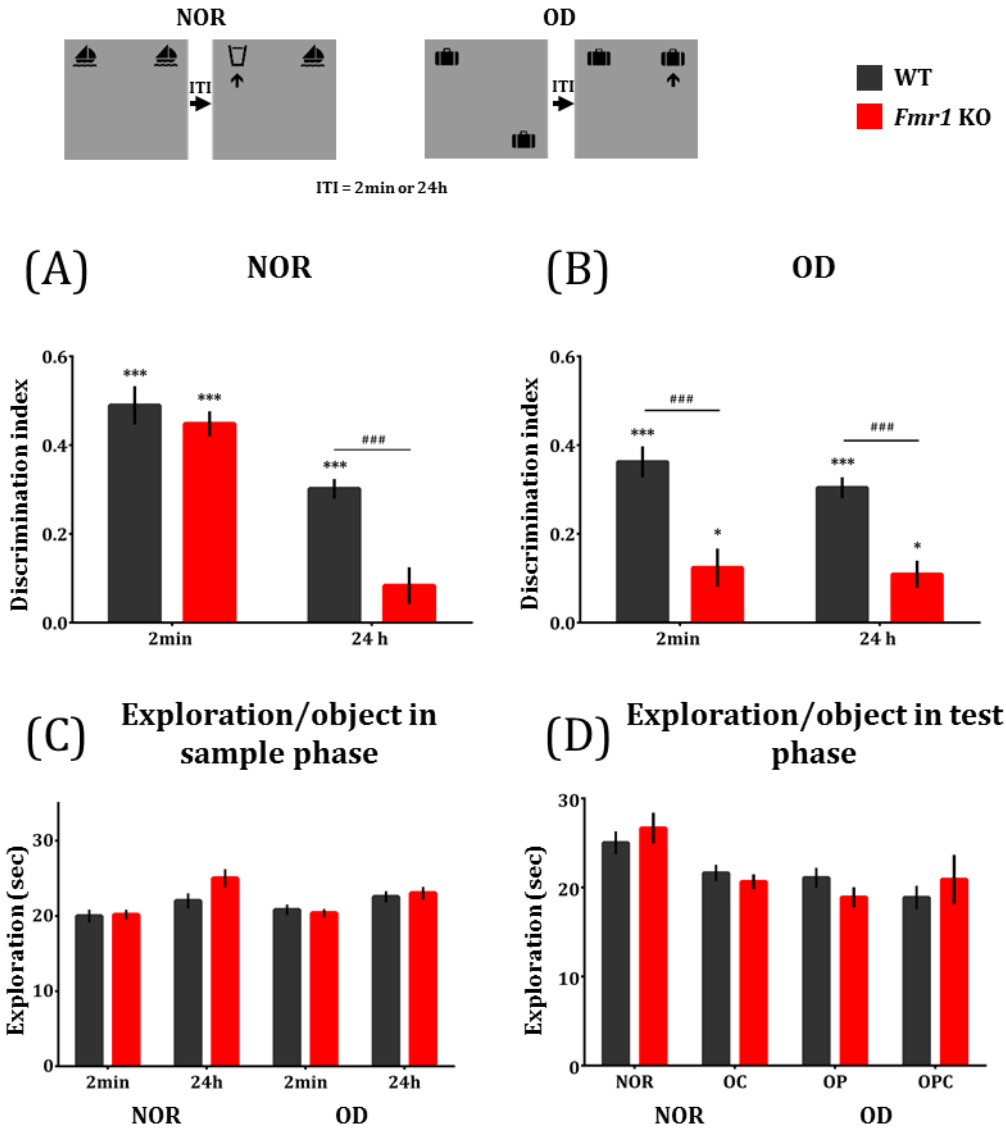



**Figure 4.14 LEH *Fmr1* KO rats show impairment in long-term object memory and reduced preference spatial novelty.** On top: a schematic of the spontaneous exploration tasks for novel object and object displacement tasks. **(A)** Both groups have intact short object memory but only WT rats are able to remember object identities over a 24h delay. Both groups of rats exhibit memory for spatial object memory at both short and long delays while *Fmr1* KO rats show diminished preference for the displaced object at both delays compared to their WT littermates **(B)**. Object exploration in sample **(C)** and test phase **(D)** of the tasks is similar between the two genotype groups. * $p<0.05$ difference from chance (DI =0) # $p<0.05$ difference between genotypes.

**Short term non-associative and associative spontaneous exploration tasks**

As described above, we used four tasks (NOR, OC, OP, OPC) to assess non-associative (NOR) and associative object memory (OC, OP, OPC) over a short (2 min) delay. As previously, both genotype groups showed significant memory in NOR, OC and OP tasks but only wildtype rats performed above chance in OPC task (WTNOR: 0.38 ±0.03, t15 = 14.18, p <0.001; WTOC: 0.33 ±0.03, t15 =11.44, p <0.001; WTOP: 0.32 ±0.03, t15 =10.23, p <0.001; WTOPC: 0.29 ±0.04, t15 =7.6, p <0.001; KONOR: 0.39 ±0.02, t15 =16.95, p <0.001; KOOC: 0.22 ±0.04, t15 =5.50, p <0.001; KOOP: 0.23 ±0.04, t15 =6.7, p <0.001; KOOPC: 0.08 ±0.03, t15 =2.79, p =0.06; all values Bonferroni corrected Fig. 4.13A). Although LEH Fmr1 KO rats showed again decreased preference for novelty in all three associative memory tasks, groups were significantly different only in OPC (NOR: t120 =0.10, p >0.99; OC: t120 =2.40, p =0.07; OP: t120 =1.99, p =0.18; OPC: t120 =4.55, p <0.001; all values Bonferroni corrected). The exploratory behaviour even though it was slightly different in LEH compared to SD, highlighting the strain differences in novelty-induced behaviour (Clemens et al., 2014), it was almost identical between genotypes in both sample (task F(3,120) =17.63, p <0.001; genotype F(1,30) =0.39, p =0.53; task × genotype F(3,120) =0.25, p =0.86; Fig. 4.13B) and test phase (task F(3,120) =7.03, p <0.001; genotype F(1,30) =0.02, p =0.90; task × genotype F(3,120) =0.93, p =0.43; Fig. 4.13C), confirming that the observed deficit is not due to reduced encoding or luck of interest.

**Short and long term spatial and non-spatial memory**

LEH rats were also tested in the previously described tasks NOR and OD at both 2min and 24h delays (Fig 4.14). Testing in short 2min delay confirmed the results from the previous group of tasks (Fig 2.13), that the short term object memory in not affected by loss of FMRP (WTNOR2min: 0.49 ±0.04, t15 = 11.31, p <0.001; KONOR2min: 0.45 ±0.03, t15 =15.93, p <0.001; Fig. 4.14A). On the other hand, only wildtype rats performed above chance in NOR at the 24h delay (WTNOR24h: 0.32 ±0.02, t15 =13.67 P <0.001; KONOR24h: 0.08 ±0.04, t15 =2.01, p =0.25; Fig. 4.14A). Furthermore, comparison between groups revealed a significant difference only at the 24h delay (NOR2min: t30 =0.80, p =0.43; NOR24h: t30 =4.65, p <0.001). In OD task Fmr1 KO rats performed significantly worse than their wildtype littermates at both delays (OD2min: t30 =4.30, p <0.001; OD24h: t30 =5.06, p <0.001) but both groups still performed significantly better than chance levels (WTOD2min: 0.36 ±0.04, t15 = 10.34, p <0.001; KOOD2min: 0.12

±0.04, t15 =2.90 p =0.04; WTOD24h: 0.30 ±0.02, t15 = 12.96, p <0.001; KOOD24h: 0.11 ±0.03, t15 =3.57 p =0.01; all values Bonferroni corrected; Fig 4.14B) indicating that even though there is a difference in novelty preference, Fmr1 KO rats still have intact spatial object memory. Analysis of the exploration times revealed a main effect of task in both sample (task F(3,120) =7.89, p <0.001) and test phase (task F(3,120) =8.01, p <0.001) and a subtle genotype effect in test (genotype F(1,30) =4.57, p =0.03) but not in sample phase (genotype F(1,30) =1.89, p =0.17), and no significant interactions in either sample (task × genotype F(3,120) =1.62, p =0.19; Fig. 4.14C) or test phase (task × genotype F(3,120) =0.29, p =0.84; Fig. 4.14D).

## 4.5 Discussion

### 4.5.1 *Fm1* KO rats exhibit distinct behavioural deficits

Recent developments in gene manipulation techniques have allowed genetic modification of mammalian species other than mice. The creation of rat models provides an invaluable opportunity of understanding the pathophysiology associated with the loss of FMRP, as well as providing cross-species validation of cellular dysfunction that will strengthen the relevance of genetic models of FXS to the human disorder. Part of the work presented in this chapter has been published on (Till et al., 2015) along with a first characterisation of cellular and plasticity abnormalities in this rat model of FXS. We find that *Fmr1* knockout rats recapitulate key aspects of hippocampal cellular and synaptic phenotypes associated with the loss of FMRP in mice, including elevated basal protein synthesis (Dölen et al., 2007), abnormal synaptic plasticity (Nosyreva & Huber, 2006) and alterations in the morphology of dendritic spines (Wijetunge et al., 2014) of hippocampal pyramidal neurons. From a translational scope, these phenotypes are commonly used to assess therapeutic efficacy for pharmacological reversal of FXS-related symptoms (Michalon et al., 2012; Osterweil et al., 2013b) therefore the fact that they do persist in the rat model is important. Taking into account that the lineage which gave rise to the two species (mouse and rat) separate more than 12 million years ago, the commonality in cellular abnormalities validates the conceptual basis of theories underlying targeted approaches to therapies and their potential relevance to the human syndrome (Wijetunge et al., 2013).

In the first group of experiments we examined whether mutant rats exhibit any behaviours resembling the elevated anxiety and hyperactivity seen in humans affected

by the disorder. Consistently with what Hamilton and colleagues had reported previously, no differences were observed in open field test that would indicate either elevated anxiety (time spent in the middle of open field), or hyperactivity (larger distance travelled). Light/dark box task did not reveal any difference between either. Some consider this task to be inadequate for identifying anxiety phenotypes in rats, based on the fact that rats are bolder when exploring a novel environment than mice (Hascoët & Bourin, 2009; Hölter et al., 2015). In our experiments, repetitive behaviours were assessed with the marble burying task (Deacon, 2006). This task is thought to access repetitive and perseverative behaviours but since the final readout is heavily influenced by novelty-induced anxiety and hyperactivity (Thomas et al., 2009), no differences were observed between groups. This result is somewhat contradicting to what Hamilton and colleagues (2014)have seen; they reported that *Fmr1* KO rats exhibit perseverative chewing behaviours when they are presented with a wood block.

Furthermore, we observed that a three-chamber social interaction test, which is commonly used to assess social interest in mice (Silverman et al., 2010), elicits a very strong preference for a social stimulus in rats. Despite the small genotype difference (Fig. 4.6A&B) both knockout rats and wildtype littermates demonstrated a strong preference for investigating the enclosure with the social stimulus (rat vs empty or novel rat vs familiar rat). This replicates previous work (Hamilton et al., 2014) showing that *Fmr1* KO rats have no differences in their preference for social stimuli in the three chamber social interaction task. The differences observed in social habituation, are consistent with absence of social odour recognition deficits (Hamilton et al., 2014); a task assessing social odour habituation is likely to support our findings (Yang and Crawley 2009). Interestingly we observed no effect in social memory test suggesting that the differences between mutants and control animals are very subtle and more demanding protocols are required in order to reveal deficits. Consistently with this idea Harony-Nicolas and colleagues (Harony-Nicolas et al., 2014) showed that by increasing the retention interval to 24h, Shank3 knockout rats (model for autism) are not distinguishing between familiar and novel conspecifics. The subtle difference we observed in USVs (Fig 4.7 C) are again consistent with previous findings (Hamilton et al., 2014) showing no differences in vocalisations between groups albeit in a different social paradigm (juvenile play). In our courtship paradigm, *Fmr1* KO rats displayed reduced vocalisations. Nevertheless, these small differences could may as well be because of limitations in our paradigm. The main limitation being our inability to differentiate between the female and male vocalisations.

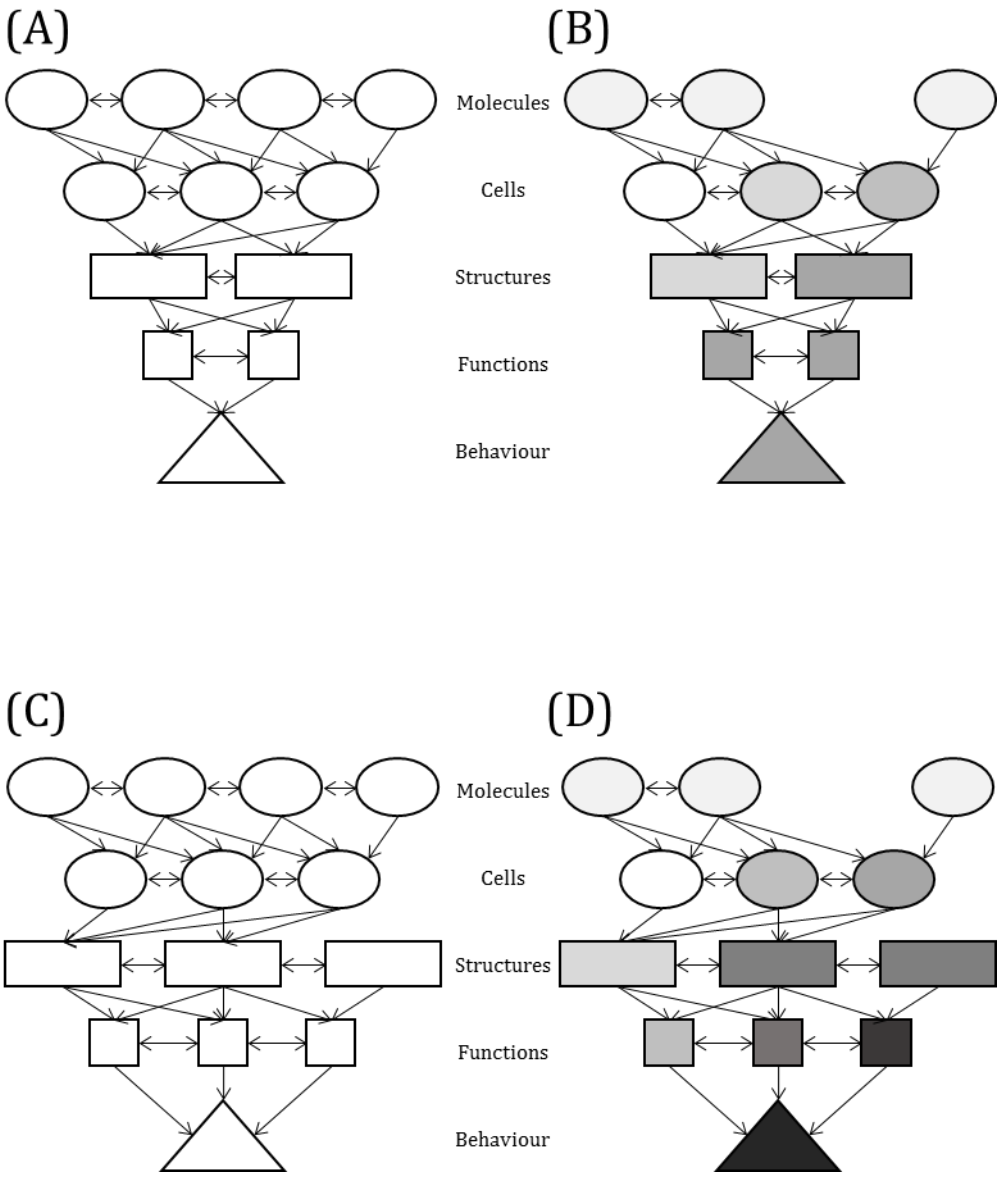



**Figure 4.15 Schematic illustration of organisational levels involved in manifested behaviour.** On top **(A)** and **(B)** the behaviour depends mainly on two brain structures, at the bottom **(C)** and **(D)** a similar but different behaviour dependent on three structures. In the presence of a molecular event such as a genetic lesion, several molecular processes and events will be affected to a different extend **(B)** and **(D).** Behaviours which require wider networks, are more likely to be affected as subtle abnormalities in each of the brain structures supporting it "accumulate", causing greater disturbances in the wider network. The intensity of grey is parallel to the disturbance of each system.

Recent studies have shown that contrary to what is widely believed, both female mice (Neunuebel et al., 2015) and rats (Börner et al., 2016) emit USVS in response to male

courtship. Therefore, it is plausible that the small difference we observe is due to females compensating for Fmr1 KOs' reduced vocalisation frequency. It would be interesting to analyse social communication in a number of other social and non-social paradigms, or even address the ontogeny of communication in *Fmr1* KO rats. Overall our results show a few consistent trends but no strong statistically significant differences in a number of parameters assessing communication and social interactions. This could simply indicate that experimental protocols and approaches used to assess the mouse model of FXS cannot be directly applied to rats. It is possible that adjusting some of these tasks by taking into account the ethological differences between the two species (rats have stronger memory expression, are more social etc.), will yield positive results.

Intellectual disability is a defining feature of Fragile X syndrome. Even though *Fmr1* knockout mice show deficits (subtle non the less) in reversal learning in the watermaze (D'Hooge et al., 1997; Van Dam et al., 2000), this form of spatial learning is not affected by the loss of FMRP in rats. This is evidence that common cellular phenotypes in the hippocampus in *Fmr1* KO mice and rats are not mirrored by the same hippocampus-dependent behavioural phenotypes. This result is consistent with previous work showing absence of deficits in hippocampus-dependent contextual fear conditioning in *Fmr1* null rats (S. M. Hamilton et al., 2014). One possibility is that these results parallel the scarcity of robust and reproducible across labs, cognitive deficit phenotypes reported for mouse models of FXS (Corinne M. Spencer et al., 2011). Another possibility is that since rats are natural swimmers (Whishaw, 1995), similar protocols used to reveal subtle deficits in *Fmr1* null mice are highly unlikely to do same for rats. In an attempt to answer this question, we decide used a more challenging paradigm in watermaze which has not been used in *Fmr1* KO mice (Fig. 2.9). This one-trial spatial learning in a hippocampus- dependent DMP water maze task was also unaffected in *Fmr1* KO rats. These findings point to an intact flexible spatial learning in *Fmr1* KO rats. Dry land spatial working memory tasks have also failed to reveal a difference between genotypes (Supp. Fig. 1) (Asiminas et al., 2014). Additional experiments further probing spatial cognitive abilities in *Fmr1* KO rats are clearly needed. Maybe a more challenging reference and reversal protocol in the watermaze (1 trial/day) could reveal a deficit as it has been shown to be more sensitive previously (Nolan et al., 2004).

Interestingly, while spatial learning in watermaze tasks was found to be unaffected in *Fmr1* knockout rats, we found significant deficits in hippocampus-dependent associative

recognition memory, but not in other types of associative memory that do not require an intact hippocampus (Fig 4.10). Complete hippocampal lesions impair performance on the OPC recognition task but do not alter performance on OR, OP and OC (Langston & Wood, 2010). We find that adult *Fmr1* null rats are able to perform similarly to wildtype litter maters in the OR, OP and OC tasks, but not the OPC task. This episodic-like memory task that requires the hippocampus as well as intact prefrontal cortex-lateral entorhinal cortex connections (Chao et al., 2016) is the most complex of the object exploration tasks used here and requires binding of multiple associations to a coherent episodic representation. The fact that only OPC reveals a deficit might be simply because of its high complexity as a task. One other explanation lies on the fact that as a task requires a number of intact circuits working as a wide range network (perirhinal cortex-hippocampus-lateral entorhinal cortex-prefrontal cortex). It is plausible that subtle synaptic abnormalities in each of these brain structures "accumulate" causing greater disturbances in the wider network (Fig. 4.15).

Further to the episodic-like memory impairment, *Fmr1* knockout rats also exhibit spatial memory impairments in an object exploration task which examines allocentric spatial memory (OD) and depends on the hippocampus (Vogel-Ciernia & Wood, 2014). The fact that spatial memory deficits were not observed in any watermaze task but were observed in this spontaneous exploration task indicates that the differences between the very nature of the tasks can hide or a reveal an impairment. Spontaneous exploration tasks do not involve any training and they have a very naturalistic approach. Tasks like watermaze, in which good performance is a "life or death" situation push the system to compensate more efficiently. One could speculate that navigation tasks which involve training, direct the system's resources (rat brain) to focus on a certain "dimension" (i.e. find salient extra-maze cues). Object exploration tasks do not require any training; rats do not learn to expect any reward or any punishment. This approach does not put any pressure on the system to compensate to the same extent. Consistently with this idea Kentros and colleagues (2004) have shown that place cells in the CA1 area of hippocampus show increased place field stability when animals performed a navigational spatial task, compared to animals which were randomly foraging in the same apparatus. Since place cell stability has been associated with better memory (Dupret et al., 2010; Kentros et al., 1998) this supports the hypothesis that navigational spatial tasks like watermaze or T-maze may force the system to utilise mechanisms that could support this type of memory and potentially mask subtle circuit malfunctions.

Overall these data suggest that the loss of FMRP selectively affects a subset of hippocampus-dependent processes that include memory/binding of complex associations. Understanding how these differences arise will require a detailed analysis of the mechanisms by which cellular dysfunction affects neuronal circuit activity to ultimately control behaviour. Moreover, these differences highlight the fact that common cellular dysfunction across species can manifest in distinct behavioural phenotypes which may result from species ethological differences (Gerlai & Clayton, 1999).

### 4.5.2 Persistent cognitive deficits across background strains

One of the limitations of the mouse model has been the inconsistent and strain specific behavioural phenotype. Most behavioural studies on the mouse model of FXS have been conducted on a pure B6 or FVB strain (Kazdoba et al., 2014; Santos et al., 2014), though relatively recent studies have explored how behavioural phenotypes manifest in hybrid background strains (Corinne M. Spencer et al., 2011). Therefore, testing whether a rat model of FXS on a different background strain (LEH) expressed the same cognitive deficits was an obvious next step. Surprisingly, the two strains almost phenocopy each other in the spontaneous exploration tasks. These robust deficits in Object-place-context and Object-displacement memory, across rat strains, provide an assay against which potential therapeutics could be tested.

In summary, this study gives valuable insight into the deficits in episodic-like memory and spatial object memory associated with the loss of FMRP. Moreover, by demonstrating that the cellular pathophysiology associated with the loss of FMRP is shared between mice and rats (Till et al., 2015), we provide the foundation for interpretation of subsequent investigations of hippocampal function that utilises the biological and technical advantages rat models permit. As a result, rat-based disease models will like complement existing model organisms and together they could provide new insight into pathophysiology and behavioural manifestations of FMRP loss in humans.

# 5. *Fmr1* KO rats exhibit abnormal development of associative memory

## 5.1 Introduction

One of the main symptoms individuals with FXS display is pronounced developmental delays. Amongst the first clinical clues in children is delayed attainment of one or several developmental milestones; on average, boys with FXS can sit without support by the time they are 10 months of age and walk and talk at 20 months compared to 6-8 and 10-18 months for neurotypical children (Maes et al., 2000). Several studies have investigated the cognitive development in children and juveniles with FXS and revealed different profiles of delay for a wide range of behavioural traits, during different developmental stages. Starting from early childhood, although the rate of growth was found to be about half of that of typically developing children, boys with FXS progress normally in general cognitive and especially language development before age 7 (Bailey et al., 1998; Frolli, Piscopo, & Conson, 2014; Tonnsen et al., 2015). Interestingly Bailey and colleagues (2001) showed that ASD comorbidity severely slows down the development in all behavioural aspects examined (cognitive development, social development, language development, motor development). Studies looking at a broader age range normally reveal a decreased cognitive development during later childhood and adolescence. Gradually, affected children and adolescents display decreased improvement in raw scores on tests assessing cognitive function, compared to neurotypical peers (Hall et al., 2008), which subsequently translates to a decline in standardized IQ scores (Fisch, Simensen, & Schroer, 2002; Kover et al., 2013; Skinner et al., 2005). More specifically, children with FXS have been shown to display sharp decreases in IQ scores before ages 8 (Skinner et al., 2005), 10 (Dykens et al., 1989), and 11 (Fisch et al., 1996). These decreases have been shown to be followed by a potential stabilization in adolescence until at least age 14 (Skinner et al., 2005). Additionally, two longitudinal studies: one in a sample of children with FXS 3 to 11 years old (Cornish et al., 2013) and a second on children and early adolescents (6-16 years old) (Quintin et al., 2015), found greater improvements in working memory compared to attention skills. Therefore, these conflicting developmental trajectories of working memory and attention may be a signature of the cognitive developmental profile of children and adolescents with FXS.

