DEPARTMENT OF BIOMEDICAL AND BIOTECHNOLOGICAL SCIENCES

Ph.D. in BIOTECHNOLOGY

CURRICULUM IN BIOMEDICAL AND PRECLINICAL BIOTECHNOLOGY

XXXIV CYCLE

Alessandra Tempio

Role of 5-HT$_7$ receptors for serotonin in mitochondrial activity and in the pathophysiology of Fragile X Syndrome

PhD Thesis

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ACADEMIC YEAR 2019-2022

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LIST OF ABBREVIATIONS

4E-BPs	eiF4e-binding proteins
5-HT	Serotonin
5-HTR	5-HT receptor
aa	aminoacid
AC	Adenylate Cyclase
Ach	Acetylcholine
ADHD	Attention-deficit/hyperactivity disorder
AMPAR	Glutamatergic a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
APP	Amyloid precursor protein
APRA	Antibody-positioned RNA amplification
Arc	Activity-regulated cytoskeletal associated protein
ASD	Autism spectrum disorders
ATP	Adenosine tri-phosphate
CA	Cornu ammonis
cAMP	Cyclic adenosine monophosphate
CamKIIa	Calcium/calmodulin-dependent protein kinase type II alpha subunit
Cdk5	Cyclin-dependent Kinase 5
CE	Capillary electrophoresis
CFC	Contextual fear conditioning
CYFIP1	Cytoplasmic FMRP interacting protein
CLIP	Crosslinking-Immunoprecipitation
CNS	Central nervous system
CoQ	Coenzyme Q
DG	Dentate gyrus
DHPG	Dihydroxyphenylglycine
DNA	Deoxyribonucleic acid
DRN	Dorsal raphe nuclei
EC	Entorhinal cortex
EF1a	Elongation factor 1a
EF2	Elongation factor 2
EF2K	Eukaryotic elongation factor 2 kinase
eIF4	Eukaryotic initiation factor 4
ERK	Extracellular-signal regulated kinase
ES	Embryonic stem cells
FAD	Flavin adenine dinucleotide
FC	Fear conditioning
FMRP	Fragile x mental retardation protein
FXAND	Fragile X-associated neuropsychiatric disorders
FXPOI	Fragile X-associated primary ovarian insufficiency
FXS	Fragile x syndrome
FXTAS	Fragile-X associated tremor/ataxia syndrome
G4 structures	G-quadruplex structures
GABA	γ-aminobutyric acid
gDNA	Genomic DNA

GPCRs	G protein-coupled receptors
GTP	Guanosine-5'-triphosphate
H3K4me3	Tri-methilation on the lysin in position 4 of the Histon H3
H3K36me3	Tri-methilation on the lysin in position 36 of the Histon H3
H4K8ac	Acetylation on the lysin in position 8 of Histone H4
H4K19ac	Acetylation on the lysin in position 19 of Histone H4
HITS-CLIP	High-throughput sequencing crosslinking-Immunoprecipitation
hnRNPs	Heterogeneous nuclear ribonucleoproteins
IA	Inhibitory avoidance
ID	Intellectual disability
IP_3	Inositol trisphosphate
Kcc2	K^+-Cl^- cotrasporter
KO	Knockout
KSRP	KH-type splicing regulatory protein
LTP	Long term potentiation
LTD	Long term depression
m^7GTP	7-methyl- Guanosine-5'-triphosphate
mAChRs	Acetylcholine (ACh) muscarinic receptors
MAP1B	Microtubule associated protein 1b
mG3P-DH	Mitochondrial Glycerol-3-Phosphate Dehydrogenase
mGluRs	Metabotropic glutamate receptors
mGluR-LTD	Metabotropic Glutamate receptor- mediated long-term depression
MAP	Microtubule associated protein
MAPK	Microtubule associated protein kinase
MK	MAP kinase-activated protein kinase
MKK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
MNK	MAP kinase-interacting serine/threonine-protein kinase
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MRN	Median raphe nuclei
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NADH	Nicotinamide adenine dinucleotide
neo	Neomycin
NES	Nuclear export sequences
NKCC1	Na-K-2Cl cotransporter isoform 1
NLS	Nuclear localization sequence
Nova	Neuro-oncological ventral antigen
NPR	Neuronal pentraxin receptor
OXPHOS	Oxidative phosphorylation
PC	Purkinje cells
PCR	Polymerase chain reaction
PF	Parallel fiber
PI3K	Phosphoinositide 3-kinases
PIKE	PI3K enhancer
PKA	Protein kinase A
PKC	Protein kinase c

PLC	Phospholipase C
PNS	Peripheral nervous system
PP	Perforant path
PP2Ac	Protein Phosphatase 2A catalytic sub-unit
PSD	Post synaptic density
RGG box	Arginine-glycine-glycine box
RIP	Ribonucleoprotein Immunoprecipitation
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RSK	Ribosomal S6 kinase
Sod	Superoxide dismutase
STEP	Striatal-enriched tyrosine phosphatase
TACE	Tumor necrosis factor-α-converting enzyme
TFC	Tone fear conditioning
tRNA	Transfer ribonucleic acid
WT	Wild-type

Key words:

Fragile X Syndrome, Autism spectrum disorders, Mitochondria, Serotonin, 5-HT7, protein translation, Akt, Cdk5, LP-211, Synaptic plasticity, mGluR-LTD.