Developmental delays and alterations during potentially critical developmental windows associated with the loss of FMRP have been studied using the *Fmr1* KO mouse model of FXS. A number of studies have reported a range of spine dysmorphologies or alterations in the pattern of synaptic protrusions and plasticity (Galvez & Greenough, 2005; Testa-Silva et al., 2012; Till et al., 2012; Wijetunge et al., 2014). These abnormalities are reported either during adulthood or more interestingly, around the second postnatal week, when both cortical and hippocampal areas undergo an extended synapse remodelling due to new functional circuit formation (Portera-Cailliau, 2012). Moreover, there are several reports of time-restricted changes in the thalamocortical circuit; namely increased NMDA to AMPA ratios and altered synaptic plasticity during the second postnatal week (Harlow et al., 2010). In addition to the deficits seen in the maturation of excitatory circuits, the development of local GABAergic inhibition is also delayed during the second postnatal week (Daw, Ashby, & Isaac, 2007) but it returns to wildtype levels by the start of the third (He et al., 2014). These phenotypes are in no way only a feature of this brain region; similar defects are displayed in other cortical and hippocampal regions (Meredith, Dawitz, & Kramvis, 2012; Pilpel et al., 2009), the amygdala (Vislay et al., 2013) and also in the other model organisms like the Drosophila model of Fragile X syndrome (Doll & Broadie, 2016; Gatto, 2009).

Besides the studies focussing on morphology and circuit function delays, there are a few focussing on biochemical/molecular evidence of a developmental abnormalities due to FMRP loss. For example, Lai, Doering and Foster (2016) examined the expression of neuroligins and neurexins in hippocampus and somatosensory cortex of *Fmr1* knockout mice. These transsynaptic proteins are key players in the maturation of two systems found to be affected by the loss of FMRP: glutamatergic (Bear et al., 2004) and GABAergic (Paluszkiewicz, Martin, & Huntsman, 2011). They observed a decrease in neurexin 3 expression in area CA1 of the hippocampus on two different postnatal days (P14 and P21) and in CA3 area only on P14. No statistically significant differences were observed for neuroligins. These changes might be linked to the delay in maturation of glutamatergic projections, seen in *Fmr1* knockout mice (Harlow et al., 2010). Moreover, the levels of certain important metabolites have been shown to be altered in the hippocampus of *Fmr1* knockouts (Gruss & Braun, 2001; Shi et al., 2012). More specifically, the levels of myo-inositol were found to be decreased on postnatal day 30 and taurine was increased in all ages tested (P18, 21, 30) (Shi et al., 2012). Taurine, which is an inhibitory amino acid derivative, is present in high concentrations in the developing

brain and decreases with age (Kulak et al., 2010). The observed increase in taurine level could therefore reflect a delay in the maturation of hippocampus. An alternative interpretation is that this difference is a part of a compensatory system mechanism, in response to excessive mGluR signalling; taurine has been shown to protect against the effects of glutamate excitotoxicity in vitro by stabilizing calcium concentration in the cytoplasm to basal levels (A El Idrissi & Trenkner, 1999). Direct comparison between the aforementioned studies can be very difficult due to differences in imaging or electrophysiological techniques used (Wijetunge et al., 2014), age, brain region, and statistical analyses (Nimchinsky et al., 2001). Nevertheless, these studies, indicate that the effects of FMRP ablation are widespread and affect brain development heavily.

What is obviously missing from the studies reviewed so far is a connection with behaviour, showing delays in related behavioural domains like memory (connection to hippocampus development deficits) or sensory integration (somatosensory cortex). The only two studies which have reported behavioural delays come from Yun and colleagues (2006) and Lai and colleagues (2014), who studied startle response and ultrasonic vocalisations (USVs) respectively. In the first study, researchers showed that *Fmr1* knockout mice showed a different developmental trajectory in their startle response, specifically after the 4$^{th}$ postnatal week. These results are somewhat consistent with abnormal developmental plasticity seen in primary auditory cortex of *Fmr1* knockouts (Kim et al., 2013). The second behavioural study focussed on pup USVs, which are considered a valid way to model communication deficits seen amongst individuals with FXS. Lai and colleagues (2014) used the maternal separation paradigm and recorded pup vocalisations in three different ages (P4, P7 and P10). They reported that *Fmr1* knockout pups showed an increased number of USVs compared to wildtype controls at P7 but not at P4 or P10. Moreover, *Fmr1* knockout mice displayed a developmental shift in the temporal distribution of vocalisations, with P10 mice calling in distinct patterns.

The lack of behavioural studies focussing on cognitive development in *Fmr1* knockout mice is evident, and there are a number of factors which have led to this. A plausible reason is that the model's unreliable cognitive phenotype (see 1.3) has discouraged researchers from attempting such laborious longitudinal studies. Another explanation lies within the very nature of the cognitive tasks routinely used in rodents; previous experience. Most of the widely used learning and memory tasks assessing cognition in rodents shape the behaviour of subjects drastically, even from the first exposure/testing

(i.e. watermaze, radial maze). Therefore, the performance profiles seen cannot be easily interpreted; improved performance on a subsequent time-point could be the effect of previous knowledge rather than a genuine behavioural recovery. Moreover, these strong, behaviour shaping experiences could abolish effects previously seen on physiological and biochemical studies. Of course the way around this stumbling block would be to conduct cross-sectional instead of longitudinal studies with the main limitation being the inability to observe the emergence of certain behaviours within the same group of subjects.

The spontaneous object exploration tasks previously described (see Chapter 2), rely on the innate preference of rodents for novelty, and do not require any training/conditioning which could drastically affect behaviour. The only parameter that could slightly affect performance is that repeated exposures to the testing apparatus and experimenter will lead to increased habituation. Recently Lyon and Langston (2014) reported that Lister Hooded rats display distinct developmental trajectories across the four object exploration tasks described in the previous chapter (NOR, OC, OP, OPC). Weaning rats, we able to perform above chance in the simplest of the four tasks (NOR), from the first time tested (P25). Object-context recognition was shown to come online at P34, and surprisingly rats developed the ability to remember both OP and OPC associations, acutely on P48.

Two of the advantages of rat over mouse model (Chapter 3) are their larger size and the fact that they can be handled much more easily. These are especially relevant to studies focussing on the cognitive development in weanling rats. Moreover, in a recent review article Semple and colleagues (2013) highlighted some of the brain development milestones and drew parallels between humans and rodents. After carefully reviewing existing literature, the authors suggested that despite the obvious enormous difference in the time scale of development between the species, the sequence of key milestones in brain development are generally quite consistent between humans and rodents.

Taken together, the advantages of spontaneous object exploration tasks in longitudinal studies, the advantages of rats as models of neurodevelopmental disorders and the similarities in brain developmental milestones between rats and humans, offer a great opportunity to study developmental trajectories in subjects that will allow a more dynamic account of how certain cognitive skills and deficits emerge in FXS.

The aim of this study is to assess the performance in the four spontaneous object exploration tasks throughout postnatal development, to examined whether *Fmr1* KO display normal developmental trajectories or exhibit any delays. Taking into account that these four tasks depend on different neuronal circuits, the exact pattern of delay would highlight circuit specific delays and give insights into brain areas which are mostly affected by the loss of FMRP.

## 5.2 Methods

### 5.2.1 Animals

Subjects were male Sprague-Dawley rats (wild-type {WT}, n=16; *Fmr1*-KO {KO}, n=16), bred in-house and kept in a 12h/12h light dark cycle. Colony founders were produced by Sigma Advanced Genetic Engineering (SAGE) Labs (St. Louis, MO, US). Ear biopsies for genotyping and identification purposes were collected on postnatal-day 14 (P14). Large litter sizes were adjusted between P10-P15 to approximately 8-10 pups in order to ensure as equal as possible maternal care during infancy (Masís-Calvo et al., 2013). Animals were weaned from their mother at (P21) (Fig. 5.1) and housed in mixed genotype cages with littermates, 4 animals per cage. Animals were provided with water, sawdust bedding and either a gnawing block of wood and/or a cardboard tube as environmental enrichment throughout the experiment. Ad libitum standard laboratory chow was provided throughout the experiment. Testing was always performed in during the light phase of the cycle. Prior to the start of the study, all experimental procedures were approved by the University of Edinburgh centenary services and complied with the Animal Care (Scientific Procedures) Act 1986.

### 5.2.2 Apparatus

Testing pparatus was identical to the one used in Chapter 4 for the four spontaneous exploration tasks previously described (Object {NOR}, Object-Context {OC}, Object-Place {OP} and Object-Place-Context recognition {OPC}) (Till et al., 2015). In brief; the apparatus consisted of a rectangular box with removable walls and floor inserts that could conform to two distinct contexts. Positions of objects were the same throughout the experiment. The box was placed on a table surrounded on 3 sides by a black fabric curtain, with one opening at the south side of the box (where subjects were placed). An

overhead light and yellow rectangular cue hanging for the black curtain were placed, (and stayed the same throughout testing), on the north west and northeast corners of the environment, serving as extra-maze cues. A variety of objects were used which had to be reasonably sized, non-porous, easily cleaned with wet wipes and not easily pushed over. Each object was only used once per animal. Objects were cleaned between trials with 70% ethanol solution and baby wipes.

### 5.2.3 Habituation/Testing

Staring from P18 (3 days before weaning), animals were handled daily in the animal house and experimental room for 5 days prior to experiments. Task-specific habituation occurred during the two days prior to testing (P23-24). On P23, the animals were habituated to both contexts in cage groups (30 minutes per context) in the morning and individually in the afternoon (10 minutes per context). Between exposures to contexts, rats were place in a holding bucket which was also used during testing. On P24, animals were habituated twice (morning and afternoon) individually but this time 2 different objects were placed in the positions in which subjects would encounter objects during testing (10 minutes per context, with objects); these objects were not used again during testing. Testing started on P25 and continued for 45 days (P71). Testing time points were 5-7 days apart except from the last two time points (P61 and P70) (Fig 5.1). In order to be able to access performance within restricted developmental time windows the testing battery described previously (Chapter 4) was compressed into a 2-day protocol for each time point. In the morning of the first day, subjects were tested in OR and during the afternoon in OC recognition. In the morning of the second day OP task took place and OPC in the afternoon (testing protocol modified from Lyon & Langston, 2014). All four tasks were carried out exactly as described before (Chapter 4).

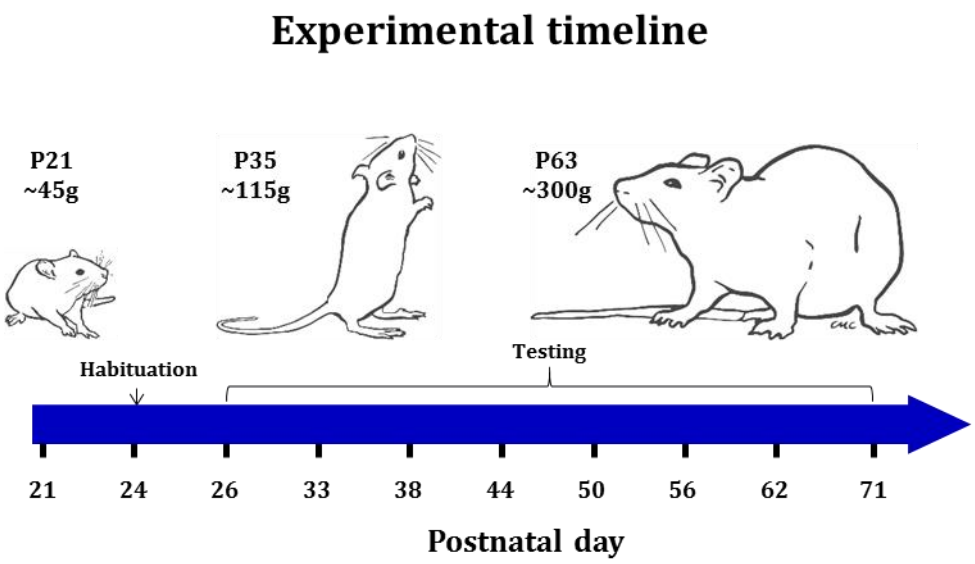



**Figure 5.1** This study used weanling rats and monitored their performance in spontaneous exploration tasks from postnatal day 26 until early adulthood (P71) to explore developmental delays in the model of FXS (Cartoons of rats taken from McCutcheon &Marinelli 2009).

### 5.2.4 Statistical analysis

As descried previously, a Discrimination Index DI [(time exploring novel object—time exploring familiar object)/(time exploring both objects)] was calculated for each test phase. The only difference was that the criterion for data exclusion was less stringent than previously since the repeated testing of (4 tasks in 2 days) had a slight impact on the subjects' interest for objects. For this experiment, trials in which a subject did not reach 15 sec of total object exploration in both the sample and the test phases and at least 5 sec of exploration for each object in the sample phase, were excluded from the analysis. This criterion is stringent enough to exclude animals who were not interested, but to include animals that explored for an adequate amount of time overall and had experienced both objects. Groups' performance in all testing time points was compared to chance levels (unless stated otherwise), and false discovery rate during multiple comparisons against chance levels, was corrected using the Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995) with the false recovery rate set to 0.05. Statistical analysis was done using IBM SPSS Statistics 22.0 and GraphPad Prism 6. All graphs were produced in GraphPad Prism 6.

## 5.3 Results

### 5.3.1 *Fmr1* KO rats show significant memory for objects throughout development

Both groups displayed significant object novelty preference in the NOR task in all testing time points used in this study (P25-P70) (Fig 5.2 A&B, Table 5.1&5.2). These results indicate that rats as young as 25-days-old can learn object identities and retrieve this information after a short 2min retention interval. Compared to adult testing were subjects were tested in each task twice and the average value was used for analysis, each time point of this study has only one testing session for each task. This difference can explain the increased variability observed across time points. Analysis of exploration times during both sample phase (Supp. Fig. 2A) and test phase (time $F_{(7,210)}$ =12.41, p <0.001; genotype $F_{(1,30)}$ =1.85, p =0.18; genotype × training day $F_{(7.210)}$ =0.33, p =0.94; Fig. 3.2C) revealed that even though exploration levels are fluctuating during the course of the experiment, both groups explore objects at a similar level.

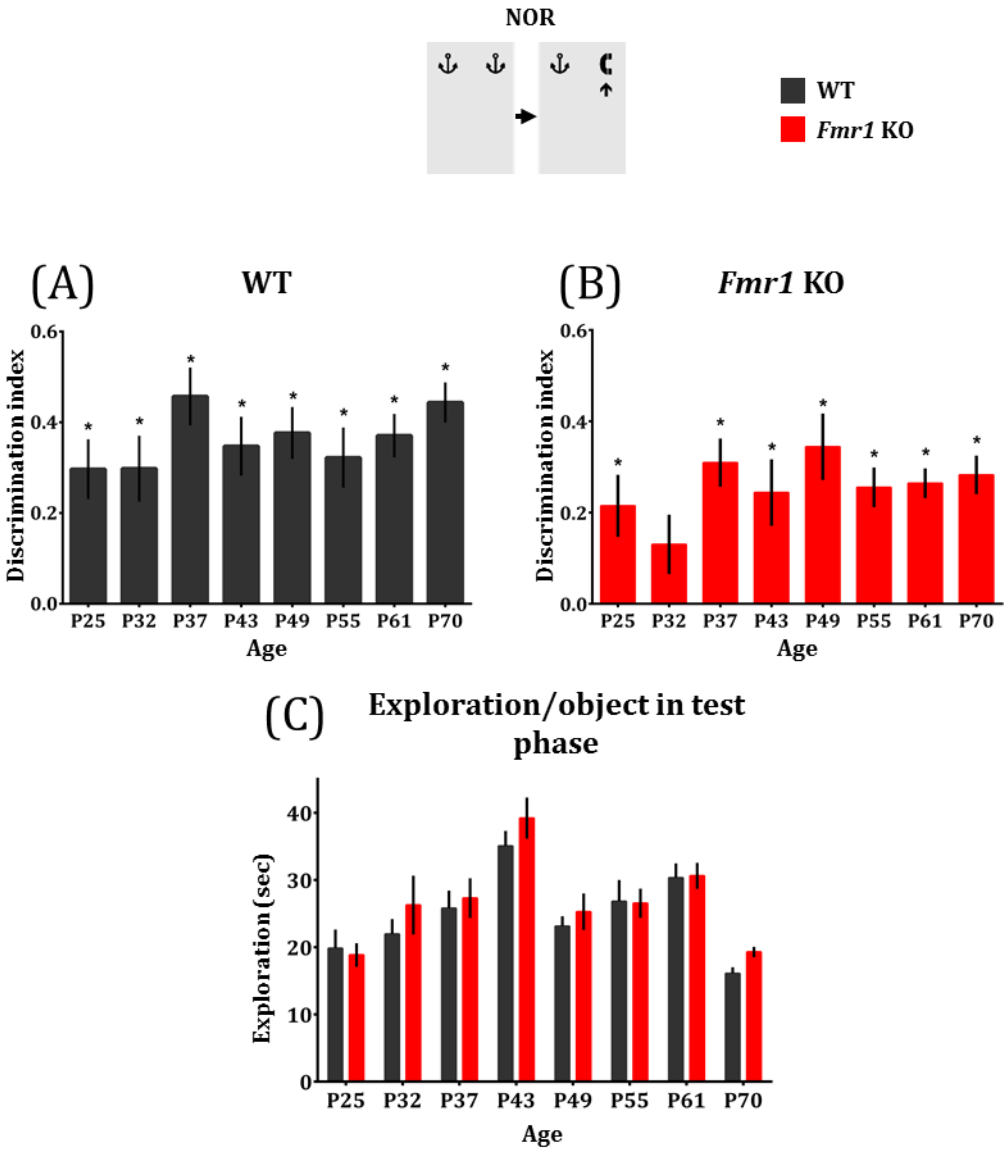



**Figure 5.2 Intact object memory in *Fmr1* KO rats.** Both wildtype **(A)** and *Fmr1* KO rats **(B)** can distinguish novel from familiar objects from the first time-point of testing. **(C)** Exploration time between the groups was similar throughout the experiment. * $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure.

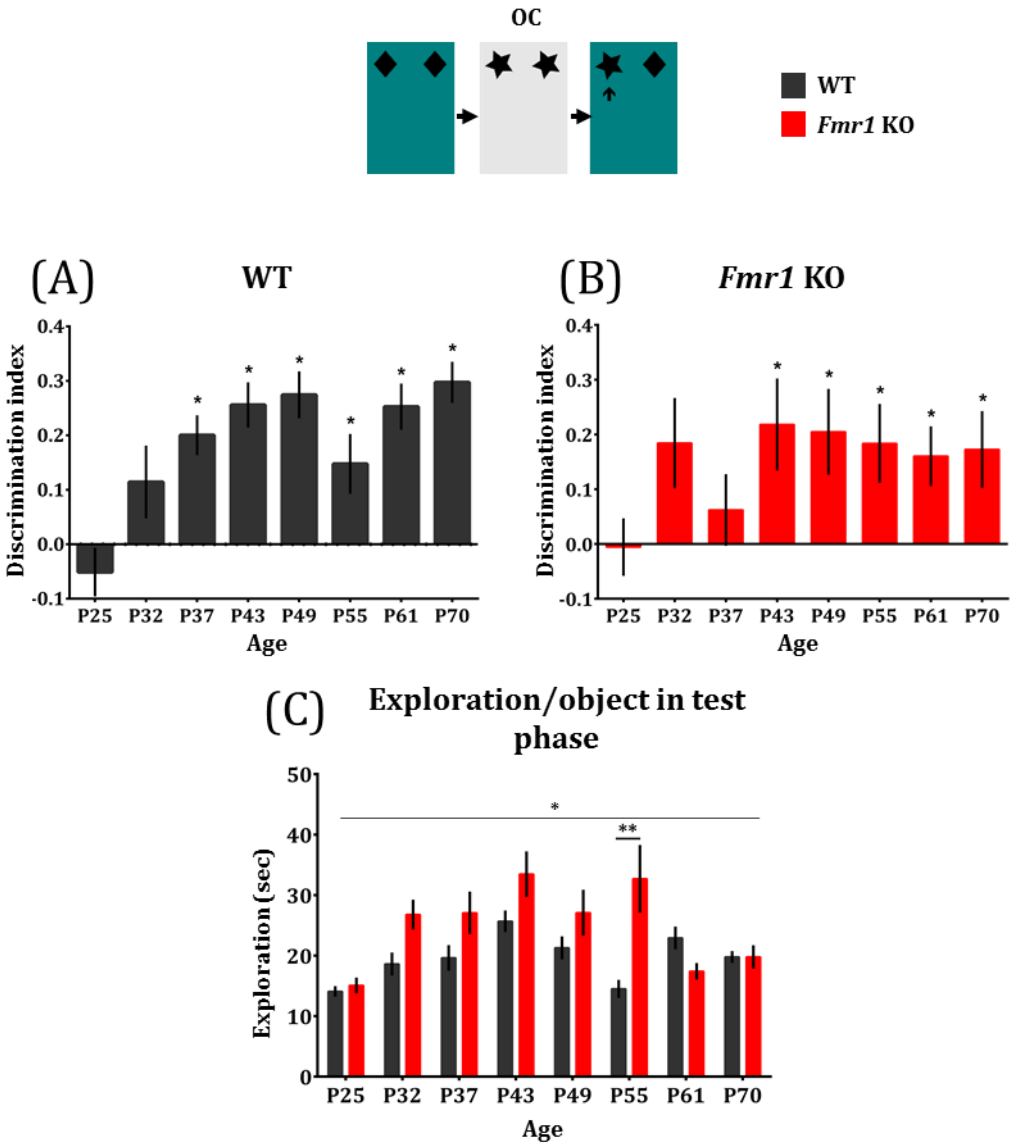



**Figure 5.3 Developmental trajectory of object-context memory is largely unaffected by FMRP loss.** Wildtype rats can distinguish novel from familiar object-context associations from P37 onwards **(A)** while *Fmr1* KO rats **(B)** show a very similar developmental profile, performing above chance from P43 onwards. **(C)** Overall Fmr1 KO rats did explore the objects more but post hoc analysis revealed that the only time point with significant difference between groups was P55. For **(A)** and **(B)*** $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure. For **(C)** * $p<0.05$ difference between groups

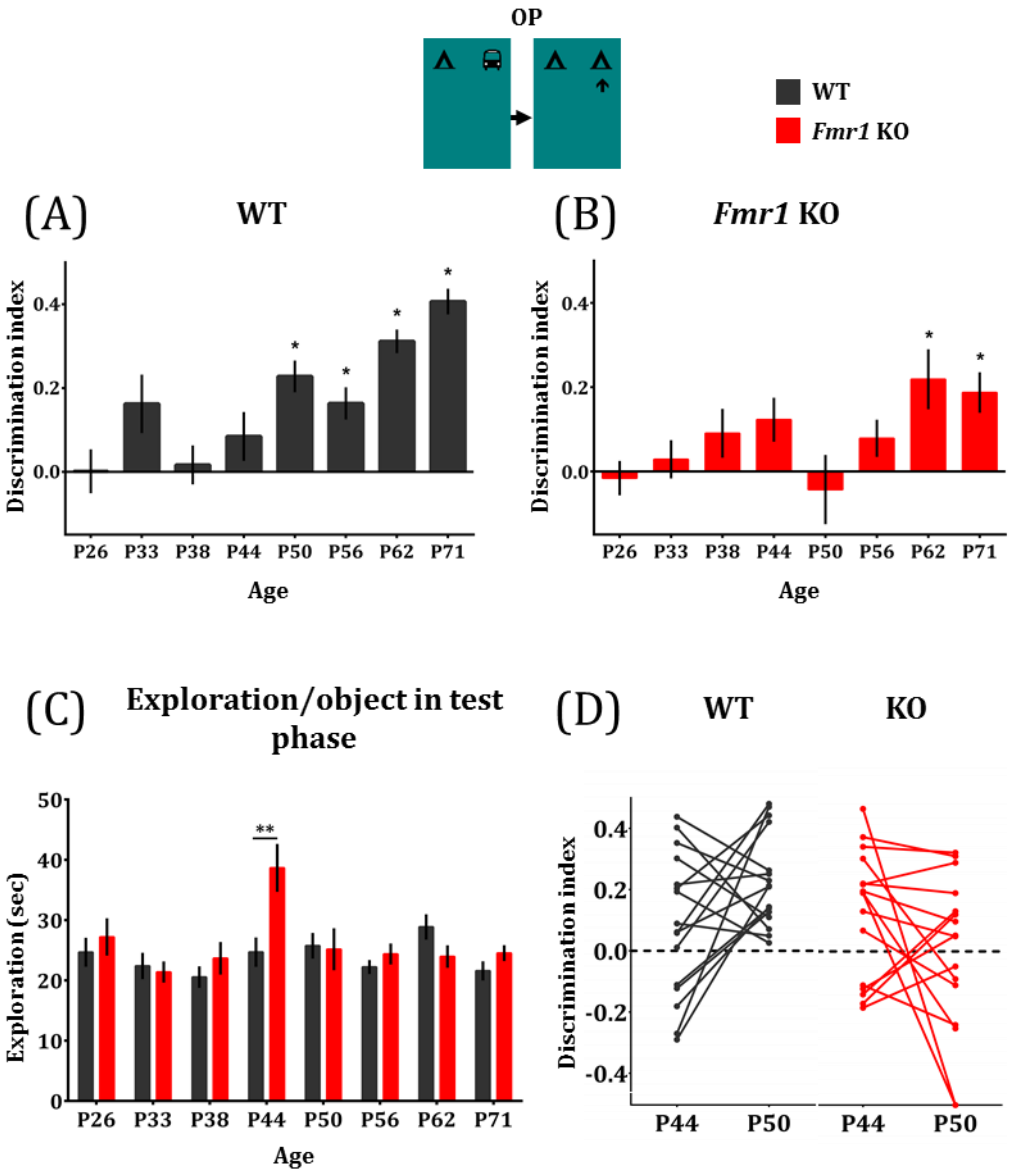



**Figure 5.4 *Fmr1* KO rats display a delay in the developmental trajectory of object-place associative memory.** WT subjects develop the ability to remember object-place associations on postnatal day 50 **(A)**. *Fmr1* KO rats start perform significantly above chance almost 2 weeks after (P62) **(B)**. Exploration time between the groups was similar throughout the experiment except from testing on postnatal day 56 when Fmr1 KO rats showed a significantly increase exploration compared to littermate controls **(C)**. Focussing on the two testing points before and after the emergence of OP **(D)**. For **(A)** and **(B)*** $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure. For **(C)** and **(D)** * $p<0.05$ difference between groups

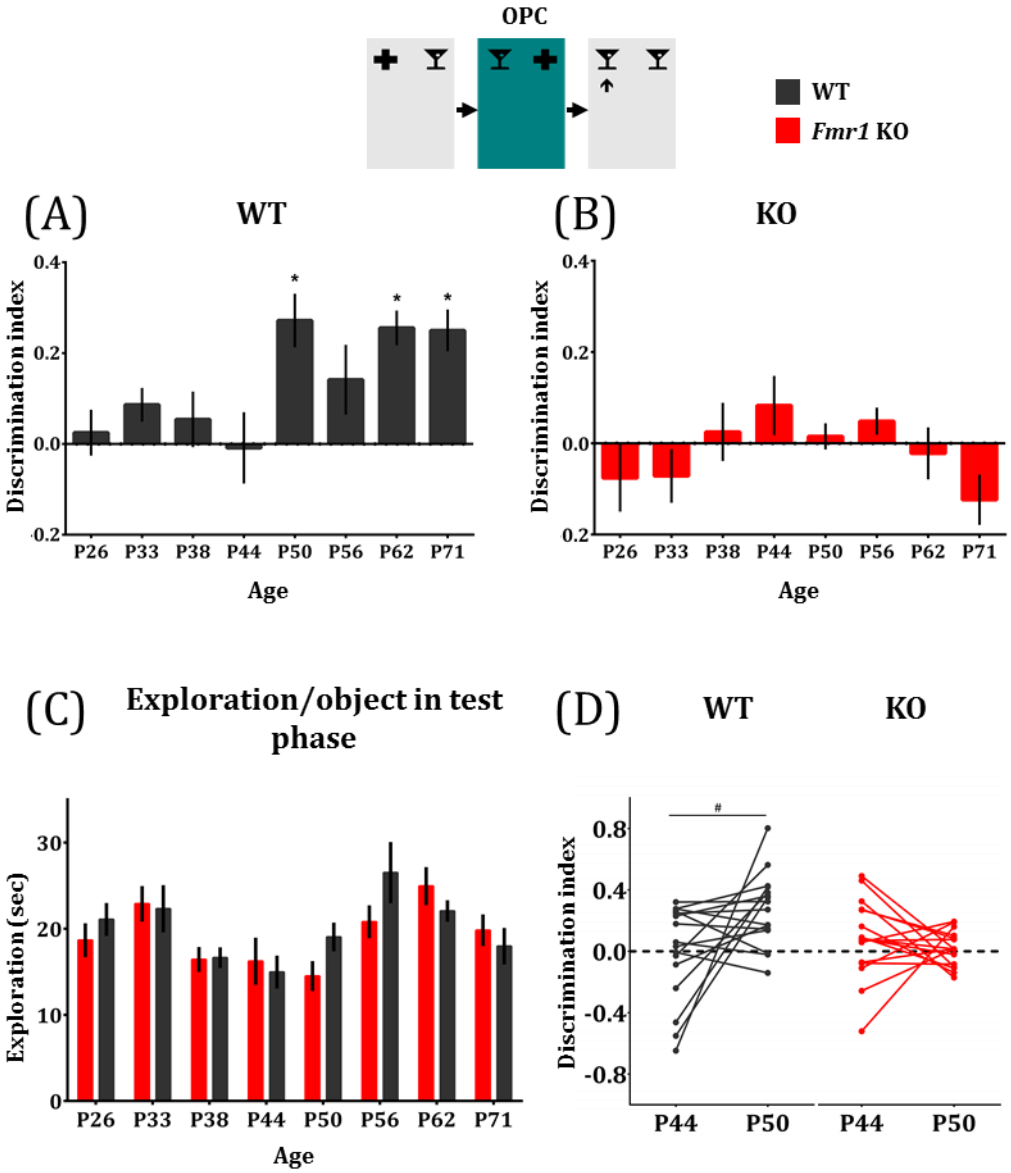



**Figure 5.5 *Fmr1* knockout rats are unable to discriminate novel from familiar object-place-context associations. (A)** WT rats show a developmental profile of performance similar to OP task, with OPC emerging at postnatal day 50. In contrast, *Fmr1* KO rats' ability to discriminate novel from familiar object-place-context (episodic-like) associations does not develop **(B)**. Exploration time between the groups was similar throughout the experiment **(C)**. Focussing on the two testing points before and after the emergence of OPC **(D)**. For **(A)** and **(B)*** $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure. For **(C)** and **(D)** * $p<0.05$ difference between groups

### 5.3.2 *Fmr1* KO rats show similar object-context memory developmental profile to wildtype littermates

The developmental trajectory of object-context associative memory seems to be largely unaffected by FMRP loss (Fig 5.3) Even though *Fmr1* KO rats display a slightly delayed trajectory (Fig 5.3B) compared to wildtype littermates (Fig. 5.3A) we cannot be certain about the precise developmental trajectory due to high variability. The ontogenetic emergence of object-context associative memory seems to happen sometime between P32 and P37 (Table 5.1&5.2). Analysis of exploration times during test phase revealed significant fluctuation (time $F_{(7,210)}$ =6.77, p <0.001) across different time points; moreover *Fmr1* KO rats were found to explore more than their wildtype littermates (genotype $F_{(1,30)}$ =7.36, p =0.01; genotype × training day $F_{(7.210)}$ =4.54, p <0.001; Fig. 5.3C) but *post hoc* t-tests revealed that the only time point when there was a group difference was at P55 (P <0.001). Analysis of exploration times during sample phase revealed no genotype effect (Supp. Fig. 2 B).

### 5.3.3 *Fmr1* KO rats display a delay in the developmental trajectory of object-place associative memory

Object-place associative memory was found to be significantly delayed for *Fmr1* KO rats (Fig. 5.4). Wildtype rats can perform significantly better than chance from P50 onwards (Fig. 5.4A, Table 5.1) whereas *Fmr1* Kos perform better than chance only from P62 (Fig. 5.4B, Table 5.2). This significant delay could be interpreted as a delay in the development of plasticity mechanisms supporting this type of memory. The ability of the subjects to discriminate between novel and familiar object-place associations seems to develop between P44 and P50 in wildtype controls. This finding contradicts previous work by Ainge and Langston (2012) who found that juvenile rats are able to remember this type of associations as early as P30. However more recently Lyons and Langston showed a surprisingly similar developmental trajectory in Lister Hooded rats, with OP coming online at P48 (Lyon & Langston, 2014). Focusing on the time points before and after the ontogenetic emergence of OP (P44 & P50) gives us a better idea of the abruptness by which this type of memory emerges (Fig. 5.4D). Even though there is not a significant difference between time for either WT ($t_{15}$ =1.91, p =0.08) or *Fmr1* KOs ($t_{15}$ =1.57, p =0.14), analysis of the individual subjects showed that 10 out of 16 WT rats (62.5%) increased performance between the two testing points whereas only 5 out of 16 (31.3%)

*Fmr1* KO rats did the same. Exploration times during test phase did fluctuate across different time points (time $F_{(7,210)}$ =3.77, p <0.001), and no differences were detected between groups (genotype $F_{(1,30)}$ =3.49, p =0.07). On the other hand, the interaction between the groups and time was found to be significant (genotype × training day $F_{(7.210)}$ =2.72, p=0.01; Fig. 3.4C) but *post hoc* 1-sample t-tests revealed that the only time point when there was a group difference was at P56 (p <0.001). Moreover, analysis of exploration times during sample phase revealed no genotype effect (Supp. Fig. 2 C).

### 5.3.4 *Fmr1* KO rats are unable to discriminate novel from familiar object-place-context associations

Analysis of the developmental trajectory of object-place-context memory confirmed our previous findings (Till et al., 2015; Chapter 4) that *Fmr1* KO rats are unable to remember complex object-place-context associations, thus *Fmr1* KOs are not able to perform significantly better than chance levels at any time point (Fig. 5.5B, Table 5.2). Interestingly, the normal emergence of OPC task is identical (at least or the testing times we used) with OP task (Fig. 5.5A, Table 5.1). Focussing on the testing times before and after the ontogenetic emergence of OPC (P44 & P50), in order to get a closer look at the changes on a single subject level, reveals that 11 out of 16 (68.8%) WT rats improved their performance between the two testing points whereas there were only 4 out of 16 (25%) *Fmr1* rats with the same change (Fig. 5.5D). Moreover, the performance of WT rats on P44 was significantly worse than on P50 (p =0.03, paired t-test) and as expected no significant difference was detected for *Fmr1* KOs. Analysis of exploration times during both sample phase (Supp. Fig. 2D) and test phase (time $F_{(7,210)}$ =4.85, p <0.001; genotype $F_{(1,30)}$ =0.56, p =0.46; genotype × training day $F_{(7.210)}$ =1.1, p =0.36; Fig. 3.5C) revealed that even though exploration levels are fluctuating during the course of the experiment, both groups explore objects at a similar level.

| Time-point | Significance | NOR | OC | OP | OPC |
|---|---|---|---|---|---|
| P25&26 | Uncorrected p | <0.001 | >0.05 | >0.05 | >0.05 |
| | Bonferroni | ** | | | |
| | B&H | * | | | |
| P32&33 | Uncorrected | <0.001 | >0.05 | <0.05 | <0.05 |
| | Bonferroni | ** | | | |
| | B&H | * | | | |
| P37&38 | Uncorrected | <0.001 | <0.001 | >0.05 | >0.05 |
| | Bonferroni | *** | *** | | |
| | B&H | * | * | | |
| P43&44 | Uncorrected | <0.001 | <0.001 | >0.05 | >0.05 |
| | Bonferroni | *** | *** | | |
| | B&H | * | * | | |
| P49&50 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.001 |
| | Bonferroni | *** | *** | *** | ** |
| | B&H | * | * | * | * |
| P55&56 | Uncorrected | <0.001 | <0.05 | <0.001 | >0.05 |
| | Bonferroni | ** | | ** | |
| | B&H | * | * | * | |
| P61&62 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.001 |
| | Bonferroni | *** | *** | *** | *** |
| | B&H | * | * | * | * |
| P70&71 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.001 |
| | Bonferroni | *** | *** | *** | ** |
| | B&H | * | * | * | * |

**Table 5.1 Development statistical overview for WT rats.** * p<0.05 for Bonferroni correction for multiple comparisons; * indicates significance from chance levels of discrimination after controlling the false discovery rate using the Benjamini-Hochberg procedure (B&H)

| Time-point | Significance | NOR | OC | OP | OPC |
|---|---|---|---|---|---|
| P25&26 | Uncorrected p | <0.01 | >0.05 | >0.05 | >0.05 |
| | Bonferroni | | | | |
| | B&H | * | | | |
| P32&33 | Uncorrected | >0.05 | <0.05 | >0.05 | >0.05 |
| | Bonferroni | | | | |
| | B&H | | | | |
| P37&38 | Uncorrected | <0.001 | >0.05 | >0.05 | >0.05 |
| | Bonferroni | *** | | | |
| | B&H | * | | | |
| P43&44 | Uncorrected | <0.01 | <0.05 | <0.05 | >0.05 |
| | Bonferroni | * | | | |
| | B&H | * | * | | |
| P49&50 | Uncorrected | <0.001 | <0.05 | >0.05 | >0.05 |
| | Bonferroni | ** | | | |
| | B&H | * | * | | |
| P55&56 | Uncorrected | <0.001 | <0.05 | >0.05 | >0.05 |
| | Bonferroni | *** | | | |
| | B&H | * | * | | |
| P61&62 | Uncorrected | <0.001 | <0.05 | <0.01 | >0.05 |
| | Bonferroni | *** | | | |
| | B&H | * | * | * | |
| P70&71 | Uncorrected | <0.001 | <0.05 | <0.01 | <0.05 |
| | Bonferroni | *** | | * | |
| | B&H | * | * | * | |

**Table 5.2 Development statistical overview for *Fmr1* KO rats.** * p<0.05 for Bonferroni correction for multiple comparisons; * indicates significance from chance levels of discrimination after controlling the false discovery rate using the Benjamini-Hochberg procedure (B&H)

## 5.4 Discussion

The disussion of the findings in this study can focus on two different directions; the developmental trajectory of object recognition and associative memory in wildtype rats and second the developmental delays and deficits related to FMRP loss.

### 5.4.1 Distinct developmental trajectories in associative memory of wildtype rats

In this study we investigated the ontogeny of associative and non-associative object recognition memory in wildtype and *Fmr1* KO juvenile rats. We showed that rats can remember novel objects form the same time tested (P25) all the way to adulthood. Moreover, we found age-related improvements in memory for, object-context, object-place and object-place-context associations, and we observed that these age-related progression differs as a function of the type of association (Fig 5.6). Consistent with the complexity of the tasks used, different age-related performance was observed, implicating the development of processes facilitating binding operations to account, at least in part, for the development of associative memory in juvenile rats (McCutcheon & Marinelli, 2009). Both wildtype and *Fmr1* KO weanling rats were able to recognise object identities from the earliest time point tested (P25) (Fig. 5.2). This result is fundamental in the interpretation of findings from more complex types of memory involving objects. Being able to remember object identities throughout the experimental time points suggests that the different developmental trajectories observed for the other 3 tasks are not due to inability to distinguish novel from familiar objects.

Consistently with previous findings by Lyn and Langston (Lyon & Langston, 2014), in our study OC recognition appears to ontogenetically emerge between P32 and P37 in wildtype rats. Contrary to this finding, Ramsaran and colleagues (2016) found that juvenile rats can remember object context associations from P17 onwards. This could be an effect of their experimental design, since they only used a specific object pair of objects, whereas in our experiments a variety of object pairs was used. Interestingly, contextual learning using a contextual fear conditioning paradigm has been shown to develop between P17 and P23 (Foster & Burman, 2010). Although the fact that fear conditioning paradigms were used in these studies, makes the comparison with non-aversive spontaneous exploration task difficult, it is possible that the late development,

seen in our OC test, could be due to the need of binding two non-aversive relevant pecies of information. Two recent reports suggest that postrhinal (POR) and lateral entorhinal (LEC) cortices are key neural substrates for object-context associative memory (Wilson et al., 2013a; Wilson, et al., 2013b); thus, these findings would appear to support the idea that POR and LEC development, or more importantly the crosstalk between two areas, may be the bottleneck in the emergence of object-context memory.

To our knowledge, this is one of the first demonstrations of a developmental trajectory of OP and OPC memory in juvenile rats. Based on the time-points tested, OP and OPC recognition both develop sometime between P44 and P50 in wildtype rats. Further studies are required to pin down the exact age these two tasks develop; Lyon and Langston (2014 and Lyon personal communication) have already shown that those two tasks seem to develop acutely and simultaneously on P48. OP associative memory has been shown to involve LEC (Wilson, et al., 2013a), prefrontal cortex (Chao et al., 2016) and is likely to involve perirhinal (PER) cortex which is an important brain area for object memory (Norman & Eacott, 2005), whereas it doesn't require an intact hippocampus (Langston & Wood, 2010). OPC associative memory has been shown to involve PER and LEC, prefrontal cortex (PFC) (Chao et al., 2016) and hippocampus (Langston & Wood, 2010). Therefore, it is unlikely that hippocampus postnatal development is dictating the emergence of these two types of associative memory. After all, hippocampus has developed almost to its adult form by P21; plasticity mechanisms are in place, stable place fields have emerged and other hippocampus dependent tasks, like object displacement task, are online (Bayer, 1980; Bekenstein & Lothman, 1991a, 1991b; Langston et al., 2010; Westbrook, Brennan, & Stanton, 2014). Nevertheless, additional complexity in dendritic morphology and overall neuron structure continues to improve; density of dendritic spines reaches a maximum on P24 in the stratum moleculare and on P48 in stratum lacunosum (Pokorný & Yamamoto, 1981).

Based on our behavioural data and what is known about the postnatal hippocampus development, we can hypothesise that: (1) OPC emergence is probably dictated by the object-place component of the task and (2) and that prefrontal cortex postnatal development timeline is the reason of the late onset of these types of associative memory. But what is known for the postnatal development of PFC? In contrast to the postnatal development of hippocampus, PFC circuits appear to develop later in life, in a timeline consistent with our findings, and continue to mature until early adulthood.

Piontkewitz and colleagues found a dramatic increase in PFC volume between P35 and P46 (Piontkewitz, Arad, & Weiner, 2011) and a subsequent mild decrease between P56-90, consistent with synaptic pruning during adolescence (Andersen, 2003). Moreover, there is a sharp increase in trypsin/EDTA resistant cortical synapses between P35 and P49, indicating increase structural stabilisation (Khaing et al 2006). These findings fully agree with the observed ontogenetic development of OP and OPC recognition (Lyon and Langston 2014). Further to this volumetric study, the functional maturation of PFC has been suggested to occur between P40 and P60. A study looking at the development of brain areas underlying foraging behaviour in rats found that prefrontal cortex is not critically involved in food and water regulation until nearly 60 days of age (Kolb & Nonneman, 1976). More detailed analysis on the development of innervation of PFC revealed that dopamine D1 receptors, mainly expressed by pyramidal cells in cortical layers III and V, continue to increase in number after P35 until early adulthood (P60), while D2 and D4 reach adult levels of density from P35 (Tarazi & Baldessarini, 2000). In contrast, GABAergic neurons appear to complete their postnatal maturation by the fourth postnatal week but the slower progression in the development of dopaminergic system dictates the maturation in interactions between pyramidal cells and GABAergic interneurons (Benes, Taylor, & Cunningham, 2000). Normal expression and development of dopamine receptors could significantly influence maturation of synaptic structures, function and behaviour, and may play an important role in the organization and connectivity of cortical and hippocampal brain systems. Consistently with the hypothesis that the development of prefrontal dopaminergic system supports OP and OPC associative memory, a recent study showed that infusion of D1/D5 receptor antagonists in prefrontal cortex, but not hippocampus or perirhinal cortex, leads to impairments in an associative spatial object memory task (Savalli, Bashir, & Warburton, 2015).

The abruptness by which OP and OPC emerge should not be a surprise. Other types of memory show the same sudden manifestation, indicating the importance of finely tuned circuits before a behaviour appears (Languille, Richer, & Hars, 2010; Westbrook et al., 2014). In contrast to this hypothesis, studies have shown that even though spatial memory is in place just before P18 it certainly continues to improve (Adams & Jones, 1984). Thus, it is possible that behaviours (types of memory in our case) which rely heavily on a single structure could emerge gradually and be refined in the process (spatial memory-hippocampus). This hypothesis is supported by studies looking at the development of spatial representation system ( Langston et al., 2010; reviewed by Ainge

& Langston, 2012). On the other hand, behaviours which require intact wide range circuits involving multiple brain areas, are more likely to show abrupt developmental emergence, simply because the circuits supporting these complex behaviours have to be fully refined. Thus, it is plausible that even though the hippocampus is functional relatively early in postnatal development, its ability to cooperate with other brain areas and integrate information from different memory and sensory systems emerges later and in a more gradual fashion depending on the neural circuits involved. This could explain why OPC recognition, which requires multiple brain areas including the hippocampus, emerges late in postnatal development.

### 5.4.2 *Fmr1* knockout rats display abnormal development of associative memory

The second major finding of this study is that *Fmr1* KO rats display delays in emergence of OP and performed no better than chance at any time point on OPC. This finding is consistent with cognitive delays seen in individuals with FXS (Bailey et al., 2001; Maes et al., 2000). While OR and OC memory development shows very similar profile between wildtype and knockout rats, differences are observed in OP and OPC. OP associative memory emergence appears to be delayed for 6-12 days and OPC memory was not observed at any time point, which is consistent with previously presented data from adult subjects (Chapter 4) (Till et al., 2015).

The vast majority of studies looking at rodent postnatal brain development have focused on the first four postnatal weeks, during which major changes take place, leading to the emergence of most basic behaviours (Hartung et al., 2016; Meredith, 2015; Sugar & Witter, 2016). The same lack of reports focussing on developmental events between the sixth and seventh postnatal week is also evident for the mouse model of FXS. Nevertheless, a handful of studies have yielded results which could be considered consistent with the idea that fully functional hippocampus, PFC and LEC are important for OP and OPC recognition. Moreover, knowledge based on adult *Fmr1* knockout mice could provide some hints on the processes which have gone awry due to FMRP loss. For example, Calabrese and colleagues (2013) have shown, using magnetic resonance histology, that hippocampus reaches its plateau volume around P50 while frontal cortex around P42. This is largely consistent with the trajectories we see in OP and OPC. Moreover, it has been shown that interneurons in the rat prefrontal cortex undergo a dramatic reduction in NMDA/AMPA ratio, a determinant of synaptic plasticity, just

before the emergence of OP and OPC (P31-P49) (H.-X. X. Wang & Gao, 2009). This could be relevant to FXS, since several studies have shown that NMDA/AMPA ratios are generally lower in cortex (Gocel & Larson, 2012; Harlow et al., 2010; Martin et al., 2015) and hippocampus (Yun & Trommer, 2011); moreover, interneurons in other cortical areas display robust deficits in excitatory drive (Gibson et al., 2008). Studies focussing on biochemical biomarkers during postnatal development have also revealed some interesting changes supporting our hypothesis and might explain the differences between *Fmr1* knockouts and wildtype littermates. Counotte and colleagues (2010) examined the synaptic proteome in rat prefrontal cortex, and found that it undergoes vast changes between P44 and P78. Keeping in mind that FMRP is a translation regulator/suppressor, controlling various genes important for synaptic function (Darnell et al., 2011), it is obvious that these natural changes in synapse proteome during sensitive developmental periods are bound to be severely affected by the loss of FMRP, leading to permanent circuit dysfunction. Lastly, the dopamine-mediated neuromodulation in prefrontal cortex of adult *Fmr1* KO mice has been shown to be decreased (Paul, Venkitaramani, & Cox, 2013; Ventura et al., 2004). It is plausible that this dysregulation could account for these observed deficits.