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This work was supported by a fellowship by the PhD Program in Biotechnology from the University of Catania (Catania, Italy).

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1 May 2021 – 15 December 2021

Abstract

Fragile X syndrome (FXS) is a genetic cause of intellectual disability and autism. *Fmr1* knockout (*Fmr1* KO) mice, a murine model of FXS, exhibit impairment in mitochondrial activity and in synaptic plasticity, with an exaggerated long-term depression induced by activation of metabotropic glutamate receptors (mGluR-LTD). Our research group has previously demonstrated that activation of serotonin 5-HT_7 receptors reverses the mGluR-LTD in the hippocampus of wild-type (WT) and *Fmr1* KO mice.

Here I highlighted some molecular mechanism involved in 5-HT_7 -mediated reversal of mGluR-LTD in the synapse between CA3 and CA1 pyramidal neurons using the patch clamp technique on hippocampal slices from wild-type and *Fmr1* KO mice. My data indicate that the blockade of cyclin-dependent kinase 5 (Cdk5) enhanced mGluR-LTD in WT hippocampal neurons to the level observed in *Fmr1* KO neurons and abolished the 5-HT_7 -mediated reversal of mGluR-LTD both in WT and *Fmr1* KO neurons, showing that Cdk5 is involved in 5-HT_7 –mediated reversal effect. In addition, my data indicate that Akt inhibition abolished the mGluR-LTD in WT, but not in *Fmr1* KO mice, pointing out that Akt is essential for mGluR-LTD only in WT slices. Moreover, in presence of an inhibitor of Akt, the effect induced by the activation of 5-HT_7 receptor on mGluR-LTD was not abolished; thereby 5-HT_7 -mediated reversal of mGluR-LTD does not require Akt activation. Then, I evaluated the role of protein synthesis on mGluR-LTD. When the inhibitor of mRNA translation anisomycin was present in the intracellular solution, mGluR-LTD was abolished in WT but not in *Fmr1* KO neurons, indicating that protein translation is necessary for mGluR-LTD only in WT neurons. Additionally, my data show that 5-HT_7 -mediated effect on mGluR-LTD was abolished in the presence of anisomycin, thus required protein translation.

Lastly, I demonstrated for the first time that 5-HT_7 receptors are present in mitochondria of a neuroblastoma cell line and the application of a 5-HT_7 inverse agonist weakly influenced the mitochondrial cytochrome c oxidase.

Sommario

La sindrome del cromosoma X fragile (FXS) è una malattia genetica ereditaria che causa disabilità intellettiva e autismo. Il modello murino della patologia, il topo *Fmr1* knock-out (KO), presenta alterazioni nella attività mitocondriale e nella plasticità sinaptica, fra cui una esagerata depressione a lungo termine mediata dall'attivazione dei recettori metabotropi per il glutammato (mGluR-LTD). Il nostro gruppo di ricerca ha precedentemente dimostrato che l'attivazione del recettore 5-HT_7 per la serotonina reverte mGluR-LTD nell'ippocampo di topi wild-type (WT) e *Fmr1* KO. Pertanto, durante il mio dottorato ho studiato alcuni meccanismi molecolari intracellulari implicati nella reversione della mGluR-LTD indotta dalla attivazione di recettori 5-HT_7 , utilizzando la tecnica del patch clamp in fettine di ippocampo di topi WT e *Fmr1* KO. I nostri dati indicano che il blocco della chinasi ciclina dipendente (Cdk5) aumentava mGluR-LTD in neuroni ippocampali WT ad un livello comparabile rispetto a quello osservato in neuroni *Fmr1* KO e aboliva la reversione della mGluR-LTD mediata dal recettore 5-HT_7 sia in neuroni WT che in *Fmr1* KO, indicando che l'attivazione di Cdk5 è necessaria per il meccanismo di reversione indotto dal recettore 5-$HT_{7.}$

Successivamente, ho valutato il ruolo della chinasi Akt nei meccanismi alla base della mGluR-LTD e nella sua reversione mediata da recettori 5-HT_7. L'inibizione di Akt aboliva la mGluR-LTD in neuroni WT ma non in neuroni *Fmr1* KO. Inoltre, l'attivazione del recettore 5-HT_7 era in grado di revertire mGluR-LTD nonostante la presenza di un inibitore della chinasi Akt, quindi l'attivazione di Akt non è necessaria per il meccanismo di reversione indotto dal recettore 5-HT_7.