### 5.4.3 Looking forward

Cognitive processes are generally supported by finely tuned interactions within large-scale neuronal networks. Performance in different cognitive tasks is obviously not inherited, but it progressively matures in parallel with the formation of functional circuits and long-range coupling in the developing brain. Overall the results of this study replicate the behavioural deficits of *Fmr1* knockout rats presented in Chapter 2 (Fig 5.7) confirming the robustness of this cognitive deficit. This study went a step forward; the use of this longitudinal paradigm revealed a delay in the ability to form OP associative memory which could not have been explored otherwise. This suggests that our approach can definitely expand our understanding of the pathophysiology associated with FXS but also other neurodevelopmental disorders. It would be interesting to see if other behaviours which appear to be unaffected during adulthood, have an abnormal developmental trajectory. Of course longitudinal studies will not be possible for a number of tasks (i.e. watermaze tasks) in which case a cross-sectional experimental design should be followed (McCutcheon & Marinelli, 2009).

Different trajectories between different types of associative memory found in this study agree with studies in children, which have also shown different developmental profiles for item-item or item-order etc. (Lee et al., 2015); this offers additional face validity to our rat model but also validates this behavioural assay. Focussing only on the developmental profile of wildtype rats, we can easily see that there is a relatively long time period (P25-P50) during which circuits supporting complex types of associative memory (OP and OPC) undergo extensive reconstruction and improvement. It is possible that pharmaceutical interventions during these period of elevated plasticity, could maximise the therapeutic effects (Andersen, 2003; Meredith et al., 2012). Therefore, these behavioural tasks could serve as powerful functional assay to help us examine the efficacy of target-based therapeutics not only in reversing established circuit and behavioural deficits but more importantly preventing their emergence.

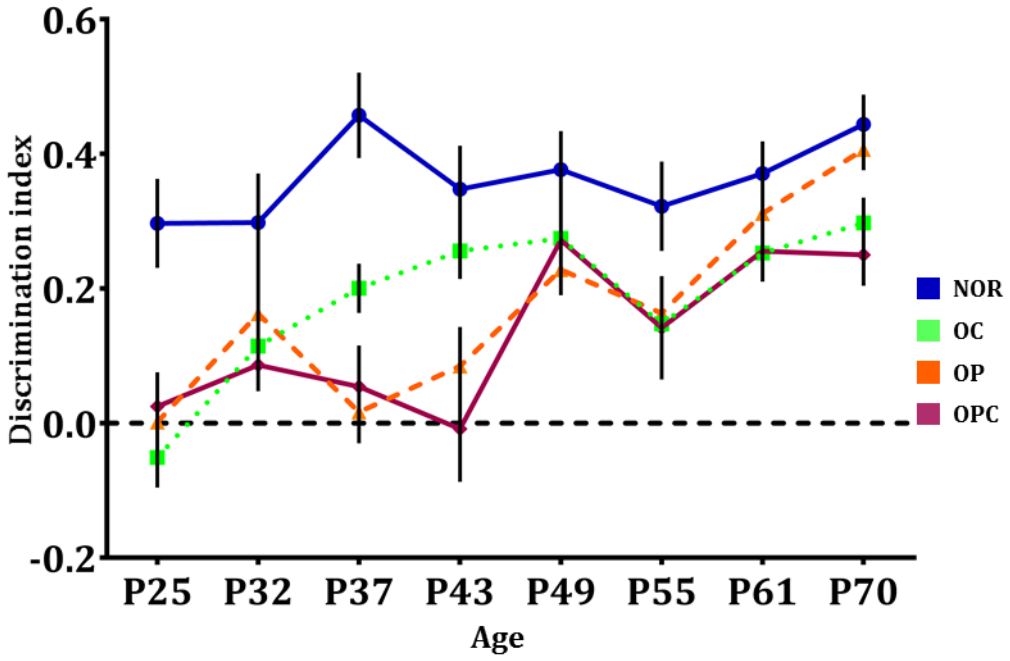



**Figure 5.6 Distinct developmental trajectory in object memory tasks for WT rats.** Significant preference for novelty appears first for NOR, then OC, and finally OP and OPC. (time: $F_{(7,420)}$ =11.31, P <0.001; task: $F_{(3,60)}$ =32.91, P <0.001; Time x task: $F_{(21,420)}$ =1.86, P =0.01).

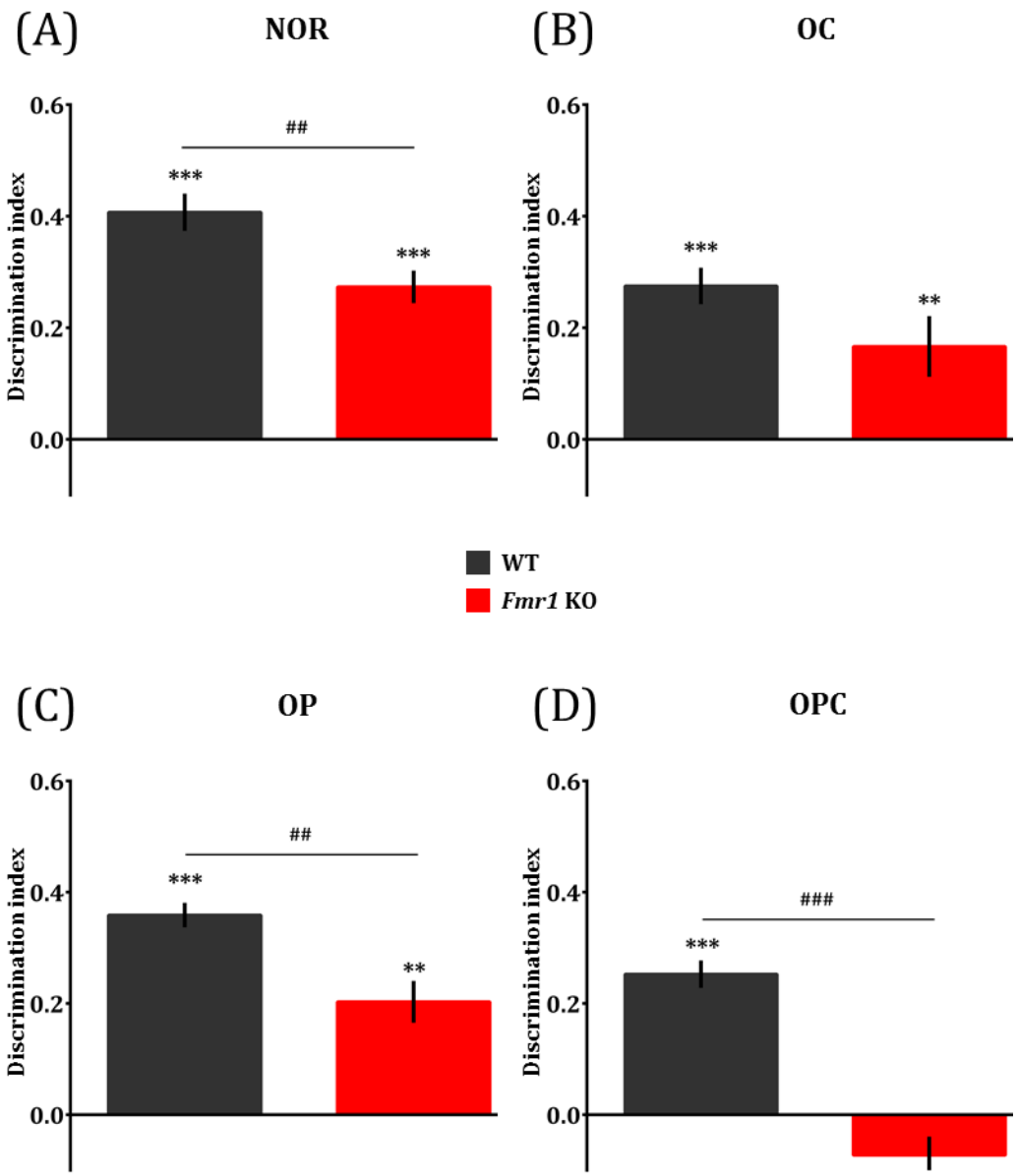



**Figure 5.7 *Fmr1* KO rats are unable to form coherent object-place-context memory associations.** Average data across the last two time-points (P61&P70) confirm previous findings in adult rats. Despite a diminished preference for novelty in NOR and OP tasks compared to their wildtype littermates, *Fmr1* KO rats are able to remember object identities **(A)**, object-context **(B)** and object-place associations **(C)**, but their ability to remember episodic-like memories **(D)** is severely impaired. * $p<0.05$ difference from chance (DI =0) # $p<0.05$ difference between genotypes.

# 6. Lovastatin treatment early in life restores cognitive development in *Fmr1* KO rats

## 6.1 Introduction

Over the last decade, multiple studies have reported that it is possible to reverse symptomatology associated with neurodevelopmental disorders in model organisms (primarily mouse models) during adulthood. The approaches utilised pharmacological or genetic rescue strategies in adulthood in different mouse models for neurodevelopmental disorders including Down syndrome (Fernandez et al., 2007), tuberous sclerosis (Ehninger et al., 2008), Rett syndrome (Guy et al., 2007) and Fragile X syndrome (Dölen et al., 2007; Osterweil et al., 2013b). Although collectively, these studies have reported correction of a wide range of phenotypic anomalies in the mouse models used, individually, the overwhelming majority of studies have reported rescue of selected physiological and behavioural deficits, with some key aspects of pathophysiology remaining unaffected. Due to the nature of these disorders, the timing of intervention could be a vital parameter to the efficacy of a given treatment; therefore, early intervention approaches in these mouse models, are currently gaining attention. For example, acute dosing with appropriate drug in new-born TS65Dn mice (a model for Down syndrome) is adequate to correct some pathophysiological and behavioural phenotypes during adulthood (Das et al., 2013). However, as mentioned earlier the therapeutic effects were somewhat limited, leaving other cerebellar pathological phenotypes unaffected (Gutierrez-Castellanos et al., 2013).

An alternative strategy to target processes during early critical periods in brain neurodevelopment, is treatment late prenatally. For example, administration of bumetanide ($Na^+$-$K^+$-$2Cl^-$ co-transporter antagonist), 24h before delivery, to pregnant *Fmr1* Het female mice and to VPA pre-treated pregnant rats (non-genetic model for autism) restored a range of physiological and behavioural deficits: namely neuronal activity of pyramidal neurons in CA3 area of hippocampus, hippocampal oscillations and abnormal pup vocalisations (Tyzio et al., 2014). Interestingly, Wang and Kriegstein (2011) showed that the same treatment (bumetanide) can cause long-lasting deficits in excitatory synaptic transmission, developmental delay and impaired sensorimotor gating in wildtype control mice, if it is given during a narrow developmental time window from E17 to P7. Drawing parallels between species (Semple et al., 2013;

Sengupta, 2013), these results could indicate that therapeutic interventions in comparable developmental stages in humans can have profound effects in pathophysiology of neurodevelopmental disorders but also highlight the importance of tight regulation in treatment applications during early development.

Other studies have gone a step further and compared different treatment timing. For example, it was shown that early pharmacological intervention in *Fmr1* knockout juvenile mice appears to be more efficient in correcting biochemical, physiological and cognitive deficits the same pharmacological treatment in adult subjects (Dansie et al., 2013; Su et al., 2011; Sun, Hongpaisan, & Alkon, 2016). The existence of time-windows sensitive to pharmacological treatment has also been reported in the Drosophila model for FXS. Gatto and Broadie (2010)reported that reintroducing the FMRP fly homolog during a critical phase in brain development led to correction of abnormal dendritic morphology. On the other hand, treatment in the adult fly or intriguingly even at an earlier stage of circuit formation was ineffective. Such experiment of timed genetic intervention, has not been translated to any other model organism of FXS but it is vital to determine if the effectiveness of treatment dependents on already defined critical periods and precise developmental stages. It appears that the weakness of treatment during adulthood or to generalise, any untimely intervention, is that the therapeutics could miss a sensitive period of elevated plasticity, important in behavioural development. If FXS is a lasting consequence of abnormal brain development with deficits in synaptic maturation, it is possible that early interventions with pharmacological agents facilitating synaptic maturation could achieve more dramatic results, unfolding in that way, the full potential of the therapeutics. Since FXS is a genetic syndrome with defined genetic aetiology, this is entirely plausible, considering that new-born screening for FXS is technically feasible (Flora Tassone, 2014).

It is obvious from the above that early detection and therapeutic intervention holds promise for maximizing the efficacy of treatment. Early interventions are likely to improve symptomatology in young children with FXS. However, adapting treatment approaches to this population requires elucidating the developmental trajectories of cognitive function during early childhood first. Another limiting factor when treating children is drug safety. Candidate therapeutics which have been used in children before and found to be effective, would significantly speed up the translation of preclinical data. It is important to keep in mind that only about 30 percent of drugs commonly prescribed

to children today, have been thoroughly tested in children; even though this can be harmless, this is not always the case. For example, 60 years ago, the antibiotic chloramphenicol was commonly prescribed in adults. But many new-borns died after receiving the antibiotic because their livers were not developed enough to metabolise the drug efficiently (FDA, 2016).

A drug group which has been approved by FDA for child use is HMG-CoA reductase inhibitors, or statins. They have been in use since 1970s and are considered a first-line pharmacologic intervention for children with familial hypercholesterolemia (Eiland & Luttrell, 2010). Over the last decade, there is a growing interest in statins with regards to their effects on brain function (Ling & Tejada-Simon, 2016). Surprisingly, studies using preclinical models of numerous neurodevelopmental disorders including Rett syndrome, Fragile X syndrome (FXS), neurofibromatosis and tuberous sclerosis have shown that statin treatment can have really positive effects against a wide range of symptoms. These promising results have led to a number of clinical trials as well. For example, in disorders such as neurofibromatosis type 1 (NF1), treatment with lovastatin reversed cognitive deficits in both a mouse model (Weidong Li et al., 2005) and children suffering from these disorders (Acosta et al., 2011; Bearden et al., 2016; Chabernaud et al., 2012; Mainberger et al., 2013). However, in a placebo-controlled trial, twelve weeks of treatment with a different statin (simvastatin) did not lead to any improvement in cognitive function of children with NF1 (Krab et al., 2008). In FXS, treatment with lovastatin has been shown to improve symptomatology. *Fmr1* KO mice which were treated with lovastatin displayed normalized protein synthesis levels in hippocampus and reduced susceptibility for audiogenic seizures (Osterweil et al., 2013b). The promising results of this study led to an open-label study in 2014. Patients who received treatment showed a significant improvement in measured behaviour output (Çaku et al., 2014).

How is lovastatin mechanism of action connected to molecular cascades affected by FMRP loss? During the early 1990s, several research groups reported that one of statins indirect mechanisms of action includes reduction of Ras farnesylation and as a result the activity of two downstream kinases, ERK-1 and ERK-2, in cells (Ling & Tejada-Simon, 2016) (Fig. 6.1B). Interestingly, two decades after, it was shown that blockade of Ras-ERK-1/ERK-2 signalling cascade, decreased some biochemical markers associated with FXS in the mouse model of the disorder (Osterweil et al., 2010). Taken together these

findings suggest that statins could be part of treatment schemes against core defects associated with FXS, but it is still unclear whether they could be used as monotherapy or in combination with other mechanism-based therapeutics or behavioural treatments. Consistent with the later though, two ongoing trials of lovastatin are focussing in the combination of lovastatin and a behavioural intervention (ClinicalTrials.gov Identifier: NCT02642653) and combination of lovastatin and minocycline (ClinicalTrials.gov Identifier: NCT02680379).

While Osterweil and colleagues (2013) focused on epileptogenesis and showed that lovastatin can reverse audiogenic seizure susceptibility in *Fmr1* knockout mice, this study aims to examine the effects of early lovastatin treatment on cognitive deficits before these have even emerged. We hypothesised that treating subjects early in life during possibly sensitive periods of postnatal development, would have strong effects in their cognitive development. In the previous chapter I have shown that *Fmr1* KO rats exhibit cognitive delays and deficits in two associative memory tasks, Object Place (OP) and Object-Place-Context (OPC) (OP delayed, OPC does not develop). Moreover, I showed that wildtype rats develop the ability to remember these complex associations not before postnatal day 49, revealing a time window of approximately 1 month from weaning (P22-P49), during which circuits supporting these times of memory are still being developed.

Cognitive attention and working memory, although delayed in FXS, reveal a developmental change, rather than a "freeze" (Cornish et al., 2013). This indicates that the system is still plastic and has a potential to change drastically once the appropriate treatment is in place. Fish and colleagues (1994) showed that it is the cognitive deficits that lag the adaptive abilities in boys with FXS. Their adaptive behaviour is sometimes sufficiently high to challenge a mental retardation diagnosis. They found that adaptive behaviours decline as a function of age. More interestingly, the average social intelligence quotient (SQ), which is indicative of adaptive behaviour, for 3 to 8-year-old affected children, is relatively high (approx. 70) (SQ follows the 'standard score' approach used in IQ tests, with a mean of 100). We propose a treatment timeline (P29-64) which would catch this period of high adaptive behaviour and possibly the last week or so in brain development (approx. a month in a rat with a lifespan of 2 years is roughly equal to 3.3 years of brain development in humans; (Semple et al., 2013; Yoon et al., 2014) before phenotypes associated with FXS are established in our rat model (Chapter 5).

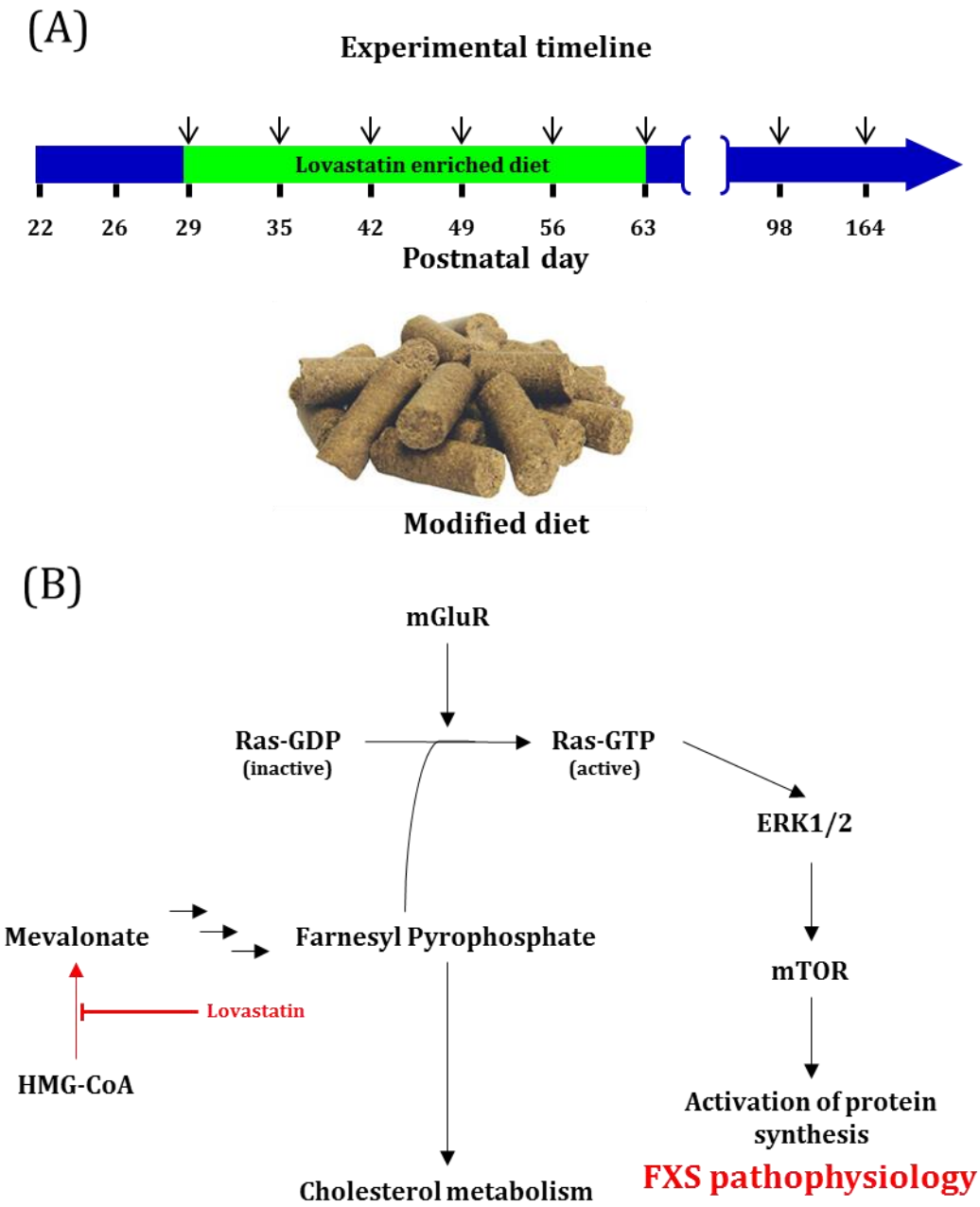



**Figure 6.1 Experimental design and lovastatin mechanism of action. (A)** Subjects were given a lovastatin enriched diet (100mg/Kg) (green) for 5 weeks (P29-P64) and tested once every week throughout this period (arrows). The same subjects were also tested approx. 5 weeks and 3 months after the end of the treatment. Throughout that period they consumed normal diet (blue). **(B)** Statins' mechanism of action and potential link to FXS pathophysiology. Based on this hypothesis, it is obvious that lovastatin acts fairly indirectly on the molecular cascades affected by FMRP loss. Its effects on cholesterol metabolism or protein farnesylation can also contribute to its therapeutic effects.

## 6.2 Methods

### 6.2.1 Animals

Subjects were male Long Evans Hooded rats (wild-type {WT}, n=25 {13 treated and 12 untreated}; *Fmr1*-KO {KO}, n=24 {12 treated and 12 untreated} *), bred in-house and kept in a 12h/12h light dark cycle. Colony founders were produced by Sigma Advanced Genetic Engineering (SAGE) Labs (St. Louis, MO, US). Animals were weaned from their mother at postnatal-day 22 (P22) and housed in mixed genotype cages with littermates, 2-4 animals per cage. Animals were provided with water, sawdust bedding and either a gnawing block of wood and/or a cardboard tube as environmental enrichment throughout the experiment. Ad libitum standard laboratory chow was provided until P29. *Note that for the last two time points (P98-105 & P164-5) numbers of animals are different due to experimental error (wild-type {WT}, n=22 {11 treated and 11 untreated}; *Fmr1*-KO {KO}, n=20 {9 treated and 11 untreated}).

### 6.2.2 Experimental Procedures

All experimental procedures, data analysis and exclusion criteria are almost identical to those described in Chapter 5 except for some minor changes. In brief; starting from weaning (P22), animals were handled daily in the animal house and experimental room for 4 days prior to experiments. Task-specific habituation was reduced to the two days prior to testing (P26-27). On P26 and P27, the animals were habituated to the apparatus as described in Chapter 3. Testing started on P28 with OR and OC and P29 with OP and OPC. On the afternoon of P29 animals switched to either control or lovastatin-enriched (100mg/kg) (Bioserv®) diet which was restocked and weighed once daily. Testing continued every 7 days until P64. At P64, animals were returned to ad libitum standard laboratory chow until the end of experiment (P164). Two more testing points 5-6 weeks and 3 months after the end of treatment were carried out (Fig. 6.1A). Rats' weight and consumption per cage was monitored throughout the dosing period (P29-P64) to ensure that diet did not have any adverse effects on their growth. Objects were used only once and were counterbalanced across tasks, genotypes and time-points to eliminate any bias. Abiding by the ARRIVE guidelines (Kilkenny et al., 2010), experimenters were always blind to the genotype and the diet of the animals. Furthermore, all experimental conditions (position of the novel object, context of test phase, order of presented contexts etc.) were counterbalanced across genotypes, treatment, age and tasks.