Inoltre ho valutato il ruolo della sintesi proteica nella mGluR-LTD utilizzando l'anisomicina (un inibitore della sintesi proteica). In presenza di anisomicina, la mGluR-LTD era abolita in neuroni WT ma non in neuroni *Fmr1* KO e la reversione di mGluR-LTD mediata dall'attivazione del recettore 5-HT_7 era abolita, dimostrando che l'effetto indotto dai recettori 5-HT_7 richiede sintesi proteica.

Infine, i miei dati hanno dimostrato per la prima volta la presenza del recettore 5-HT_7 nei mitocondri isolati da una linea cellulare di neuroblastoma. L'attività di un agonista inverso per il recettore 5-HT_7 influenzava l'attività dell'enzima citocromo c ossidasi, conosciuto come il complesso IV della catena respiratoria mitocondriale.

CHAPTER 1: Introduction

Fragile X Syndrome (FXS) is a genetic form of intellectual disability associated with autism, mood disorders and epilepsy in about one third of patients (Berry-Kravis et al., 2011; Hagerman et al., 2017b). In FXS, the FMR1 gene is silenced; as a consequence, the expression of its gene product, the Fragile X Mental Retardation Protein (FMRP), is strongly reduced or entirely absent. FMRP rules the expression of a large number of synaptic proteins (Sidorov et al., 2013), which are essential for the correct function of cerebral circuits and for synaptic plasticity. Synaptic plasticity represents the ability of the nervous system to remodel the connectivity between neurons, modifying the functionality of synaptic networks. Among the different forms of synaptic plasticity described in the hippocampus, long-term depression induced by activation of metabotropic glutamate receptors (mGluR-LTD) plays an important role in learning and behaviour (Lüscher and Huber, 2010). In FXS, the congenital lack of the FMRP protein causes abnormalities in the morphology of synapses and in synaptic plasticity in brain areas responsible for learning and memory, including the frontal cortex and hippocampus. In *Fmr1* knockout (KO) mice, a murine model of this disease, dendritic spines display abnormal and immature morphology (Bakker et al., 1994; Comery et al., 1997), mGluR-LTD is exaggerated (Huber et al., 2002) and cortical mitochondria show an altered oxidative phosphorylation (D'Antoni et al., 2020; Griffiths et al., 2020; Licznerski et al., 2020).

Serotonin, or 5-hydroxytriptamine (5-HT), is a neurotransmitter involved in many physiological processes such as mood, perception, aggression, anxiety, appetite and respiratory stability (Abela et al., 2020; Cervantes-Durán et al., 2013; Cummings and Leiter, 2020; Hannon and Hoyer, 2008; Nichols and Nichols, 2008; Paulus and Mintz, 2016). 5-HT activates several receptor subtypes that influence the excitability of hippocampal neurons (Ciranna, 2006) and modulates hippocampus-dependent cognitive functions (King et al., 2008; Perez-Garcia and Meneses, 2008). The research group with whom I have worked during my PhD has demonstrated that activation of serotonin 5-HT7 receptors is able to reduce the excessive mGluR-LTD in *Fmr1* KO hippocampal neurons (Costa et al., 2012) and rescue learning and behaviour impairment in *Fmr1* KO mice (Costa et al., 2018), thus might became a novel pharmacological strategy for FXS therapy.

In the experimental work for my PhD thesis, I have studied the effects of activation of serotonin 5-HT7 receptors on long term synaptic plasticity in a murine model of FXS and on mitochondrial functions in an immortalized neuronal cell line.

1.1 Fragile X syndrome: genetic view

Fragile X Syndrome (FXS, OMIM #300624), also known as Martin-Bell Syndrome, was first described by Martin and Bell in 1943 as a form of intellectual disability (ID) following an X-linked inheritance (Martin and Bell, 1943). The disease takes its name from a rare fragile site called FRAXA (Xq27.3) on the X chromosome (Sutherland and Baker, 2000). A chromosome fragile site is a chromosomal locus that tends to form a gap or break in condensed metaphase chromosome following exposure of cells to DNA replication stress (Bjerregaard et al., 2018; Durkin and Glover, 2007). Using folate deficiency, it was possible to recognise the FRAXA fragile site at the tip of the X chromosome long arm, in Xq27.3 locus. Folic acid plays a critical role in maintaining genomic stability; it is required for DNA repair, to prevent chromosome breakage and to reduce DNA methylation. In case of folate deficiency, the incorporation of uracil into DNA rather than thymine leads to accumulation of dUMP, causing single- and double-stranded DNA breaks, chromosome breakage, and micronucleus formation (Leopardi et al., 2006; Lindberg et al., 2007). In 1969, Lubs reported a fragile site on the X chromosome and the association of the Xq27.3 fragile site with X-linked intellectual disability was confirmed in 1991 (Lubs, 1969; Verkerk et al., 1991).

Fragile X mental retardation protein (FMRP), the protein coded by FMR1 gene silenced in FXS, is an RNA binding protein with a prominent role in the regulation of many mRNAs in neuronal post-synaptic membranes (Hagerman et al., 2017a). The absence of FMRP is due to the expansion of a CGG triplet repeat in the 5′ untranslated region of the fragile X mental retardation 1 (FMR1) gene. This expanded CGG repeat coincided with the fragile site at the end of the X chromosome (Verkerk et al., 1991; Xie et al., 2016).

The FMR1 gene has about 40 kilobases (kb), encoding an mRNA of 3.9 kb, consisting of a ∼0.2 kb 5′ untranslated region, a 1.9 kb protein coding region, and a 1.8 kb 3′ untranslated region (Verkerk et al., 1991). It is composed of 17 exons and its pre-mRNA transcript is subjected to alternative splicing of exons 12 and 14 (Ashley et al., 1993; Verkerk et al., 1993): those lacking the exon 12 sequences are major products, while those lacking exon 14 appear expressed at a very low level (Sittler et al., 1996). The longest human *FMR1* mRNA, which shares 97% sequence identity with the mouse *Fmr1* ortholog, at the amino acid level encodes for a protein of 71 kDa (kilodaltons) with 632 aminoacids that contains a variety of functional sequences and domains, many of which are influenced by alternative splicing of the pre-mRNA (Denman et al., 2004; Dolzhanskaya et al., 2006; Sittler et al., 1996).

The expansion of the trinucleotide CGG located in the 5'-untranslated promoter region of FMR1 gene above normal range (greater than 54 repeats) is responsible for the development of fragile X-associated disorders in individuals carrying the premutation (55–200 CGG repeats), including fragile-X associated tremor/ataxia syndrome (FXTAS) (Hagerman et al., 2001; Jacquemont et al., 2003), fragile X-associated primary ovarian insufficiency (FXPOI) (Sherman, 2000) and fragile X-associated neuropsychiatric disorders (FXAND) (Hagerman et al., 2018) and causes Fragile X Syndrome (FXS) in patients carrying the full mutation (greater than 200 CGG repeats) leading to methylation, transcriptional silencing and to either the absence or a deficiency of FMRP (Salcedo-Arellano et al., 2020). *FMR1* premutation is associated with disorders that are caused by excessive transcription of *FMR1* (Tassone et al., 2007), in contrast to the gene silencing caused by the full mutation in individuals with FXS (Tassone et al., 2000).

Some individuals with FXS have mosaicism of CGG repeat lengths, with some cells harbouring full mutation alleles and others harbouring premutation alleles (Hagerman et al., 2017a). Other individuals with FXS have methylation mosaicism, with some cells containing methylated *FMR1* alleles and others with unmethylated *FMR1* alleles. Both types of mosaicism will support the production of some FMRP, so those individuals might have less-severe cognitive and behavioural defects than patients with a full mutation that is completely methylated, in whom FMRP is absent. With the frequent use of high-throughput targeted screening techniques and whole-exome sequencing in clinical practice, an increasing number of individuals with a deletion or point mutation in *FMR1* have been reported, which represent <1% of individuals with FXS (Myrick et al., 2014; Quartier et al., 2017). These mutations lead to a dysfunction or absence of FMRP, inducing FXS features which can be either different or similar to those of FXS patients with the full mutation.

Males with a full mutation almost invariably express some features of FXS, whereas females with a full mutation have a broad spectrum of symptoms ranging from severe impairment to apparently normal function (Zeesman et al., 2004). Although the repeat is highly stable when transmitted from individuals with normal alleles (6–44 CGGs), premutation alleles (55–200 CGGs) are unstable and tend to increase in size when passed from generation to generation and frequently expand to the full mutation in one generation (Fu et al., 1991; Nolin et al., 2003). This risk of full mutation expansion increases with maternal CGG repeat length to nearly 100% for mothers with >90 CGGs (Nolin et al., 2011), whereas a 56-repeat allele is the smallest known to expand to a full mutation in one generation (Fernandez-Carvajal et al., 2009). Nevertheless, the AGGs interspersed within the

FMR1 repeat region increase the stability of the gene (Eichler et al., 1994). The presence of even a single AGG significantly reduced the risk of full mutation expansions for alleles with <80 repeats (Nolin et al., 2015). This effect is most evident for alleles <70 repeats. As the total repeat length increases beyond 70, the allele instability is substantial despite the presence of the AGG interruptions they may contain. Once the repeat length exceeds 90 repeats, there is no apparent effect of AGG interruptions.