### 6.2.3 Statistical analysis

As described previously, a Discrimination Index DI [(time exploring novel object—time exploring familiar object)/(time exploring both objects)] was calculated for each test phase. The only difference was that the criterion for data exclusion was less stringent than previously. As the repeated testing of (4 tasks in 2 days) had a slight impact on the subjects' interest for objects. For this experiment, trials in which a subject did not reach 15 sec of total object exploration in both the sample and the test phases and at least 5 sec of exploration for each object in the sample phase, were excluded from the analysis. This criterion is stringent enough to exclude animals who were not interested, but to include animals that explored for an adequate amount of time overall and had experienced both objects. Groups' performance in all testing time points was compared to chance levels (unless stated otherwise), and false discovery rate during multiple comparisons against chance levels, was corrected using the Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995). Due to missing data (rats didn't reach object exploration criteria), a mixed linear model with time as a repeated measure was used to examine the effects of the treatment. Statistical analysis was done using IBM SPSS Statistics 22.0 and GraphPad Prism 6. All graphs were produced in GraphPad Prism 6.

## 6.3 Results

### 6.3.1 Lovastatin does not impede normal physical development of rats

A promising drug candidate should have minimal side effects on the physiology of the subjects receiving the treatment. To determine whether lovastatin treatment has negative impacts on normal growth, we tracked weight changes in rats while they consumed lovastatin enriched diet, daily for the course of the treatment (35 days) (Fig 6.1). Lovastatin did not affect normal weight gain in either WT (time $F_{(35,770)}$ =1453, p <0.001; treatment $F_{(1,23)}$ =0.44, p =0.51; treatment × time $F_{(35,770)}$ =0.71, p =0.89; Fig. 6.2A) or *Fmr1* KO rats (time $F_{(35,770)}$ =1037, p <0.001; treatment $F_{(1,22)}$ =3.42, p =0.08; Fig. 6.2B). Even though a significant interaction was found in *Fmr1* KO rats (treatment × time $F_{(35,770)}$ =2.97, p <0.001), *post hoc* t-tests revealed no significant differences between groups. The average food intake per subject was similar for both diets throughout the course of the treatment, with both treatment groups reaching a plateau in food consumption (approx. 29gr/day) after postnatal day 57 (time $F_{(14,476)}$ =53.29, p <0.001; diet $F_{(114)}$ =1.33, p =0.27; treatment × time $F_{(14,476)}$ =1.68, p =0.01; Fig. 6.2C) with *post hoc*

1-sample t-tests revealing no differences at any time point. Finally, analysis of the daily treatment intake revealed that there was a peak of approx. 15mg lovastatin/Kg rat, between P35 and P42 (before the emergence of object-place and object-place-context memory) for both WT and *Fmr1* KO rats but overall no effect of genotype (time $F_{(34,748)}$ =21.47, p <0.001; genotype $F_{(1,22)}$ =0.03, p =0.87; genotype × time $F_{(35,770)}$ =0.27, p >0.99; Fig. 6.2D).

### 6.3.2 Lovastatin does not affect normal cognitive development in Object and Object-Context recognition

Treatment with lovastatin early in life did not affect object recognition memory in either WT (time $F_{(5,112.7)}$ =0.52, p <0.05; treatment $F_{(1,22.7)}$ =0.52, p =0.48; treatment × time $F_{(5,112.7)}$ =0.76, p =0.58; Fig 6.3A) or *Fmr1* KO rats (time $F_{(5,108.1)}$ =0.40, p =0.85; treatment $F_{(1,21.2)}$ =0.55, p =0.47; treatment × time $F_{(5,108.1)}$ =1.12, p =0.36; Fig. 6.3B). Both treatment groups in both genotypes were able to perform significantly above chance levels from the first testing time point. Analysis of exploration times during both sample (Supp. Fig. 3A) and test phase (time $F_{(5,220)}$ =1.41, p =0.22; test group $F_{(3,44)}$ =0.99, p =0.40; test group × time $F_{(15.220)}$ =0.84, p =0.63; Fig. 6.3C) revealed no difference in exploratory behaviour as a result of treatment. Dividing the 6 testing times in 2 epochs (before and after the ontogenetic emergence of object-place and object-place-context memory) reduces the variability and confirms that object recognition memory is not affected by FMRP loss (genotype $F_{(1,45)}$ =1.21, p =0.28) and that lovastatin treatment does not affect performance (treatment $F_{(1,45)}$ <0.05, p =0.95; treatment × genotype $F_{(1,45)}$ =1.18, p =0.28; Fig. 6.3D). The performance of individual subjects shows clearly that there was no substantial improvement between the time points. Only 7 out of 13 (approx. 50%) WT untreated rats improved their performance but overall there was no statistical difference between the two epochs ($t_{12}$ =1.42, p =0.17, paired t-test; Fig. 6.3E). On the other hand, WT rats who received treatment showed a small but significant improvement ($t_{11}$ =3.11, p =0.01, paired t-test;). Moreover, both untreated and treated *Fmr1* KO did not show any improvement ($KO_{control}$: $t_{11}$ =0.31, p =0.77; $KO_{lova}$: $t_{11}$ =1.10, p =0.29, paired t-test; Fig. 4.3F).

Object context developmental was not affected from lovastatin treatment either. Both genotype groups could perform significantly above chance from P35 onwards either they received control or lovastatin enriched diet (WT: time $F_{(5,114.4)}$ =3.50, p <0.01; treatment $F_{(1,22.7)}$ =0.22, p =0.65; treatment × time $F_{(5,114.4)}$ =0.48, p =0.79; Fig. 6.4A. *Fmr1* KO: time

$F_{(5,106.7)}$ =2.25, p >0.05; treatment $F_{(1,21.6)}$ =1.83, p =0.19; treatment × time $F_{(5,106.7)}$ =0.51, p =0.77; Fig. 6.4B). Exploration did not differ between groups either in sample (Supp. Fig. 3B) or test phase of testing (time $F_{(5,220)}$ =4.35, p <0.001; test group $F_{(3,44)}$ =0.50, p =0.68; test group × time $F_{(15.220)}$ =0.66, p =0.82; Fig. 6.4C). Collapsed data points in two epochs (P28-42 & P49-63) confirm that there are no differences between groups in response to the treatment (genotype $F_{(1,45)}$ =0.33, p =0.57; treatment $F_{(1,45)}$ =2.0, p =0.16; treatment × genotype $F_{(1,45)}$ =0.44, p =0.51; Fig. 6.4D). Analysis of individual subject novelty preference verify our previous observations that object-context memory develops normally before P42 and that lovastatin does not alter this trajectory ($WT_{control}$: $t_{12}$ =3.54, p <0.01; $WT_{lova}$: $t_{11}$ =1.42, p =0.18; $KO_{control}$: $t_{11}$ =0.53, p =0.61; $KO_{lova}$: $t_{11}$ =0.99, p =0.34, paired t-test; Fig. 6.4E&F).

### 6.3.3 Lovastatin treatment corrects developmental delay in Object-Place associative memory

Lovastatin treatment did not impede normal development of object-place memory in WT subjects (time $F_{(5,111.7)}$ =13.44, p <0.001; treatment $F_{(1,21.8)}$ =1.50, p =0.23; treatment × time $F_{(5,111.7)}$ =0.43, p =0.83; Fig. 6.5A). Previous data (Chapter 5) suggested that *Fmr1* KO rats exhibit a developmental delay in the ontogeny of object-place associative memory. This study verified this delay; even though after correction for false discovery *Fmr1* KO rats did not perform significantly above chance at any testing point during treatment, there is a strong trend on P64 (Table 6.2). Lovastatin treated KO subjects displayed a significantly improved developmental trajectory (time $F_{(5,107.1)}$ =6.08, p <0.001; treatment $F_{(1,22.3)}$ =8.38, p <0.01; treatment × time $F_{(5,107.1)}$ =1.05, p =0.40; Fig 6.5B). Exploratory activity was identical between all groups in both sample (Supp. Fig. 3C) and test phase (time $F_{(5,220)}$ =5.32, p <0.001; test group $F_{(3,44)}$ =0.21, p =0.89; test group × time $F_{(15.220)}$ =0.66, p =0.82; Fig. 6.5C). Collapsed data into pre-emergence and post-emergence epochs shows clearly that all groups except untreated *Fmr1* KO rats improved significantly (genotype $F_{(1,45)}$ =8.99, p <0.01; treatment $F_{(1,45)}$ =9.0, p <0.01; treatment × genotype $F_{(1,45)}$ =2.70, p =0.11; time × treatment × genotype $F_{(1,45)}$ =3.13, p =0.08;Fig. 6.5D). Moreover, performance of individual subjects confirms this robust improvement in object-place memory between the two epochs for WT and KO treated subjects. All WT on control diet ($t_{12}$ =7.23, p <0.001) and 11 out of 12 WT treated animals ($t_{11}$ =4.76, p <0.001; Fig. 6.5E) displayed improved performance between the two epochs. On the other hand, only *Fmr1* KO rats treated with lovastatin improved their performance ($t_{11}$

=3.49, p <0.01; Fig. 6.5F); even though 10 out of 12 *Fmr1* KO on control diet improved their performance this chance was not robust enough to lead to significant difference between epochs ($t_{11}$ =2.02, p =0.07 Fig. 6.5F).

### 6.3.4 Lovastatin treatment prevents the emergence of cognitive deficits in Object-Place-Context recognition

The most striking cognitive deficit described in this thesis, is the inability of *Fmr1* KO rats to form coherent episodic-like memories (Chapter 4). This type of associative memory develops at P50 in WT rats (Chapter 5). Interestingly lovastatin treatment early in life, starting at a pre-symptomatic period can, not only, prevent the emergence of the deficit but also totally restore its developmental trajectory to WT levels (Fig. 6.6). Consistent with all 3 previously described tasks, WT rats are not affected by lovastatin treatment and show very similar developmental trajectory with the untreated group (time $F_{(5,109.5)}$ =4.33, p <0.01; treatment $F_{(1,21.9)}$ =1.13, p =0.30; treatment × time $F_{(5,109.5)}$ =0.85, p =0.52; Fig. 6.6A). More importantly, lovastatin treated *Fmr1* KO rats display normal development, while untreated subjects can never perform significantly above chance levels (time $F_{(5,106.7)}$ =3.12, p <0.05; treatment $F_{(1,21.9)}$ =9.32, p =0.06; treatment × time $F_{(5,106.7)}$ =3.06, p <0.05; Fig. 6.6B). The exploration levels are identical between groups in both sample (Supp. Fig. 3D) and test phase of the task (time $F_{(5,220)}$ =1.00, P =0.42; test group $F_{(3,44)}$ =0.70, P =0.56; test group × time $F_{(15.220)}$ =1.18, P =0.29; Fig. 6.6C). Collapsed data points into the pre-emergence and post-emergence epochs confirms the effects of lovastatin treatment (genotype $F_{(1,45)}$ =2.05, p =0.16; treatment $F_{(1,45)}$ =8.91, p <0.01; treatment × genotype $F_{(1,45)}$ =2.11, p =0.15; time × treatment × genotype $F_{(1,45)}$ =9.26, p <0.01; Fig. 6.6D). Analysis of the individual values further validates our findings. Almost every WT rat in both treatment groups improved their performance between the two epochs ($WT_{control}$: $t_{12}$ =2.75, p <0.05; $WT_{lova}$: $t_{11}$ =2.83, p <0.05; Fig. 6.6E). On the other hand, only *Fmr1* KO rats who received lovastatin treatment improved their performance ($KO_{control}$: $t_{11}$ =0.52, p =0.61; $KO_{lova}$: $t_{11}$ =4.35, p <0.01, paired t-test; Fig. 6.6F).

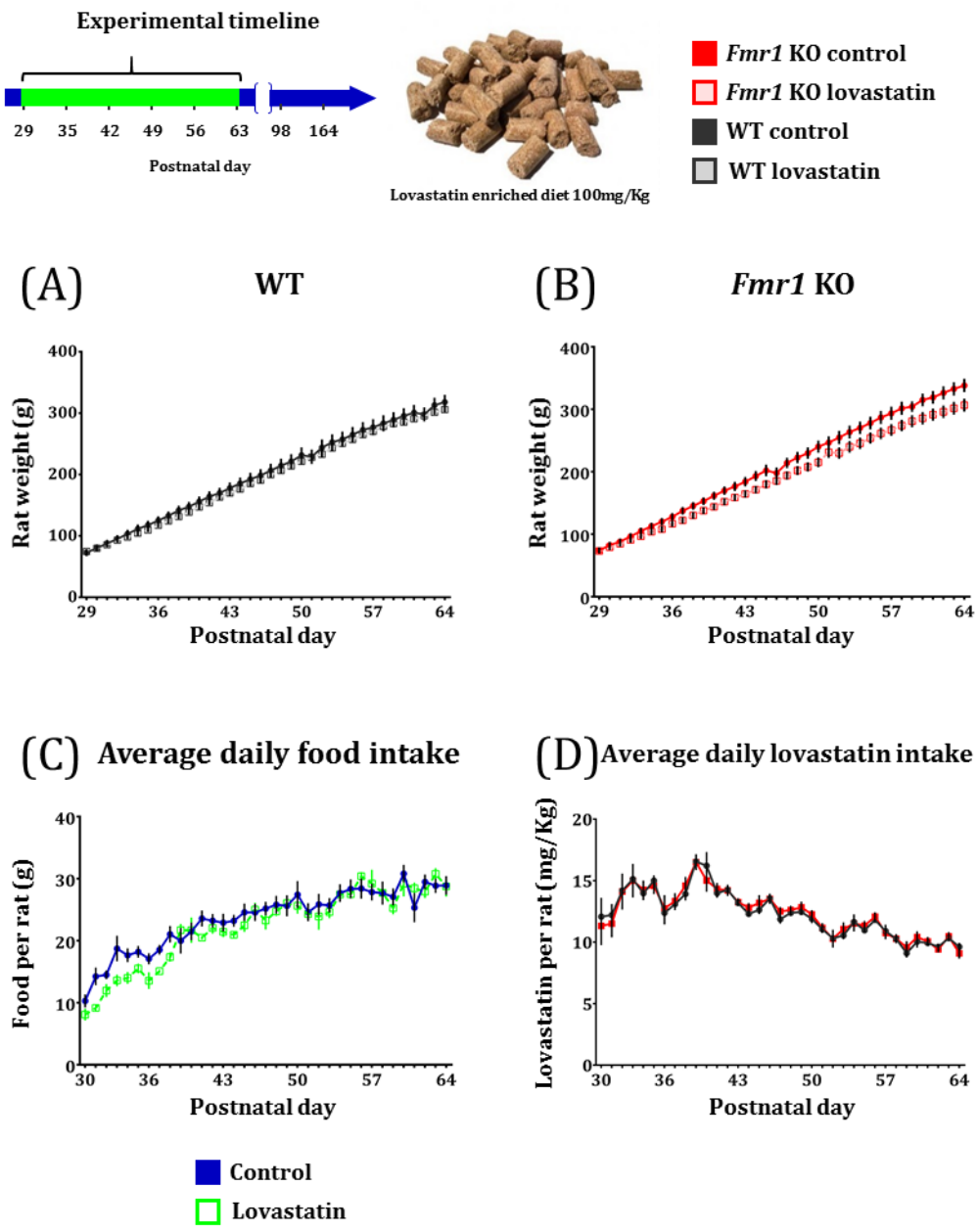



**Figure 6.2 Lovastatin has no effects on normal physical development of rats.** Both WT **(A)** and *Fmr1* KO **(B)** rats have normal development during lovastatin treatment compared to untreated same genotype controls. Average daily food intake, based on cage consumption is similar between the two used diets **(C).** Normalising the daily food intake to rats' weight reveals no difference between genotypes **(D)**.

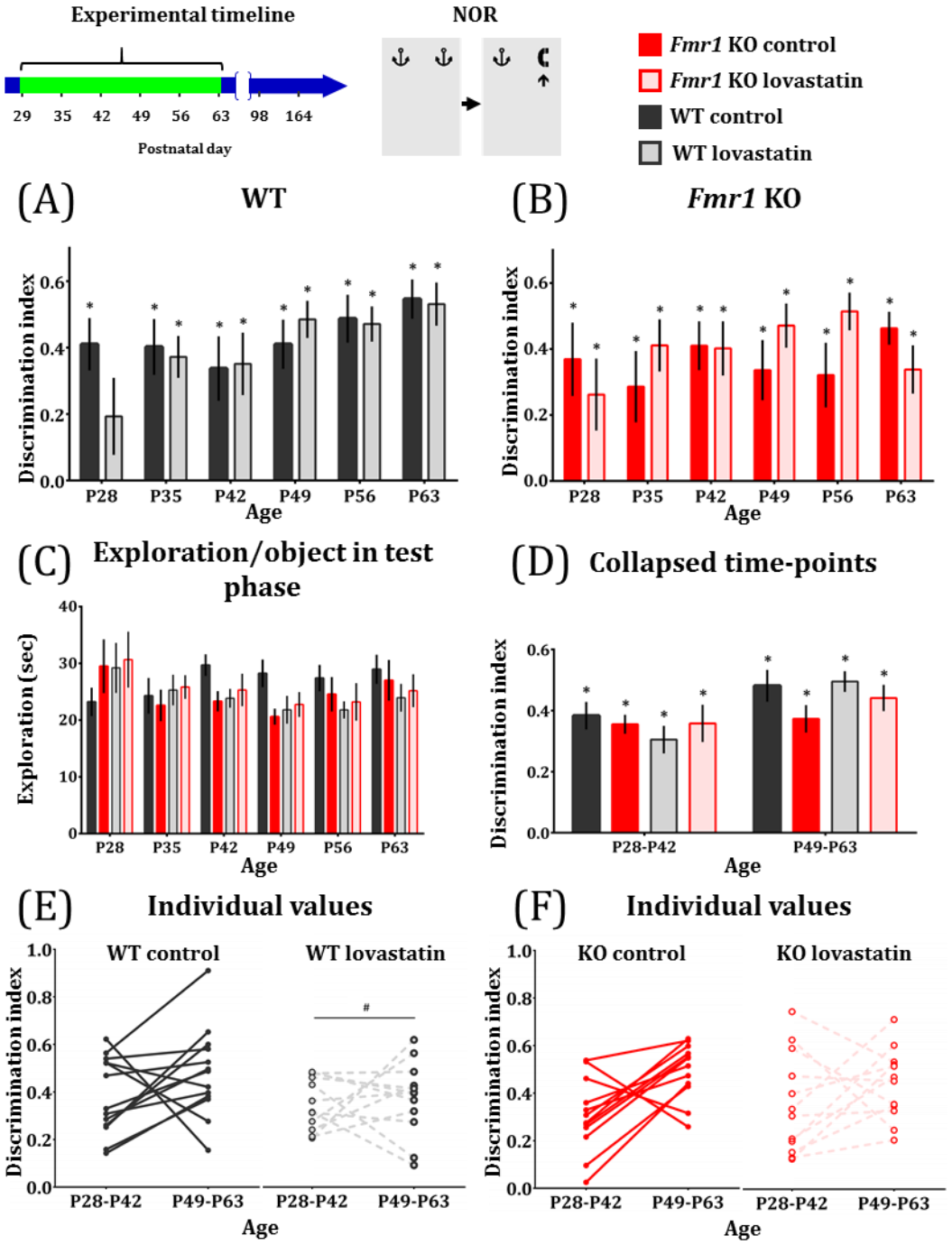



**Figure 6.3 *Fmr1* KO and WT rats experience normal development of object recognition memory.** WT **(A)** and Fmr1 KO **(B)** rats' ability to discriminate novel from familiar objects is unaffected by lovastatin treatment. Exploration time between the groups was similar throughout the experiment **(C)**. Averaging performances before and after the critical age for the ontogenetic emergence of object-place and episodic memory (P49, Chapter 3) reveals no differences between groups **(D)**. Focussing on the individual subjects reveals no improvement for *Fmr1* KO **(F)** and WT untreated rats but a small improvement for WT treated subjects **(E)**. For (A), (B) and (D) * $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure. For (E) and (F) # $p<0.05$ difference between groups.

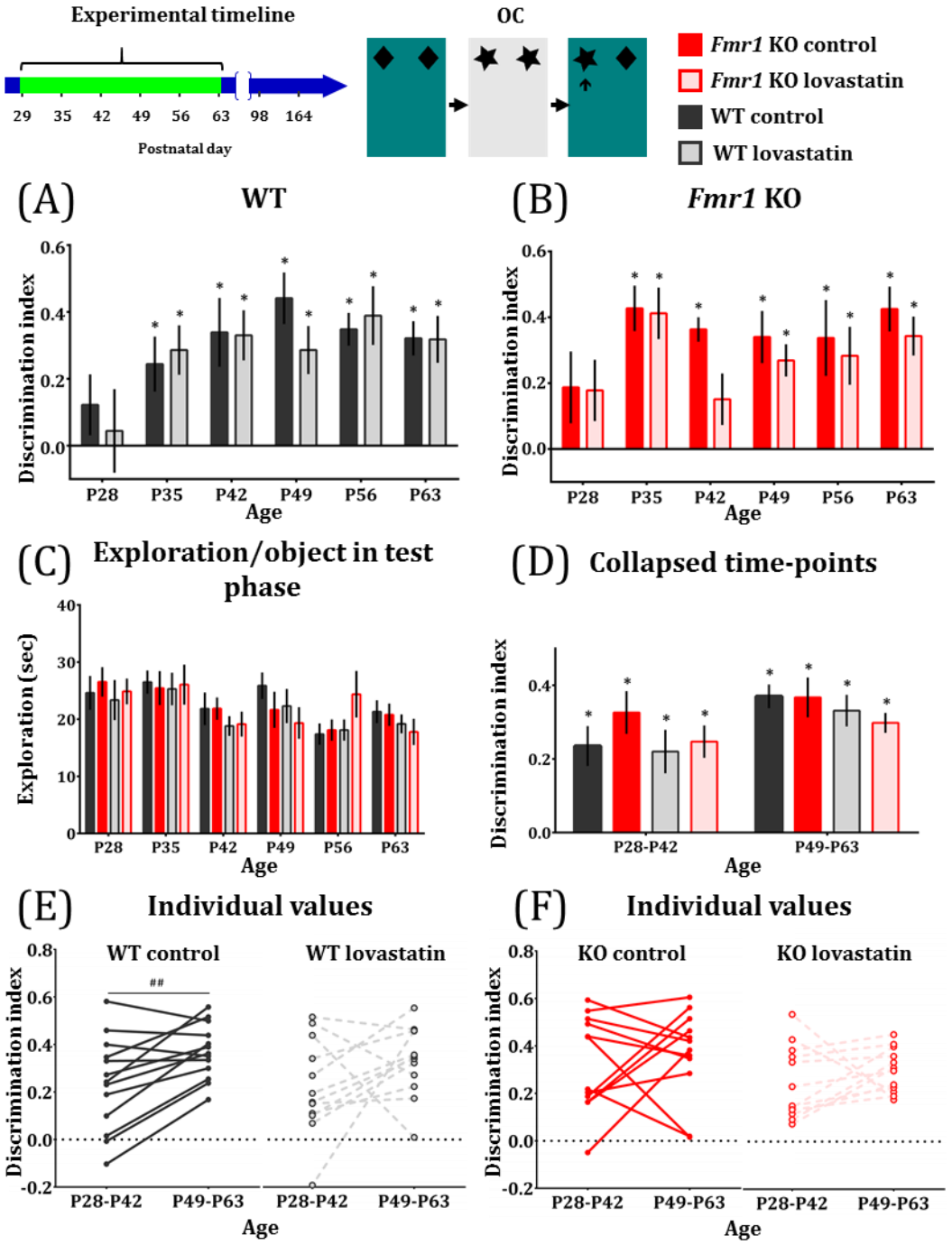



**Figure 6.4 Normal development of object recognition memory for *Fmr1* KO and WT rats treated with lovastatin.** WT **(A)** and Fmr1 KO **(B)** rats' ability to discriminate novel from familiar objects is unaffected by lovastatin treatment. Consistently with our previous observations both *Fmr1* KO and WT rats develop the ability to discriminate novel from familiar object-context associations on P35. Exploration time between the groups was similar throughout the experiment **(C)**. Averaging performances before and after the critical age for the ontogenetic emergence of object-place and episodic memory (P49, Chapter 3) reveals no differences between groups **(D)**. Focussing on the individual subjects reveals no improvement for *Fmr1* KO **(F)** and WT treated rats but a small improvement for WT untreated subjects **(E)**. For (A), (B) and (D) * $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure. For (E) and (F) # $p<0.05$ difference between groups.