The gender of the transmitting parent is an important factor in the transmission of fragile X syndrome. It has long been accepted that individuals with fragile X syndrome have received their mutant allele from their mothers: the expansion to a full mutation occurs in maternal transmissions; virtually all premutation alleles from males are passed to daughters as premutation alleles (Nolin et al., 2003; Nolin et al., 2008; Nolin et al., 2019) although two rare examples of full mutation transmissions from fathers have been reported (Alvarez-Mora et al., 2017; Zeesman et al., 2004). Males with full mutations have full mutation alleles in their somatic cells, but only premutation size alleles are present in sperm (Reyniers et al., 1993; Willems et al., 1992).

1.2 Clinical aspects of Fragile X Syndrome

In FXS, the lack of FMRP, a regulator of translation, leads to dysregulation of hundreds of proteins that affect synaptic plasticity and connectivity in the developing brain, leading to intellectual disability (ID) and other clinical features of the syndrome (Danesi et al., 2018; Gatto et al., 2014; Higashimori et al., 2013; Pilaz et al., 2016; Wang et al., 2004).

The manifestations of FXS are variable and depend on sex, age, background genetic effects, environmental influence, level of gene methylation and presence of mosaicism, which lead to differences in FMRP level production (Dyer-Friedman et al., 2002; Hagerman, 2002; Loesch et al., 2004). Females typically have less-severe manifestations than males, as *FMR1* on the other X chromosome can produce FMRP. Cognition impairment involves 30% of individuals with an IQ less than 70 (intellectual disability), 30% with an IQ in the borderline range (Kates et al., 1997; Reiss et al., 1994) and 30% with an IQ in the normal range (above 80), but anxiety and attentional problems frequently occur (Hagerman et al., 2017a).

The physical features of FXS include long face, broad forehead, high palate, prominent ears, and in males macroorchidism that develops during the puberty (Hagerman, 2002; Heulens et al., 2013; Kidd et al., 2014). However, classic facial characteristics have differences inherent to age and ethnicity (Lubala et al., 2018). In addition to commonly recognized characteristics, patients can present variable alterations of connective tissue, attributed to FMRP dysregulation of essential components of the extracellular matrix, including elastin. Other manifestations of FXS related to loose connective tissue include hernias, joint dislocations and flat feet with pronation (Hagerman, 2002; Kidd et al., 2014). Phenotypic findings related to connective problems include soft velvet-like skin, joint hyperextensibility, particularly in the fingers, double jointed thumbs, flat feet with pronation, mitral valve prolapse, dilated aortic root and occasional scoliosis (Ramírez-Cheyne et al., 2019).

Infants with FXS are often affected by hypotonia, emesis due to frequent reflux events, an initial poor latch or suck with breastfeeding (Hagerman, 2002). Most patients present delays in language development and emerging hyperactivity, anxiety and sensory over-reactivity in the second year of life (Berry-Kravis et al., 2010; Cordeiro et al., 2011; Hogan et al., 2017). Recurrent otitis media is observed in >60% of patients in the first few years of life and usually requires the insertion of ventilation tubes (pressure-equalization tubes) to normalize hearing. After the first year of life, tactile defensiveness begins to emerge, individuals have poor eye contact and a tendency to hand-flap with excitement; hand biting or chewing on clothes are also common. Up to 20% of patients have crossed eyes or lazy eyes and if this persists after the first year of life, ophthalmological treatment is needed (Hagerman, 2002). Many children with FXS have emerging anxiety and sensory hyperarousal in their second year of life, and once they are able to walk, they typically become hyperactive (Verkerk et al., 1991). Indeed, 80% of boys with FXS have substantial hyperactivity by 3–4 years of age and are diagnosed with attention-deficit/hyperactivity disorder (ADHD), whereas only 40% of girls with FXS are diagnosed with ADHD by school age (Cornish et al., 2013; Cornish et al., 2007). Subjects with FXS have stronger and more frequent responses and reduced habituation to sensory stimulations (*e.g.*, olfactory, auditory, visual, tactile, and vestibular stimuli) as measured by electrodermal responses (Miller et al., 1999). Children begin overstuffing their mouth with food because of sensory deficits by 3 years of age, and obesity is reported in ~35% of patients by adolescence (McLennan et al., 2011). If hypotonia is a substantial problem during infancy, motor delays in sitting and walking might occur. Seizures occur in ~8–16% of males and 3–7% of females with FXS, typically present in the first 5 years of life, and are the most substantial medical problem

for children with FXS (Berry-Kravis et al., 2010; Kidd et al., 2014; Musumeci et al., 1999). Seizures are most commonly partial complex seizures but can also be generalized tonic–clonic or absence seizures. Symptoms of autism spectrum disorder (ASD) can develop during early childhood, and ~50–60% of males and 20% of females with FXS also have ASD (Harris et al., 2008; Kaufmann et al., 2004; Kaufmann et al., 2017; McDuffie et al., 2015; Roberts et al., 2009). Intellectual disability is common in males with FXS, although ~15% of males (predominantly those with mosaicism) and 70% of females have an IQ in the borderline to normal range but have learning and emotional problems (De Vries et al., 1996; Loesch et al., 2004). After puberty, there is a tendency for improvement of the most problematic behaviours during childhood, including aggression, hyperactivity and irritability. Nevertheless, many of the initial symptoms of FXS, such as anxiety and poor attention, persist into adulthood, and ~86% of males and 77% of females with FXS meet the diagnostic criteria for an anxiety disorder (Cordeiro et al., 2011). During adulthood, patients with FXS seem to have an increased risk of hypertension, obesity, gastrointestinal disorders, mood disorders and anxiety. 17% of patients with FXS can present with symptoms of parkinsonism and dementia (Sauna-Aho et al., 2018; Utari et al., 2010). However, patients with FXS have a normal life span. Individuals with FXS can also have sleep disturbances, mainly waking up in the middle of the night and not being able to go back to sleep, especially in the first 3–4 years of life (Hagerman, 2002).

1.3 Fragile X Syndrome diagnostic criteria

The diagnosis of FXS can only be confirmed using genetic testing through the identification of the CGG expansion. Prior to the identification of the *FMR1* gene, culturing cells in a folate-deficient medium followed by cytogenetic analysis was the method of choice for FXS diagnosis. However, this approach, while assessing for the presence of “fragile sites” (visualized as discontinuity of staining in the region of the gene) on the long arm of the X chromosome, proved to be difficult (Sutherland et al., 1985) as the fragile site was often seen only in small percent of cells. This was not as much as of a problem in males, where the fragile site could generally be seen in at least 10% of cells, but rather in female, where the mutation often could not be visualized.

The gold standard DNA methodologies for the diagnosis of FXS use a combination of polymerase chain reaction (PCR), particularly useful for CGG sizing within the premutation range and, Southern blot analysis for sizing larger alleles and for determining their methylation status (Tassone, 2015).

The genomic DNA (gDNA) can be isolated from whole blood, tissue, saliva or culture cells. Isolated gDNA can be amplified by PCR or digested with methylation sensitive restriction enzymes for

Southern blot analysis. Conventional PCR using primers that flank the CGG repeat can amplify *FMR1* alleles containing CGG repeat usually up the lower premutation range. The alleles can be visualized either on an agarose gel, on an acrylamide gel or by capillary electrophoresis (CE).

In particular, the use of the triplet-primed PCR assay is the preferred test worldwide, because it detects alleles throughout the expanded range, including the premutation in both males and females, and provides a much more accurate determination of allele size within the premutation range. Triple primer PCR assay utilizes two *FMR1* specific primers that flank the CGG repeat as well as a third primer that is complementary to the CGG repeat element (CGG primer). The PCR produces both full-length gene-specific *FMR1* amplicons as well as triplet repeat-specific products visualized on CE as a series of peaks. In addition, triplet-primed PCR enables the mapping of AGG interruption sequences, which are interspersed and present within the CGG region of *FMR1.*

Although several methodologies can amplify alleles throughout the full mutation range (Chen et al., 2010; Lyon et al., 2010; Saluto et al., 2005; Strom et al., 2007) they cannot determine methylation status, the epigenetic modification leading to FXS. This is of relevance for the diagnosis of FXS as the degree of methylation has been shown to be associated with the degree of intellectual disabilities and/or of the clinical involvement (Hagerman, 2002; Hagerman et al., 1994; McConkie-Rosell et al., 1993; Pretto et al., 2014; Snow et al., 1993). Methylation specific PCR approaches using bisulfite modification of the CGG repeat sequence are based on the conversion of unmethylated cytosine into uracil residues, with methylated cytosine remaining resistant to this modification (Susan et al., 1994). When amplified and sequenced, this “modified DNA” can provide information about methylation at specific CpG sites within the amplified DNA sequence (Laird, 2010).

1.4 Fragile X mental retardation protein (FMRP)

The origin of all changes that lead to the molecular, pathological and clinical symptoms shown by individuals with FXS is the absence or the deficiency of Fragile X mental retardation protein (FMRP). FMRP is the *Fmr1* gene product that belongs to the family of the heterogeneous nuclear ribonucleoproteins (hnRNPs), whose function is the regulation of mRNA metabolism (Bassani et al., 2013). FMRP is distributed in neurons throughout the mouse brain at all ages (Gholizadeh et al., 2015). The expression patterns of FMRP during development shows a decrement in mice, with high levels of expression at PN 7–14 and thereafter a progressive reduction (Bonaccorso et al., 2015; Gholizadeh et al., 2015; Lu et al., 2004). During this period, FMRP is mainly present in neurons of cingulate cortex, hippocampus, striatum and cerebellum but it is also present in astrocytes,

microglia and oligodendrocytes precursor cells in the developing brain (Gholizadeh et al., 2015). The correlation of peak levels of FMRP expression with synaptic formation, consistent with FMRP localization in synaptic structures, highlight a crucial role for FMRP in the formation, maturation, stabilization and elimination of synapses. Consistent with this idea, loss of FMRP results in increased synaptic number and morphological differences during early postnatal development (Antar et al., 2006; Bilousova et al., 2009; Nimchinsky et al., 2001).