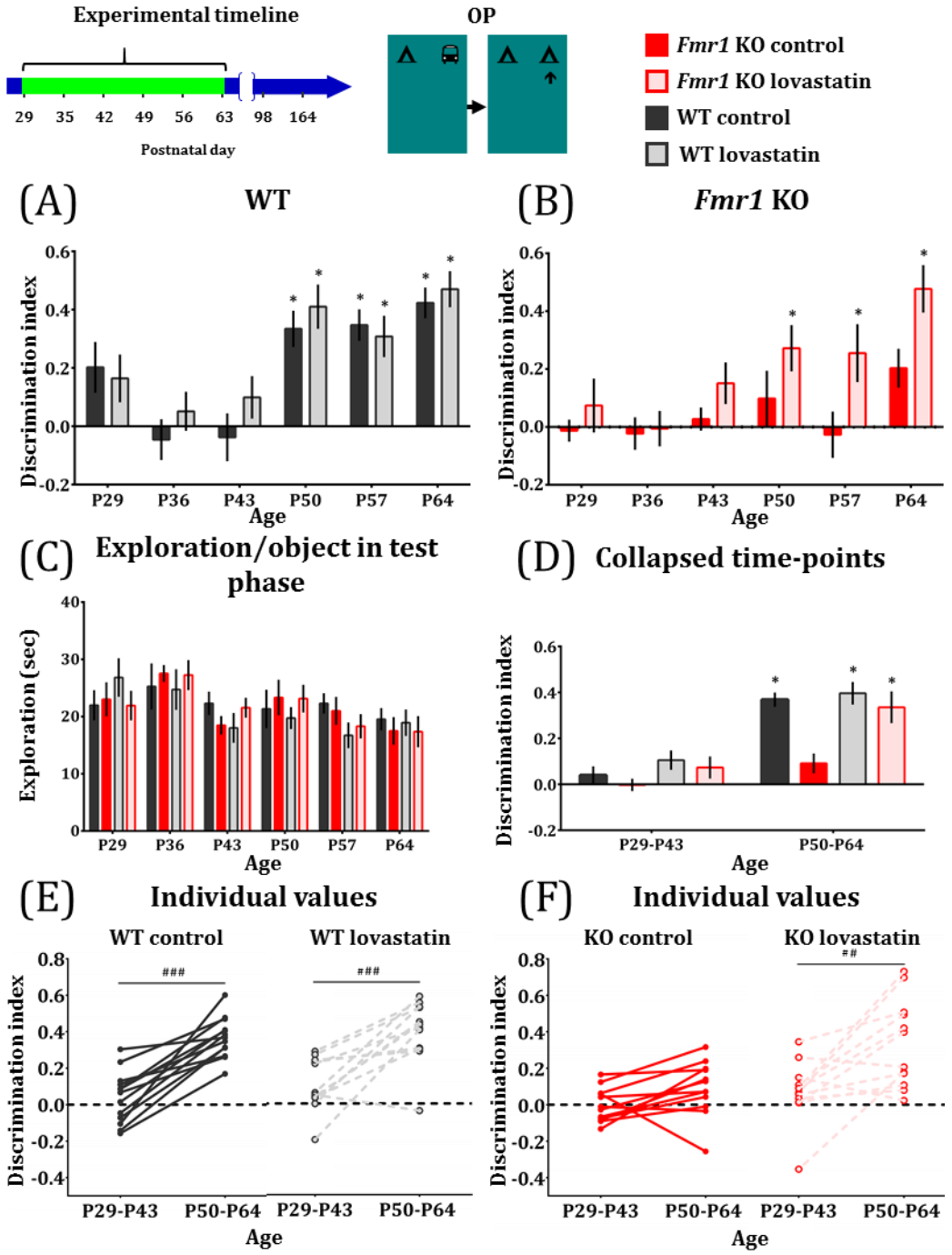



**Figure 6.5 Lovastatin restores normal developmental trajectory of object-place memory in *Fmr1* KO rats.** WT rats which received lovastatin treatment experience identical cognitive development to untreated controls and begin to discriminate novel from familiar object-place associations on P50 **(A).** Fmr1 KO rats' ability to discriminate novel from familiar object-place associations develops normally with lovastatin treatment early in life **(B).** Exploration time between the groups was similar throughout the experiment **(C).** Averaging performances before and after the critical age for the ontogenetic emergence of the task further confirms our observations **(D).** Focussing on the individual subjects reveals no improvement for *Fmr1* KO untreated subjects **(F)** but a significant improvement for both WT groups and treated *Fmr1* KO subjects **(E)**. For (A), (B) and (D) * $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure. For (E) and (F) # $p<0.05$ difference between groups.

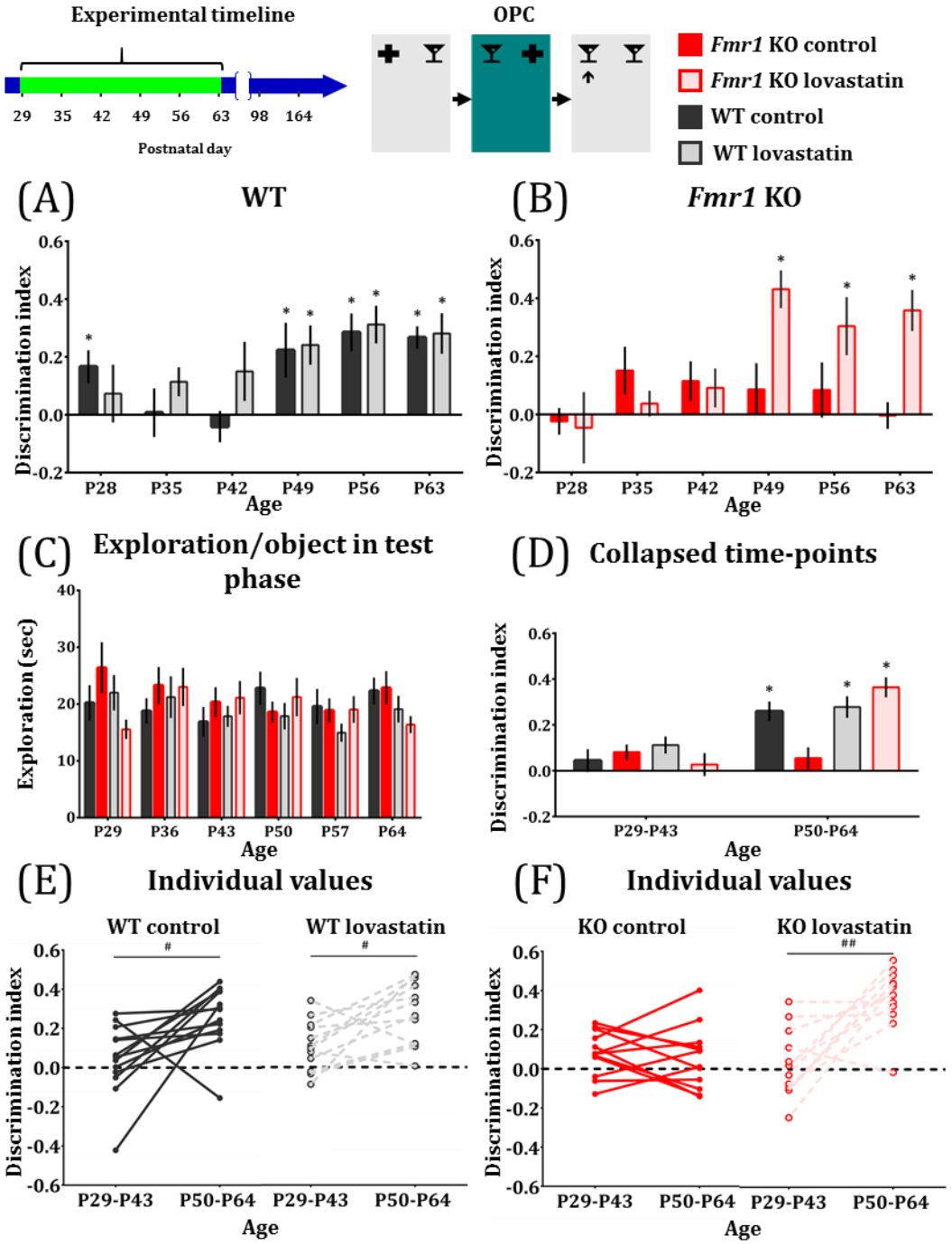



**Figure 6.6 Lovastatin restores normal developmental trajectory of Object-Place-Context associative memory in *Fmr1* KO rats.** WT rats who received treatment experience identical cognitive development to untreated controls and start to discriminate novel from familiar object-place associations on P50 **(A).** Even though untreated *Fmr1* KO rats perform at chance level throughout testing, lovastatin treatment early in life can lead to normal development of object-place-context associative memory **(B)**. Exploration levels between the groups was similar throughout the experiment **(C)**. Averaging performances before and after the critical age for the ontogenetic emergence of the task confirms our previous observations **(D)**. Focussing on the individual subjects reveals no improvement for *Fmr1* KO untreated subjects **(F)** but a significant improvement for both WT groups and treated Fmr1 KO subjects **(E)**. For (A), (B) and (D) * $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure. For (E) and (F) # $p<0.05$ difference between groups.

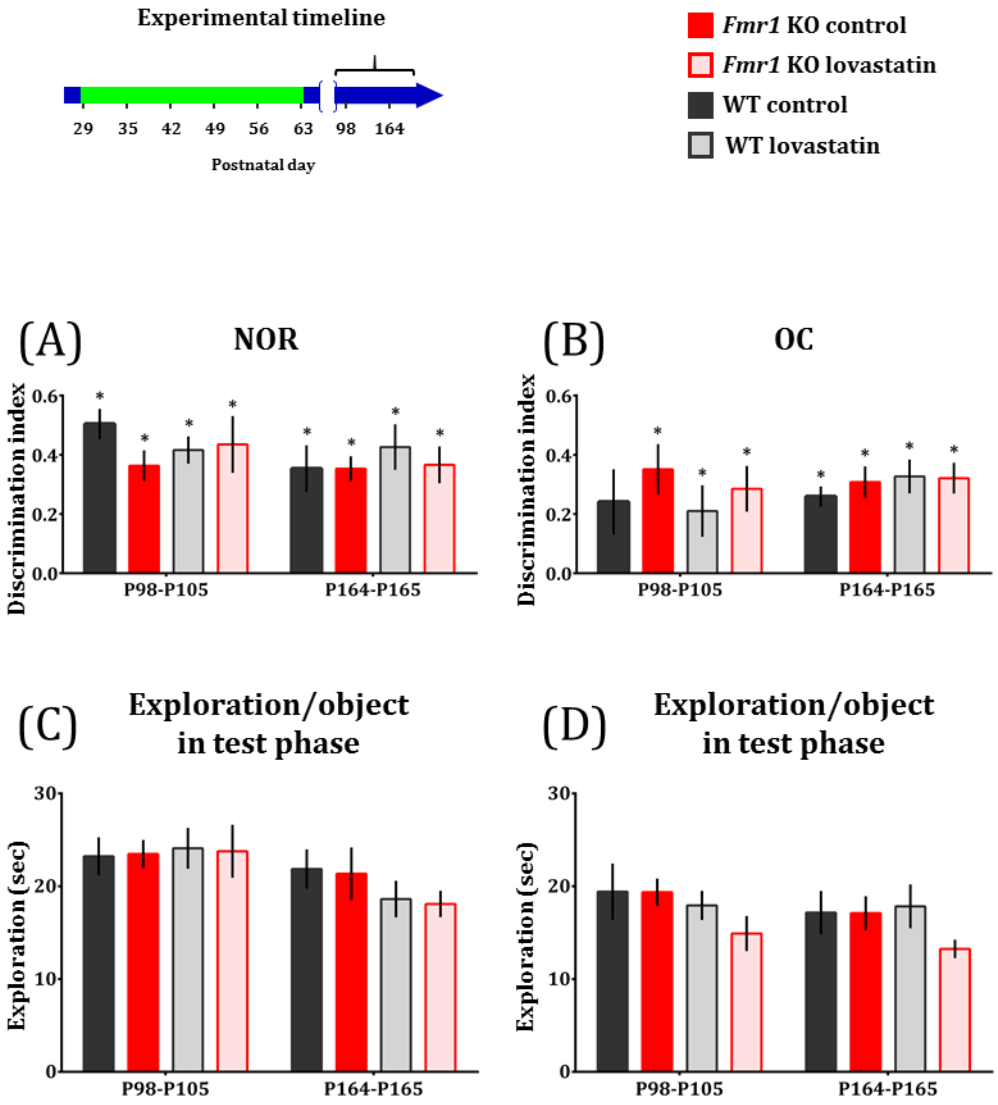



**Figure 6.7 Performance in NOR and OC 5 weeks and 3 months after the end of treatment.** All groups perform significantly better than chance levels in NOR **(A)** and OC **(B)**. Exploration levels between the groups was similar for both NOR **(C)** and OC **(D)** in both testing points. For (A), (B) and (D) * $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure.

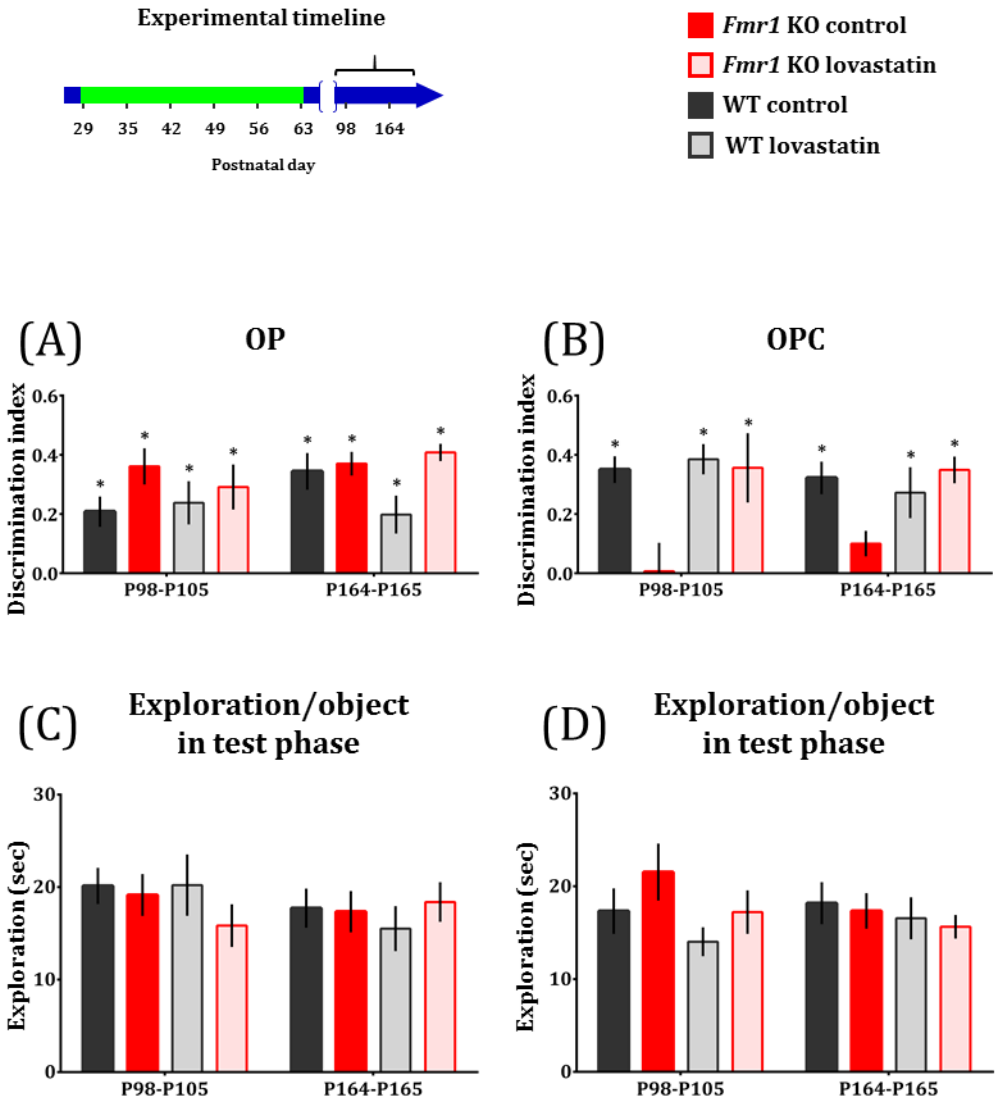



**Figure 6.8 Lovastatin treatment early in life leads to long lasting cognitive improvements.** Performance in OP 5 weeks and 3 months after the end of treatment is similar for all experimental groups confirming the developmental delay rather than a deficit **(A)**. Surprisingly *Fmr1* KO rats treated with lovastatin display WT levels of performance in **(B)**. Exploration levels between the groups was similar for both OP **(C)** and OPC **(D)** in both testing points. For (A), (B) and (D) * $p<0.05$ difference from chance (DI =0); significance values have been controlled for the false discovery rate using the Benjamini–Hochberg procedure.

### 6.3.5 Early lovastatin treatment has profound long lasting effects on cognition

Perhaps the most interesting and at the same puzzling result in this study come when the same subjects which received lovastatin treatment for 5 weeks during adolescence, were tested approx. 5 weeks and more than 3 months after the end of the treatment (Fig. 6.7 & Fig. 6.8). Performance in both NOR and OC was identical to the last testing point during treatment (NOR: genotype $F_{(1,35.5)}$ =1.05, p =0.31; treatment $F_{(1,35.5)}$ =0.27, p =0.61; treatment × genotype $F_{(1,35.5)}$ =0.51, p =0.48; Fig 6.7A. OC: genotype $F_{(1,36.9)}$ =1.15, p =0.29; treatment $F_{(1,36.9)}$ =0.01, p =0.92; treatment × genotype $F_{(1,36.9)}$ =0.16, p =0.69; Fig 6.7B). As previously, we found no differences between groups in exploration during sample (Supp. Fig 3A&B) or test phase (NOR: time $F_{(1,32)}$ =6.91, p <0.05; group $F_{(3,32)}$ =0.23, p =0.88; time × group $F_{(3,32)}$ =0.64, p =0.60; Fig 6.7C. OC: time $F_{(1,32)}$ =1.22, p =0.29; group $F_{(3,32)}$ =1.99, p =0.14; time × group $F_{(3,32)}$ =0.13, p =0.94; Fig 6.7D). Furthermore, *Fmr1* KO displayed fully developed ability to discriminate novel from familiar object-place associations (genotype $F_{(1,36.9)}$ =4.52, p <0.05; treatment $F_{(1,36.9)}$ =1.17, p =0.29; treatment × genotype $F_{(1,36.9)}$ =0.05, p =0.83; Fig 6.8A), while exploration times stayed again unaffected (time $F_{(1,32)}$ =0.97, p =033; group $F_{(3,32)}$ =0.19, p =0.91; time × group $F_{(3,32)}$ =0.88, p =0.46; Fig 6.8C). OPC task revealed the most intriguing result; *Fmr1* KO rats which received lovastatin early in life retained the profound effects of treatment for more than 3 months after the end of it (genotype $F_{(1,39.2)}$ =5.36, p <0.05; treatment $F_{(1,39.2)}$ =6.77, p <0.05; treatment × genotype $F_{(1,39.2)}$ =7.34, p <0.05; Fig 6.8B) without any effects on exploratory activity (time $F_{(1,32)}$ =0.13, p =073; group $F_{(3,32)}$ =1.58, p =0.21; time × group $F_{(3,32)}$ =0.75, p =0.53; Fig 6.8B).

| Time-point | Significance | NOR | OC | OP | OPC |
|---|---|---|---|---|---|
| P28&29 | Uncorrected p | <0.001 | >0.05 | <0.05 | <0.05 |
| | Bonferroni | ** | | | |
| | B&H | * | | | * |
| P35&36 | Uncorrected | <0.01 | <0.05 | >0.05 | >0.05 |
| | Bonferroni | ** | | | |
| | B&H | * | * | | |
| P42&43 | Uncorrected | <0.01 | <0.01 | >0.05 | >0.05 |
| | Bonferroni | * | | | |
| | B&H | * | * | | |
| P49&50 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.05 |
| | Bonferroni | ** | *** | ** | |
| | B&H | * | * | * | * |
| P56&57 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.001 |
| | Bonferroni | *** | *** | *** | ** |
| | B&H | * | * | * | * |
| P63&64 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.001 |
| | Bonferroni | *** | *** | *** | *** |
| | B&H | * | * | * | * |
| P98-105 | Uncorrected | <0.001 | >0.05 | <0.01 | <0.001 |
| | Bonferroni | *** | | | *** |
| | B&H | * | | * | * |
| P164-165 | Uncorrected | <0.01 | <0.001 | <0.001 | <0.001 |
| | Bonferroni | ** | *** | ** | ** |
| | B&H | * | * | * | * |

**Table 6.1 Development statistical overview for WT rats on control diet.** * p<0.05 for Bonferroni correction for multiple comparisons; * indicates significance from chance levels of discrimination after controlling the false discovery rate using the Benjamini-Hochberg procedure (B&H)

| Time-point | Significance | NOR | OC | OP | OPC |
|---|---|---|---|---|---|
| P28&29 | Uncorrected p | <0.01 | >0.05 | >0.05 | >0.05 |
| | Bonferroni | * | | | |
| | B&H | * | | | |
| P35&36 | Uncorrected | <0.05 | <0.001 | >0.05 | >0.05 |
| | Bonferroni | | *** | | |
| | B&H | * | * | | |
| P42&43 | Uncorrected | <0.001 | <0.001 | >0.05 | >0.05 |
| | Bonferroni | ** | *** | | |
| | B&H | * | * | | |
| P49&50 | Uncorrected | <0.01 | <0.01 | >0.05 | >0.05 |
| | Bonferroni | * | * | | |
| | B&H | * | * | | |
| P56&57 | Uncorrected | <0.01 | <0.05 | >0.05 | >0.05 |
| | Bonferroni | * | | | |
| | B&H | * | * | | |
| P63&64 | Uncorrected | <0.001 | <0.001 | <0.05 | >0.05 |
| | Bonferroni | *** | *** | | |
| | B&H | * | * | | |
| P98-105 | Uncorrected | <0.001 | <0.01 | <0.001 | >0.05 |
| | Bonferroni | *** | * | ** | |
| | B&H | * | * | * | |
| P164-165 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.05 |
| | Bonferroni | *** | ** | *** | |
| | B&H | * | * | * | |

**Table 6.2 Development statistical overview for Fmr1 KO rats on control diet**. * p<0.05 for Bonferroni correction for multiple comparisons; * indicates significance from chance levels of discrimination after controlling the false discovery rate using the Benjamini-Hochberg procedure (B&H)

| Time-point | Significance | NOR | OC | OP | OPC |
|---|---|---|---|---|---|
| P28&29 | Uncorrected p | >0.05 | >0.05 | <0.05 | >0.05 |
| | Bonferroni | | | | |
| | B&H | | | | |
| P35&36 | Uncorrected | <0.001 | <0.01 | >0.05 | <0.05 |
| | Bonferroni | ** | * | | |
| | B&H | * | * | | |
| P42&43 | Uncorrected | <0.01 | <0.01 | >0.05 | >0.05 |
| | Bonferroni | * | ** | | |
| | B&H | * | * | | |
| P49&50 | Uncorrected | <0.001 | <0.01 | <0.001 | <0.01 |
| | Bonferroni | *** | * | ** | * |
| | B&H | * | * | * | * |
| P56&57 | Uncorrected | <0.001 | <0.01 | <0.01 | <0.01 |
| | Bonferroni | *** | ** | ** | * |
| | B&H | * | * | * | * |
| P63&64 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.01 |
| | Bonferroni | *** | ** | *** | * |
| | B&H | * | * | * | * |
| P98-105 | Uncorrected | <0.001 | <0.05 | <0.01 | <0.001 |
| | Bonferroni | *** | | | *** |
| | B&H | * | * | * | * |
| P164-165 | Uncorrected | <0.001 | <0.001 | <0.05 | <0.05 |
| | Bonferroni | ** | ** | | |
| | B&H | * | * | * | * |

**Table 6.3 Development statistical overview for WT rats on lovastatin diet.** * p<0.05 for Bonferroni correction for multiple comparisons; * indicates significance from chance levels of discrimination after controlling the false discovery rate using the Benjamini-Hochberg procedure (B&H)

| Time-point | Significance | NOR | OC | OP | OPC |
|---|---|---|---|---|---|
| P28&29 | Uncorrected p | <0.05 | >0.05 | >0.05 | >0.05 |
| | Bonferroni | | | | |
| | B&H | * | | | |
| P35&36 | Uncorrected | <0.001 | <0.001 | >0.05 | >0.05 |
| | Bonferroni | ** | ** | | |
| | B&H | * | * | | |
| P42&43 | Uncorrected | <0.001 | >0.05 | >0.05 | >0.05 |
| | Bonferroni | ** | | | |
| | B&H | * | | | |
| P49&50 | Uncorrected | <0.001 | <0.001 | <0.01 | <0.001 |
| | Bonferroni | *** | ** | * | *** |
| | B&H | * | * | * | * |
| P56&57 | Uncorrected | <0.001 | <0.01 | <0.05 | <0.05 |
| | Bonferroni | *** | | | |
| | B&H | * | * | * | * |
| P63&64 | Uncorrected | <0.001 | <0.001 | <0.001 | <0.001 |
| | Bonferroni | ** | ** | *** | ** |
| | B&H | * | * | * | * |
| P98-105 | Uncorrected | <0.01 | <0.01 | <0.05 | <0.05 |
| | Bonferroni | * | * | | |
| | B&H | * | * | * | * |
| P164-165 | Uncorrected | <0.01 | <0.001 | <0.001 | <0.001 |
| | Bonferroni | * | ** | *** | ** |
| | B&H | * | * | * | * |

**Table 6.4 Development statistical overview for *Fmr1* KO rats on lovastatin diet.** * p<0.05 for Bonferroni correction for multiple comparisons; * indicates significance from chance levels of discrimination after controlling the false discovery rate using the Benjamini-Hochberg procedure (B&H)

## 6.4 Discussion

This study builds upon observations described in the previous chapter about altered developmental trajectories of associative memory displayed by *Fmr1* KO rats. The three issues addressed in this study were: (1) the effects of lovastatin in the normal development of WT rats; (2) the effectiveness of lovastatin treatment at early age in restoring normal development of cognitive abilities; and (3) the dependence of behavioural improvements on the continuous administration of treatment. The main findings of this early-onset lovastatin intervention in weanling rats can be summarized in the following points. Firstly, lovastatin appears to have minimum impact on rat growth; *Fmr1* KO subjects which received the treatment seem to have slightly lower weight but they display the same growth curve, whereas WT animals, both treated and untreated, were identical in their physical development. This can easily be explained by the fact that lovastatin affects cholesterol metabolism. Translating the average daily drug intake (approx. 12mg/Kg for rats equals 2.4mg/kg for humans Fig. 6.2D) is very close to the suggested dosage by FDA for children and teenagers with hypocholesteraemia (40mg daily) (Çaku et al., 2014; Reagan-Shaw, Nihal, & Ahmad, 2008). This is very important because it simply means that the observed behavioural improvements are not due to unrealistic drug quantities that would reduce the translational value of these results. Moreover, this also explains the very limited adverse effects (slight weight change) and the absence of effects on normal development of cognition in wildtype rats. This is particularly important as it means that other physiological mechanisms are not significantly altered, making transition to clinical trials even smoother. Moreover, lovastatin treatment starting from the 4$^{th}$ week can prevent the emergence of cognitive deficits (seen from P49 onwards) and lead to normal cognitive development. This is the first study to my knowledge reporting not just reversal but the prevention of a cognitive deficit in a model of FXS. What is really important and adds validity to this results, is that recent unpublished work in the lab has shown that plasticity deficits in prefrontal cortex (an important area for the two affected types of associative memory, OP and OPC)(Chao et al., 2016) are also reversed as a result of the treatment (Supp. Fig. 4) (Adam Jackson personal communication). Finally, the most surprising results of this study came from testing the same experimental subjects 5 to 6 weeks and more than 3 months after the end of treatment. Even though the rats were fed normal diet for all this time, the therapeutic effects of lovastatin seen at the end of the treatment persist even 100 days after. This the first demonstration of such an exciting but also puzzling effect. Moreover,

unpublished work has revealed that basal levels of protein synthesis are reduced to wildtype level in animals treated with lovastatin (Supp. Fig. 4) (Susana Ribeiro dos Louros personal communication). Putting aside the unique experimental design and the suppressing persistence of therapeutic effects of this study, overall these results are consistent with previous studies showing that lovastatin can reverse phenotypes associated with FXS (Osterweil et al., 2013b), other disorders (Y. S. Lee et al., 2014; Weidong Li et al., 2005).

### 6.4.1 How does lovastatin alleviate FXS symptomatology?

Lovastatin, like other statins, inhibits HMG-CoA reductase, an enzyme that is part of mevalonate pathway (Fig. 6.1B) and acts several steps upstream of ERK-1 and ERK-2 cascades. Therefore, any molecular changes introduced by lovastatin treatment inhibit ERK-1 and ERK-2 activity fairly indirectly. This mechanism of action for lovastatin is consistent with mGluR theory for FXS (Bear et al., 2004) as ERK-1 and ERK-2 signalling is activated by mGluR5 (Krab, Goorden, & Elgersma, 2008; Osterweil et al., 2010). Nevertheless, explaining the effects of lovastatin on behaviour and physiology, only based on fact that it can modulate ERK-1 and ERK-2 activity is limiting. While mGluR antagonists which are thought to target Fragile X syndrome-relevant translational mechanisms more directly, showed similar therapeutic effects to lovastatin when tested in mice (Michalon et al., 2012), clinical trials did not yield any promising results. This outcome does not conclusively answer the question of whether or not selective mGluR5 antagonists can alter FXS symptomatology and definitely does not rule out the validity of the mGluR5 theory, however, these results do suggest that selective pharmacological decrease of mGlur5 signalling, alone, in humans with FXS is not sufficient in order to ameliorate behavioural abnormalities, when used as a short-term treatment in the ages studied. There is still a distinct lack of mechanistic supportive evidence to justify the use of statins in the prevention or treatment of pathophysiology associated with various neuropsychiatric disorders. While the precise molecular mechanism of lovastatin action is beyond the scope of this thesis, it is tempting to speculate about possible pleiotropic effects of lovastatin treatment contributing to its therapeutic function.

There are several possible experiments which will elucidate further the mechanism of lovastatin action. An obvious line of investigation is cholesterol metabolism since cholesterol is vital to normal brain function including learning and memory (Schilling et

al., 2014; Schreurs, 2010). Cholesterol metabolism alterations have been documented in individuals with FXS (Elizabeth Berry-Kravis et al., 2015) and recently Pietropaolo and colleagues (2014) have reported that dietary supplementation with omega-3 fatty acids from weaning reversed a wide range of behavioural deficits associated with loss of FMRP in mice. Moreover, cholesterol metabolism has been shown to be affected in a different neurodevelopmental disorder, Rett syndrome (Nagy & Ackerman, 2013). Consistently with studies on FXS, both omega-3 fatty acids and lovastatin treatment improved cholesterol homeostasis but also partially alleviated motor impairments, and generally improved the life of mice modelling the disease (Buchovecky et al., 2013; De Felice et al., 2012). A way to examine whether cholesterol homeostasis normalisation is even partially, related to lovastatin effects on pathophysiology associated with FXS, would be to use a similar statin which cannot cross the blood brain barrier (something that lovastatin can do). In that way any effects in symptomatology would be only due to other systemic effects of the drug.

Another function or lovastatin which should be addressed in relation to FXS and other neurodevelopmental disorders is its effects on neuroinflammation. The role of immune system on brain function under healthy and diseased conditions is becoming to be a major line of investigation especially in the field of neurodevelopmental disorders (Derecki, Cronk, & Kipnis, 2013; Gupta et al., 2014; Malkki, 2016). Along these lines, offspring of perinatally infected mothers is a widely used model of autism (Knuesel et al., 2014). In FXS, it has been shown that the mouse model of the syndrome displays some neuroinflammatory imbalances (IL-1β, CD45, CD11b), which can be rescued by omega-3 fatty acids supplementation (Pietropaolo et al., 2014). Interestingly Gouveia and colleagues (2011) showed that lovastatin reduces expression of the mRNA of interleukin-1β, amongst other cytokines, in animals with status epilepticus; remember that one of the main behavioural deficits rescued by lovastatin treatment was increased susceptibility in audiogenic seizures (Osterweil et al., 2013b). Moreover, statins have been shown to decreases inflammation by reducing the expression the cytokines IL-1β and TNF-α, in the kainic-induced animal model of epilepsy (Lee et al., 2008) and modulate immune T-cell function (Zhao et al., 2015). The connection between immune system and neurodevelopmental disorders is further validated by the finding of abnormally active neuroinflammatory processes in autistic individuals (Ashwood et al., 2011; Vargas et al., 2005). Taken together, the above could suggest a possible additional route for lovastatin to have positive effects on the FXS symptomatology. Obviously

further work is needed in order to elucidate the relations between statins, core FXS pathophysiology and immune system. Focussing on the resident immune cell of CNS, microglia, could be a potential route, since there is already a defined connection between microglia function, synapse formation and function and neurodevelopment. (Schafer et al., 2012; Zhan et al., 2014). A possible first experiment would be to explore translational changes in microglia during different age points, using Translating ribosome affinity purification (TRAP) (Heiman et al., 2014), and examine if lovastatin treatment early in life can have effects on possible translational abnormalities.

Looking at the seminal work from Osterweil and colleagues (2013), certain results point to the hypothesis of additional (possibly systemic) ways lovastatin exerts its effects. For example, while lovastatin had a dramatic effect on improving audiogenic seizure susceptibility, the effects on Ras-ERK-1/ERK-2 signalling were only moderate. Taking into account that farnesylation depletion affects not only Ras signalling (Sebti, 2005), it is fair to believe that downregulating ERK-1 and ERK-2 is just one of lovastatin's (and other statins) mechanisms of action. In order to get a better idea on the importance of Ras farnesylation in FXS symptomatology, the farnesyl group could be supplemented during lovastatin treatment and examine whether or not the beneficial effects of lovastatin diminish. Another approach could be a more direct downregulation of Ras farnesylation, using bisphosphonates (Bergstrom et al., 2000). In that way we could bypass additional effects from mevalonate pathway products upstream Ras signalling (Fig. 6.1B).

Focussing on our behavioural results, one might argue that the effects we're seeing cannot be interpreted as direct effect of lovastatin. Repeated exposures to the testing environment, experimenter and the overall procedure could have easily served as environmental enrichment. Environmental enrichment, especially early in life, has been shown to alleviate some cellular and behavioural abnormalities in *Fmr1* knockout mice (Lauterborn et al., 2015; Oddi et al., 2014), so it could be possible that an enhanced plasticity due lovastatin treatment was enough to amplify the effects of environmental enrichment. Even though there are no behavioural data to support or dispute this hypothesis, the preliminary electrophysiology results, showing rescue of plasticity deficits, came from animals which were only dosed in their home cage, without any additional manipulation. Of course the most direct way to examine the synergistic effects of lovastatin and environmental enrichment would be to use the same treatment duration but only test the subjects at the end of it. In line with this idea, an ongoing

clinical trial is testing the effects of lovastatin combined with behaviour intervention (ClinicalTrials.gov Identifier: NCT02642653).

### 6.4.2 Are all statins alike?

Over the recent years, statins have gain significant attention in relation to their effects against core pathophysiological mechanisms of a wide range of neuropsychiatric diseases (Ling & Tejada-Simon, 2016). Until this point, it is still questionable and poorly examined whether the preclinical and clinical outcomes documented, are a result of the statins' cholesterol-dependent or cholesterol-independent effects. One has to keep in mind that even though statins seem to share a common mechanism of action and have similar potency, and safety profile, they are not the same (Pedersen & Gaw, 2001; Tobert, 2003). Despite the similarities in the molecular structures of lovastatin and simvastatin, newer statins like fluvastatin and rosuvastatin are structurally diverse. The seemingly small structural differences can alter drastically the mechanism of action of a compound and, ultimately, its effect on physiology. This is particularly interesting as lovastatin, simvastatin and fluvastatin have been shown to inhibit Ras/ERK pathway (Osterweil et al., 2013b; Tsubaki et al., 2016) while rosuvastatin has been reported to enhance it (Z. Zhang et al., 2013). A difference between statins, highly related to their effects on the central nervous system (CNS) is concerning their relative lipophilicity and hydrophilicity. This parameter is important since lipophilicity is needed in order a compound to cross the blood brain barrier. Another important difference between statins is that even though all are predominantly metabolised by cytochrome P450, this is done by different isoforms of the enzyme (Pedersen & Gaw, 2001). This is particularly important when we consider early interventions, since during postnatal liver maturation, different P450 isoforms display different temporal patterns of expression (Cui, Renaud, & Klaassen, 2012; Ince et al., 2013). Therefore, it is important to improve our understanding as to the precise mechanism differences between statins. Until more comparative evidence is produced, clinicians and researchers should consider the effects on CNS for each statin individually, instead of assuming that other statins will have similar effects.

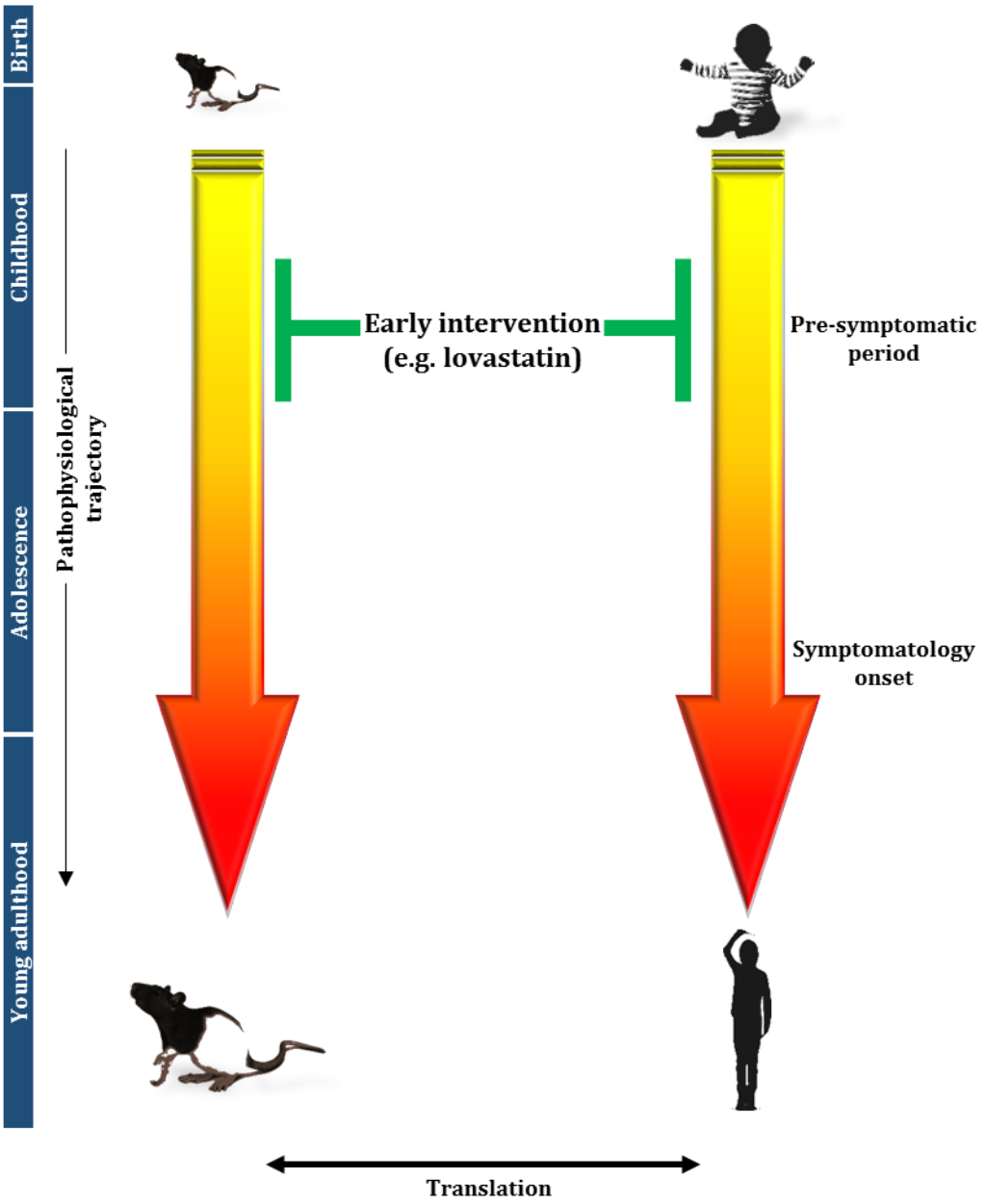



**Figure 6.9 Paradigm shift in therapeutic intervention.** After several failed clinical trials, both clinicians and basic researchers have suggested that the age of the participants requires further consideration. Animal models can be utilized to address biological questions on the pathological trajectory to full onset of symptomatology in neurodevelopmental disorders.

### 6.4.3 How can early intervention have such long lasting results?

Whilst in some ways this study replicates previous studies examining the efficacy of lovastatin (Lee et al., 2014; Osterweil et al., 2013) or the augmented effects of early intervention (Dansie et al., 2013; Oddi et al., 2014; Sun et al., 2016), the most striking and novel result is that early pharmacological intervention can have very long lasting effects on cognition, on a genetic rat model of intellectual disability and autism. This result besides impressive, is also quite puzzling. How can a relatively short pharmacological treatment, during a period of no observed major brain development milestones, lead to effects which persist for more than three months after the end of the treatment?

A first obvious suggestion is that this period (P29-P64) does indeed include important cellular events which affect synaptic formation and function later in life. I mentioned earlier that almost all research on postnatal brain development has been focusing on the four first postnatal weeks. Following the results of this study, there is a need to focus on discovering brain development hallmarks in the following weeks ($4^{th}$-$7^{th}$). It is possible that FMRP loss has a profound effect during this critical period which subsequently contribute to cognitive deficits but its effects during adulthood alone are minimal. This hypothesis agrees with the spatiotemporal expression pattern of FMRP (Rhiannon M. Meredith et al., 2012) and previous studies showing that treatment early in life can lead to more dramatic and persisting effects than treatment during adulthood (Dansie et al., 2013; Sun et al., 2016). Contrary to the latter hypothesis, a relatively recent study showed that ablation of FMRP in adulthood leads to decrease neurogenesis in the hippocampus and hippocampal dependent cognitive deficits (Guo et al., 2011). Repeating our study with the same duration of treatment but in adult animals, after the behavioural deficits in OPC have been established, is needed in order to reveal if: (1) established cognitive deficits can be reversed in adult *Fmr1* KO rats, and (2) if the treatment has long lasting effects.

In this absence of relevant physiological evidence that could explain the results of our study based on the more "mainstream" proposed mechanisms of action for lovastatin (Ras-ERK1/2 activity downregulation)(Krab, Goorden, et al., 2008), I think it is important to consider alternative ways by which lovastatin exerts its effects. A recent interesting study looked at the effects of early treatment with antioxidants in a rat model of schizophrenia (Cabungcal et al., 2014). What the authors showed is that when pre-symptomatic juveniles and adolescent rats were treated with the antioxidant N-acetyl

cysteine, physiological and behavioural deficits' onset was prevented. One of the pathophysiological features of this model is altered prefrontal function, including inhibitory interneurons during adolescence and augmented oxidative stress especially affecting parvalbumin (PV)-positive interneurons in prefrontal cortex.

Could these data be relevant to FXS? Research in *Fmr1* KO mice has revealed major defects in cortical inhibitory circuits (Selby, Zhang, & Sun, 2007). Moreover, a few studies in *Fmr1* knockout mice have demonstrated that FMRP loss leads to an augmentation in oxidative stress and related markers in the brain that it is possible to contribute to the pathophysiology of the Fragile X syndrome (de Diego-Otero et al., 2009; El Bekay et al., 2007). In agreement with these findings, FMRP has been shown to positively regulate the expression of the mitochondrial and cytosolic Superoxide Dismutase (SOD) (Bechara et al., 2009). Thus FMRP loss could lead to an increase in mitochondrial oxidative stress and subsequent abnormalities in mitochondrial function. Interestingly, lovastatin and other statins have been found to possess anti-oxidative properties (Kumar, Srivastava, & Gomes, 2011; Lee et al., 2016). Taken together these studies may suggest an additional mechanism for lovastatin, explaining its long lasting effects. It is plausible that specific neuronal types which are part of key circuits for learning and memory (Lipina et al., 2015) are highly susceptible to elevated oxidative stress (Liang et al., 2016), especially during early periods in life. Agents like lovastatin and other antioxidants, which can reduce oxidative stress and subsequently protect these circuits from decay, will lead to long lasting or even permanent results. Of course testing this hypothesis is a relatively straightforward task; dosing with an antioxidant during the same time period should produce the same or similar results to ours.

Besides pharmacological approaches, it is important to remember that intervention strategies might not be limited to pharmacological. For example, a different research group, using the same rat model of schizophrenia that was utilised for the antioxidant treatment rescue (Cabungcal et al., 2014), reported that cognitive training during adolescence is enough prevent the emergence of cognitive impairments normally seen in adulthood (Lee et al., 2012). In the case of FXS. Several studies have shown the beneficial effects of environmental enrichment (Lauterborn et al., 2015; Restivo et al., 2005) but only recently Oddi and colleagues showed that early social enrichment can have long lasting results in physiology and behaviour which persist through adulthood (Oddi et al., 2014). This is yet another testament to the fact that combination of

pharmacological treatments and cognitive interventions holds a great promise in curing neurodevelopmental disorders.

### 6.4.4 Paradigm shift in future clinical trials?

The current optimism, shared amongst some researchers, that treatment for FXS during adulthood is possible, is not unreasonable; several preclinical studies in adult mice showed reversal of symptomatology (Henderson et al., 2012; Michalon et al., 2012; Osterweil et al., 2013b). Nevertheless, after several failed clinical trials, both clinicians and basic researchers have suggested that the age of the participants requires further consideration. Like all other neurodevelopmental disorders, the behavioural and cognitive characteristics of FXS, such as social communication deficits, intellectual disability, and cognitive inflexibility, manifest early in infancy and continue to unfold during life. Failed trials of pharmacological interventions in adults may echo therefore, the fact that the fully matured nervous system has irreversibly suffered the pathophysiological consequences of FMRP loss (exacerbated LTD and abnormal synaptic morphology and function). Our results, support this paradigm shift in FXS clinical trials (Fig. 6.9). We showed that early pharmaceutical intervention during a pre-symptomatic period, can not only prevent the emergence of cognitive deficits associated with the loss of FMRP, but also have persistent benefits on cognitive function.

# 7. General discussion

FXS is the most common monogenic cause of intellectual disability and autism spectrum disorder. The mouse model of the syndrome (Ce E Bakker et al., 1994) has been proven invaluable in FXS research so far, however the subtle and strain-specific behavioural phenotype and recent failures of clinical trials which failed to replicate the promising results of preclinical studies, have raised questions over its validity. The recent generation of a rat model of FXS paves the way for determining whether certain phenotypes are species specific or persist across mammalian species. In this thesis, we have demonstrated that this novel rat model is promising, recapitulating key pathophysiological features of the disease (Till et al., 2015), but further work is needed to utilise all the advantages of rats as model organism. We have identified a robust cognitive deficit, which persists across two background strains (Chapter 4) (Asiminas et al., 2015) and we further explored its developmental trajectory in a longitudinal study (Chapter 5) (Asiminas et al., 2014). The next step was to attempt to reverse that deficit using a known pharmaceutical agent (lovastatin) (Chapter 6) (Asiminas et al., 2016). Utilising knowledge gained from the longitudinal study we decided to begin treatment over a pre-symptomatic, for the observed deficit, period. Unexpectedly, treatment could not only prevent the emergence of the deficit, but also fully restore normal developmental trajectory in all other developmentally delayed but unimpaired in adulthood tasks. Furthermore, when the same animals were tested 3 months after the end of the treatment showed the same behavioural profile compared to the end of the treatment. Our results show that not only we can prevent the emergence of cognitive deficits associated with FXS but also that therapeutic interventions in potentially critical developmental windows can have long lasting or even permanent effects.