The FMR1 gene encodes a total of 11 known FMRP isoforms in humans, as a result of alternative splicing (Zhang et al., 2019). These FMRP isoforms share a highly conserved N-terminal block of ~400 residues and variable C-terminal sequences with varying mRNA-binding affinities.

The most prevalent form of FMRP in humans contains 632 amino acids and is a classic RNA binding protein containing at least three canonical RNA binding motifs : two hnRNP K homology (KH1 and KH2) domains and an arginine-glycine-glycine (RGG) box in the C-terminal region (Nelson et al., 2013). A GXXG loop in the KH1 and KH2 domains of FMRP is conserved in many RNA-binding KH domains, such as the KH-type splicing regulatory protein (KSRP) and neuro-oncological ventral antigen (Nova-1 and 2) proteins, further suggesting that FMRP KH domains play a role in binding-specific RNAs (Hollingworth et al., 2012; Nicastro et al., 2015). A third KH domain was discovered upstream of the KH1 domain through x-ray crystallography, termed KH0 (Fu et al., 1991; Hu et al., 2015; Myrick et al., 2015). FMRP predominantly binds long mRNA (Li et al., 2020; Sawicka et al., 2019; Van Driesche et al., 2019) to the coding regions of mRNAs rather than 5' or 3'-UTRs, unlike most other RNA binding proteins (Richter and Zhao, 2021).

The amino terminal region of FMRP contains two Agenet/Tudor domains that interact with RNA, chromatin and other proteins (Adinolfi et al., 2003; Myrick et al., 2015; Myrick et al., 2014). FMRP also contains nuclear localization (NLS) and nuclear export sequences (NES) that direct its shuttling between nucleus and cytoplasm (Eberhart et al., 1996). At steady state, however, the protein is predominantly cytoplasmic.

Unlike other KH motif-containing RNA binding proteins, all three FMRP KH domains weakly bind single stranded RNAs and may require higher order secondary structures to confer specificity (Athar and Joseph, 2020). In an initial study, 432 FMRP-bound RNAs were identified through a ribonucleoprotein Immunoprecipitation (RIP) and 8 of the 12 top ranked recognized targets have a

G-quadruplex (G4) structure (Brown et al., 2001; Schaeffer et al., 2001). The RNA G-quadruplex is a secondary structure formed by sequences where guanine is the predominant base (Fay et al., 2017). G-tetrads are formed when guanines are organized into planar quartets where each base is connected to two other bases. When three or more G-quartets stack onto one another, they form a stable righthanded helical structure and in such vertical stacking, metal ions such as monovalent cations can intercalate into the central anionic core of a G-tetrad to coordinate, stabilize hydrogen-bonded tetrads, and enhance base-stacking interactions. The G-quadruplex regulates different steps of RNA metabolism; concerning FMRP interaction, it has also been shown to be involved in not only the regulation of translation but also mRNA transport along dendrites and axons (Beaudoin and Perreault, 2013; Melko and Bardoni, 2010). The FMRP RGG box can bind the G-quadruplex (Melko and Bardoni, 2010) which is present in several mRNAs, among which mRNAs coding for MAP1B (MicrotubuleAssociated Protein 1B), PP2Ac (Protein Phosphatase 2A catalytic sub-unit), APP, CamKIIa and Semaphorin3F (Darnell et al., 2001; Melko and Bardoni, 2010; Schaeffer et al., 2001).

The interaction between FMRP and the RNAs through a G-quadruplex structure was confirmed by another study where FMRP antibody-directed amplification of mRNA sequestered in FMRP-containing mRNPs (APRA) found G4 structures in many FMRP-bound mRNAs (Miyashiro et al., 2003). Nevertheless, RIP and APRA are lacking specificity and do not recognize the mRNA binding sites. In order to identify RNA-FMRP interaction, a crosslinking-immunoprecipitation (CLIP) was performed and a very large number of FMRP mRNA targets were identified in the mouse brain, most of which have been previously linked to autism (Maurin et al., 2018a).

Through a cumulative distribution of analysis, mRNA targets of FMRP carry a G4 forming motif. Thus FMRP regulates translation of mRNAs with G-quadruplex Structures (Edwards and Joseph, 2022) but not all mRNA targets harbour this motif (Melko and Bardoni, 2010).