Curing cognitive deficits associated with FXS or other intellectual disability syndromes is one of the holy grails in translational neuroscience. We believe that with the right medication during the appropriate developmental windows, which could enhance synaptic plasticity, in conjunction with the other types of educational and behavioural interventions, including relevant computer technologies, we will be able to achieve such a goal. This is a very exciting time in translational neuroscience not only for FXS but other conditions as well, due the extensive commonality amongst different types of neurodevelopmental disorders.

## 7.1 So you want to be a model (of FXS): what rats have to offer

This thesis is amongst the first few to examine the validity of new genetically modified rat models of neurodevelopmental disorders. Along with recently published work (Berzhanskaya et al., 2016a; Berzhanskaya et al., 2016b; Engineer et al., 2014; Hamilton et al., 2014; Kenkel et al., 2016; Ruby, Falvey, & Kulesza, 2015; Till et al., 2015), we showed that rats have the potential to expand our knowledge of pathophysiology associated with FXS significantly but also validate phenotypes across mammalian species. Rat's size, behavioural repertoire and physiology (Chapter 3) make it a very suitable model organism for FXS and other neurodevelopmental syndromes, but is it better than the mouse model? As is the case with any neurodevelopmental disease model, the *Fmr1* KO rat should not be seen as faithfully reproducing the disease (there is no such thing as a rat with FXS). However, this and other neurodevelopmental disease models are extremely useful to testing specific hypotheses about developmental trajectories of molecular, electrophysiological and behavioural phenomena of relevance to the disease of interest. Perhaps the biggest promise of rat models is bridging the gap between pre-clinical and clinical research. Due to their physiology which is closer to human, rats have been used in the pharmaceutical industry for years, to predict how human patients will metabolise medication, and to identify and study potential side effects. The results of these studies are essential before Phase I trials, addressing tolerance, can begin in humans. Moreover, during the past decades, the standard approach in drug discovery research, included drug efficacy screening in genetically modified mice, models of disorders, and then safety and toxicity assessment in rats, mainly due to the large volume of historical safety data in the rat and the physiology similarities between rats and humans. This methodology has relied heavily on extrapolations of used mouse dosing to rat, and the assumption that this dose would have similar efficacy in the rat. The recently developed genetically modified rat models, can now address this problem, enabling researchers to conduct both drug efficacy and safety studies in the same species, increasing coherence between efficacy and toxicity studies and speeding up the process significantly.

## 7.2 Time after time: the value of studying developmental trajectories

In Chapter 5 in this thesis I described a longitudinal study exploring the developmental trajectory of associative and non-associative object memory. Although these type of studies have been reported previously (Green & Stanton, 1989) very little is known about the emergence of behavioural phenotypes in rodent models of neurodevelopmental disorders. We showed that *Fmr1* KO rats showed a developmental delay in a type of memory which is unaffected during adulthood (OP) and they were nether able to develop the ability to remember more complex episodic-like memories (OPC). To our knowledge, this was the first demonstration of a cognitive delay in a rodent model of FXS and the first identification of a pre-symptomatic period during which potential interventions could be introduced. On a more general note, our findings highlight the value of studying behavioural developmental trajectories especially through carefully designed longitudinal rather than cross-sectional studies.

Longitudinal studies are fundamentally different from, cross-sectional studies, and have the power to answer important questions about cognitive development. While cross-sectional studies compare data from different groups, longitudinal studies track the same subjects over time. They therefore control for two problems: the lack of comparability across different groups, and the inability to answer questions regarding continuity in individual development. Although longitudinal studies only evaluate one group over time, which means that any findings might simply reflect conditions relevant at the time of data collection, they are nevertheless considered far superior to cross-sectional ones. But, they are also logistically more difficult to handle. Since longitudinal studies involve data collection from long periods of time, they can be really helpful in determining patterns.

Focussing on neurodevelopmental disorders, longitudinal studies can serve as a useful tool to test possible developmental pathophysiological hypotheses, in particular to investigated mechanisms involved in delayed emergence of behavioural anomalies, driven by developmental dysregulation. Taking into account that the sequence of key milestones in brain development are generally quite consistent between humans and rodents (Semple et al., 2013; Sengupta, 2013), longitudinal studies in rodent models can help us investigating how and when developmental sequences are disrupted in animal models, predict symptoms based on developmental markers identifiable during a pre-

symptomatic period, and help us identify critical periods suitable for intervention. What our data shows is that there is period during adolescence (P35-50) when neuronal networks supporting complex types of associative memory are still being refined. Despite indications that extensive synaptic changes take place during that period (Counotte et al., 2010), the vast majority of literature looking at synaptic maturation has been focussing so far in the first four postnatal weeks. There is a need for more in depth behavioural and physiological longitudinal studies in order to elucidate changes during adolescence which precede later behavioural deficits.

## 7.3 The early bird gets the worm

*"No one calls in question the fact that the experiences of the earliest years of our childhood leave ineradicable traces in the depths of the mind"*

Sigmund Freud 1899

Our findings clearly demonstrate that early pharmaceutical intervention with lovastatin fully restores cognitive development in *Fmr1* KO rats and leads to long lasting beneficial effects (Chapter 6). It is difficult to explain the mechanism by which a pharmacological (Dansie et al., 2013) or behavioural intervention (Oddi et al., 2014) leads to sustained effects, especially in the case of disorders with defined genetic aetiology, pre-existing any intervention such as FXS. It is possible that FMRP loss primarily has a profound effect during critical plasticity periods (Rhiannon M. Meredith et al., 2012), which precede cognitive deficits in adulthood. These developmentally coordinated changes in structural and plasticity phenotypes (Harlow et al., 2010; Till et al., 2012) and their consequences have not yet been fully investigated. However, it is possible that they illustrate the necessity of specific misregulated proteins, like FMRP, on specific neuronal circuits during development and maturation. Focussing on the spatiotemporal expression profiles of misregulated genes, important for neuronal function will expand our knowledge regarding which neuronal circuits and underlying behaviours are more susceptible to impairments in FXS and other neurodevelopmental disorders. An obvious consequence of the very existence of early time-windows, critical for phenotypic deficits is that they may constitute early periods of high susceptibility to therapeutic treatment. By comparing outcomes of treatment designs focussing on either young pre-symptomatic periods and during adulthood in animal models, the potential translational advantages from such an approach can be evaluated.

Another critical point when discussing the application of treatment order to correct neurodevelopmental impairments, during identified critical periods is that the brain undergoes multiple critical plasticity periods, spread across different brain areas, each of which has a unique underlying mechanism. Even within the same region of the nervous system, the same developing neuronal network could go through a sequence of critical periods, supported or triggered by distinct neurotransmitter or ion channel-dependent mechanisms. For instance, retinal circuits go through a series of simultaneous network activation from perinatal development onwards, which involve first gap junction coupling, followed by nicotinic cholinergic receptor signalling and lastly, glutamatergic neurotransmission to mediate activity during the later postnatal stages (Blankenship & Feller, 2010). Taking into account this complexity, the pathophysiology associated with a neurodevelopmental disorder could depend on abnormalities in the sequence of these critical periods or even synapse-specific impairments in a regional circuit. Intervention strategies will probably need a careful fine-tuning in order to correct abnormal molecular mechanisms and restore a balance in the affected networks.

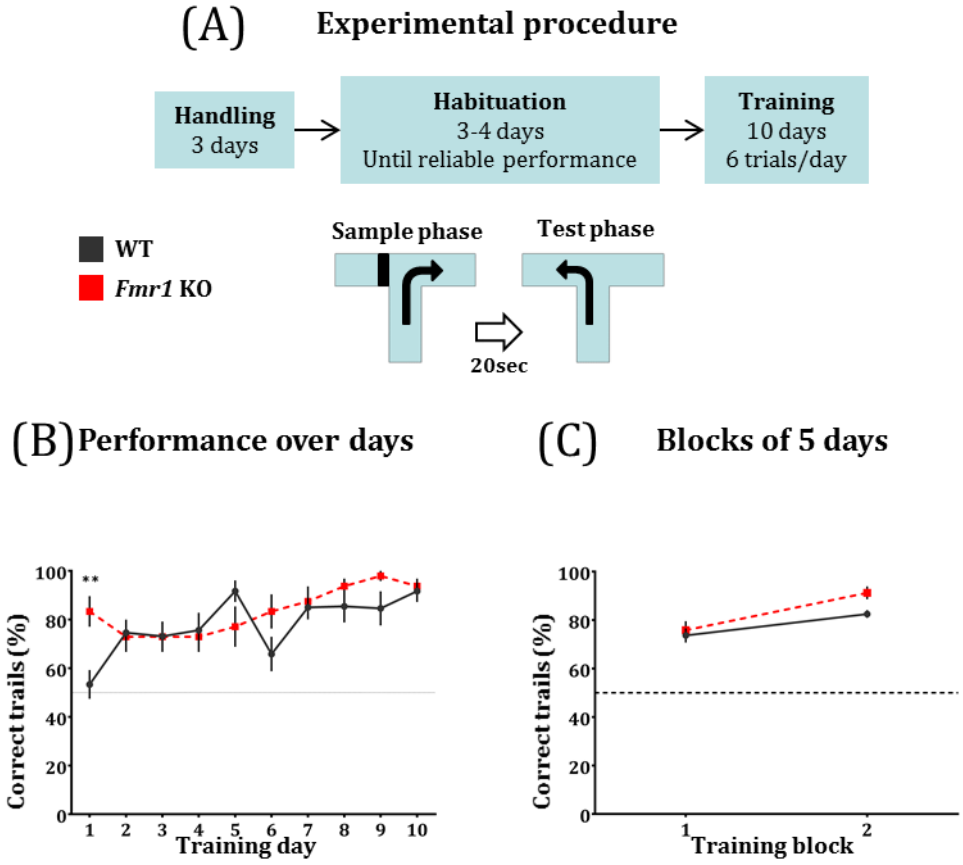
The extent to which late-stage pharmacological interventions could rescue the established deficits at specific neuroanatomical sites in FXS patients requires additional investigation. Although adult pharmacological interventions may be enough to reverse synaptic function, it seems unlikely that they will be enough to rewire abnormally formed circuits; this hypothesis could perhaps explain the limited efficacy of treatments over the relatively short duration of a clinical trial, seen so far. As a result, the FXS treatment field is moving toward pharmacological treatment trials with participants at the youngest possible age, in an effort to correct synaptic plasticity deficits early on in postnatal development; the hope is that normalizing circuit formation mechanisms will help improve behavioural impairments of FXS, on a long-term course. Future waves of treatment trials in FXS will likely deviate from previous studies, as researchers begin to use the information collected from earlier unsuccessful trials, in an attempt to eventually optimize study design specifically for FXS participants. For example, a case study of two young children with FXS showed that aggressive early combination treatment combined with intense educational intervention can lead to profound behavioural and cognitive improvement (Winarni et al., 2012). It's obvious that, for medications designed to be used by infants and young children, tolerability/toxicity will be a critical factor in determining which are appropriate for intervention that early in life. One of the potential

medications discussed earlier, lovastatin, has already been approved by FDA for the treatment of hypercholesterolemia in children (Tobert, 2003).

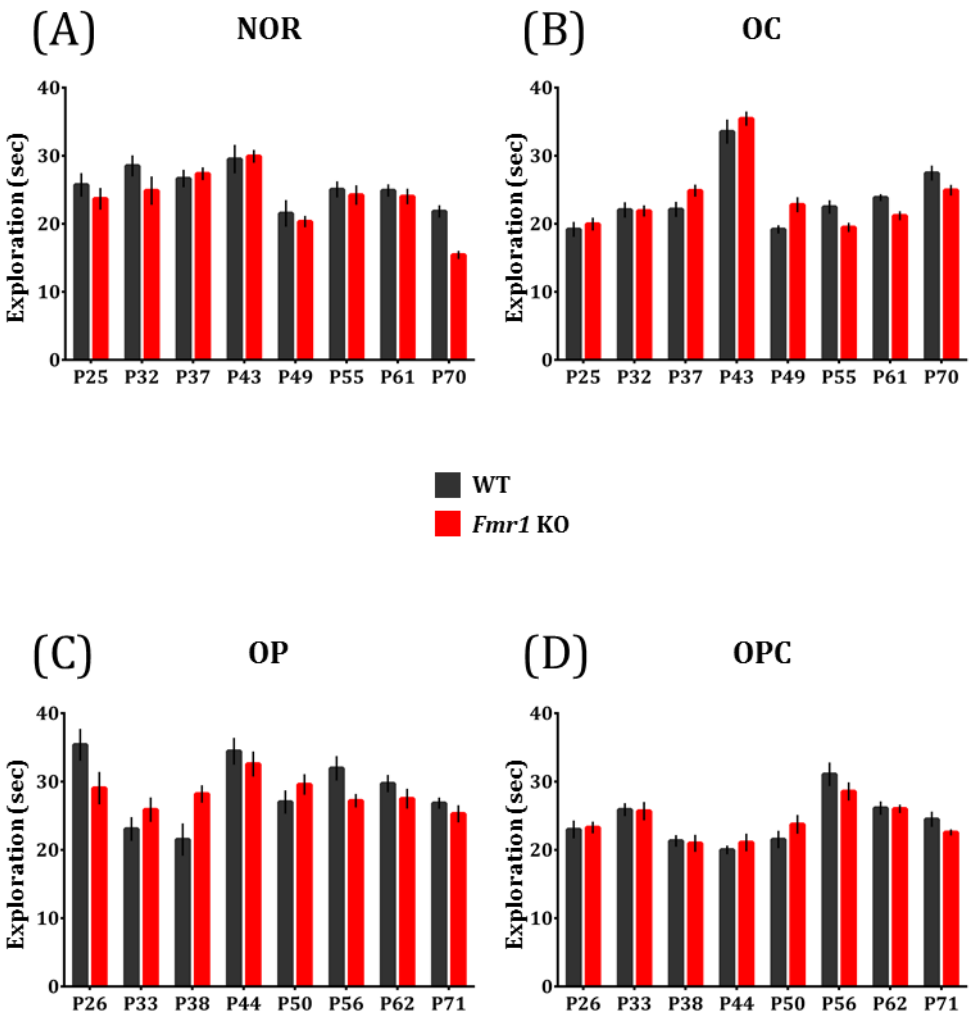
## 7.4 Changing direction in translational research

The low predictive validity of current animal models of FXS, as seen by the recent failure of many clinical trials, is at least partially an effect of the current direction in translational research. Animal model validity is currently being judged largely based on the identification of behavioural output which could be considered analogous to the multifaceted human phenotype. What this thesis is proposing is that animal models should be defined based on affected circuit mechanisms instead. We saw that common cellular pathophysiology between mouse and rat models of FXS leads to distinct behavioural deficits in rats (Till et al., 2015). Whether or not these behavioural deficits are consistent with human symptomatology is somewhat irrelevant; as long a human genetic lesion associated with a disease, leads to defined impairments from a molecular all the way to a behavioural level that makes an animal model valid and valuable to test the efficacy of candidate therapeutics.

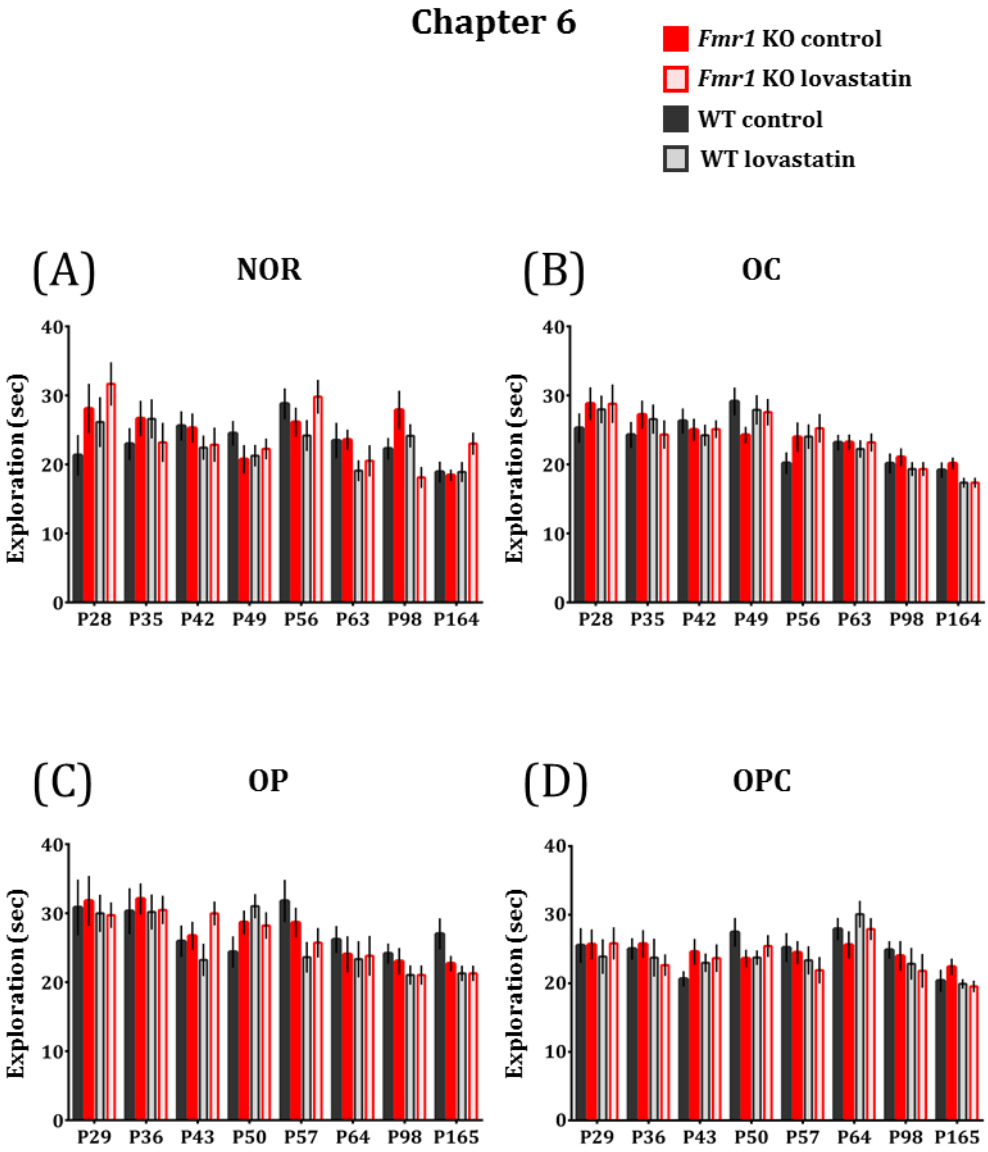
# 8. Supplementary figures



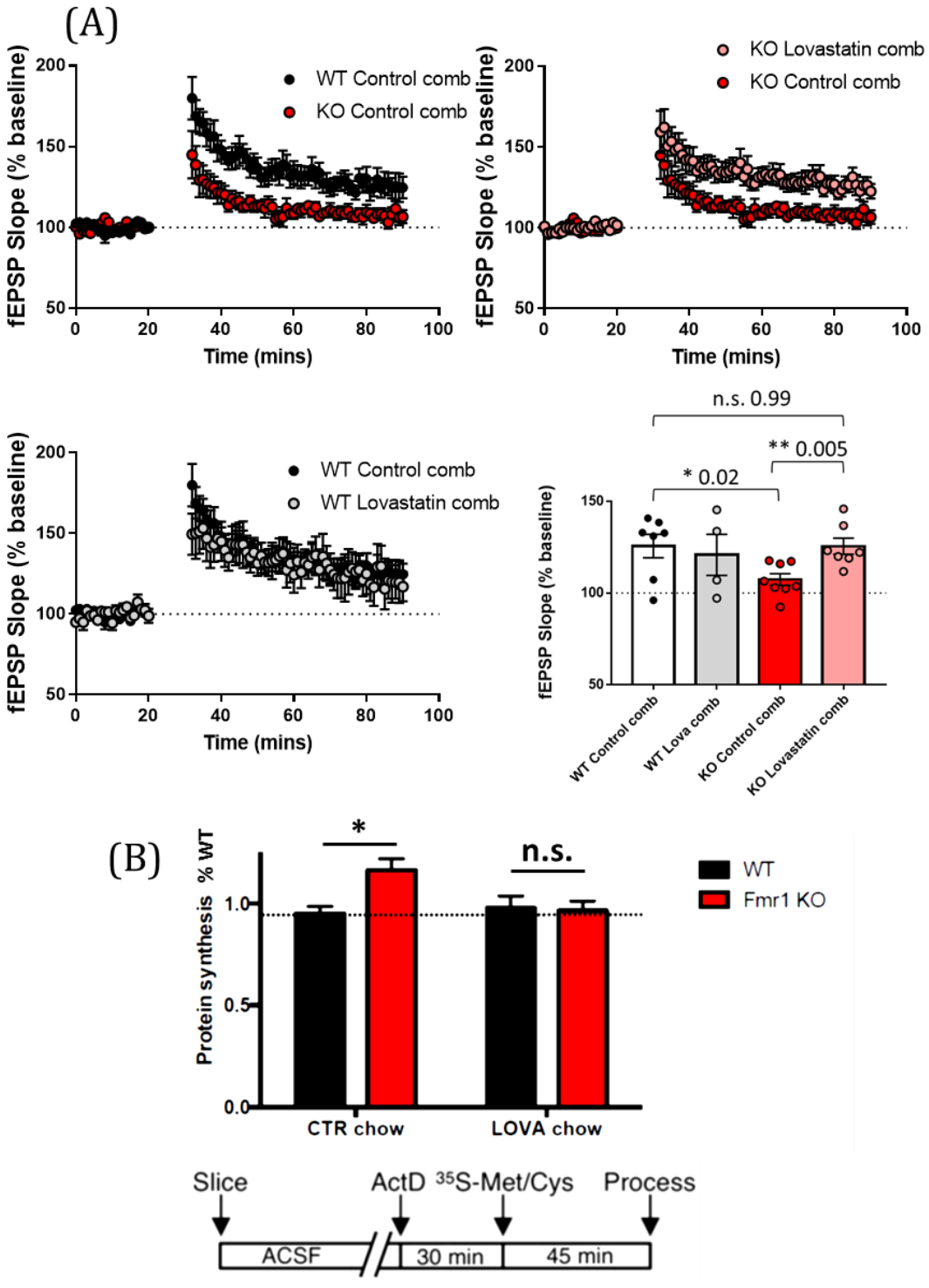

**Supplementary Figure 1. (B)** 2W ANOVA: Day x genotype $F(9,126) = 2.41$, $P = 0.01$; genotype $F(1,14) = 2.62$, $P = 0.13$; Day $F(9,126) = 4.94$, $P < 0.0001$ **(C)** 2W ANOVA: Block x genotype $F(1.14) = 2.33$, $P = 0.15$; genotype $F(1,14) = 2.62$, $P = 0.13$; Block $F(1,14) = 31.6$, $P < 0.0001$. Fmr1 KO (n = 8) WT (n = 8) Results are means ± s.e.m




**Supplementary Figure 2. Sample phase exploration** for NOR **(A)**, OC **(B)**, OP **(C)**, OPC **(D)** throughout testing. No differences between genotype groups. Fluctuation between different time points could be attributed to rats' different interest to objects used.




**Supplementary Figure 3.** Sample phase exploration for NOR **(A)**, OC **(B)**, OP **(C)**, OPC **(D)** throughout testing. No differences between genotype or treatment groups.




**Supplementary Figure 4.** Lovastatin treatment reverses long term plasticity deficits in prefrontal cortex after 5 weeks of treatment (A). 3 months after the end of treatment Fmr1 KO rats treated with lovastatin show corrected levels of basal protein synthesis in dorsal hippocampal slices from Fmr1 KO rats compared with WT littermate controls (B).

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