The molecular bases of interaction between FMRP and RNA are still unknown. The sequence ACUK (K = G or U) and WGGA (W = A or U) are enriched in FMRP targets (Ascano et al., 2012) but they are essential but not sufficient to mediate the FMRP-RNA interactions (Maurin and Bardoni, 2018; Maurin et al., 2015; Suhl et al., 2014). Maurin and collegues identified a consensus sequence CTGKA bound by FMRP and two other less prominent motifs TAY and GWRGA (Maurin et al., 2018a). All the sequences can negatively modulate FMRP translational regulation consistent with the repressor function on translation made by FMRP. Nevertheless, FMRP was also found to stimulate translation of some target mRNAs. In addition to the RNA G-quadruplex secondary structure, FMRP can bind

mRNA through a SoSLIP (*Sod1* Stem Loop Interacting with FMRP) structure (Bechara et al., 2009). SoSLIP is a triple stem-loop structure and acts as an FMRP-dependent translational enhancer and as a mild internal ribosome binding site (IRES) in an FMRP-independent manner. FMRP enhances translation of the superoxide dismutase 1 (*Sod1)* mRNA when it interacts with the SoSLIP structure. Sod1 is an oxidative-stress-mitigating enzyme and in absence of FMRP, the enzyme expression is reduced, leading to an increased oxidative stress in the brain.

FMRP target mRNAs encode for proteins involved in cell signalling and in cell communication (Miyashiro et al., 2003), synaptic transmission and neuronal activity (Darnell et al., 2011; Maurin et al., 2018a; Van Driesche et al., 2019), transcription signalling (Sawicka et al., 2019), microtubule organization for axon transport (Maurin et al., 2018a; Sawicka et al., 2019; Van Driesche et al., 2019), mechanisms of circadian rhythm (Sawicka et al., 2019), neurogenesis and both axonal and dendritic morphogenesis (Li et al., 2020; Sawicka et al., 2019).

FMRP is widely detected in all mammalian tissues, with the highest expression levels in the brain and testes (Devys et al., 1993). In the adult brain, FMRP is highly expressed in the hippocampus, nucleus basalis and in the granule layer of the cerebellum (Bardoni et al., 2001; Cornish et al., 2007; Kim et al., 2009). In neurons, FMRP is detectable in the nucleus as well as in dendrites and axons, at both pre- and postsynaptic sites (Christie et al., 2009). In addition, FMRP was detected in the developing processes of oligodendroglia progenitor cells (OPCs) and immature oligodendrocytes in the neonatal brain, in primary cultures of oligodendrocytes, as well as in oligodendrocyte cell lines. FMRP belongs to the fragile X-related (FXR) family proteins, together with fragile X-related protein 1 (FXR1) and fragile X-related protein 2 (FXR2), which are the highly homologous RNA binding proteins (Majumder et al., 2020). The genes codifying for these proteins are Fragile X mental retardation 1 (*FMR1*), FMR1 autosomal homolog 1 (*FXR1*) and FMR1 autosomal homolog 2 (*FXR2*), and are located on different chromosome, respectively in Xq27.3, 3q26.33 and 17p13.1. The FXR proteins share approximately 60% amino acid sequence identity (Siomi et al., 1996). All three proteins have conserved regions for nuclear localization (NLS) and nuclear export (NES), which suggests a function in shuttling between cytoplasm and nucleus (Eberhart et al., 1996; Feng et al., 1997; Siomi et al., 1995; Zhang et al., 1995). They are involved in RNA binding by their two KH domains and an RGG box (Siomi et al., 1995; Zhang et al., 1995). The paralogs FXR1 and FXR2 are expressed in the same tissue and share the cellular profile of FMRP with only slight differences

(Agulhon et al., 1999; Bakker et al., 2000). FXR1 is expressed more abundantly in cardiac and skeletal muscle compared with FMRP and FXR2 (Bakker et al., 2000; Mientjes et al., 2004). In adult human brain, the FXRs protein have a cytoplasmic localization and a high expression in Purkinje, cortical and brainstem neurons (Tamanini et al., 1997). They have been observed in the nucleus of hippocampal neurons (Bakker et al., 2000). In foetal human brain, FXR2, like FMRP, is expressed in the cytoplasm of the neurons, but the FXR2 expression is lower than in adult brain. In adult brain FXR1 is only found in the cytoplasm of the neurons, while in foetal brain a substantial number of neurons also showed a nuclear localization (Tamanini et al., 1997). In brain tissues of FXS patients, FXR1 and FXR2 expression is unchanged compared to the normal control (Tamanini et al., 1997). The three proteins have RNA-binding properties and a ribosomal association, which indicates a role in the ribosomal and RNA metabolism of neurons. (Tamanini et al., 1997) However, the absence of FMRP in FXS leads to mental retardation despite the normal expression of FXR1 and FXR2 in neurons of FXS patients. Therefore, FXR1 and FXR2 are not able to compensate the lack of FMRP, having independent, although similar, cellular functions.

FMRP loss caused by *FMR1* gene mutation leads to an alteration of translation. The observation that hippocampal slices derived from *Fmr1* knockout mice, an animal model of FXS, incorporate 15–20% more ^{35}S-methionine into protein compared to wild type mice has supported the suggestions that FMRP is primarily a translational inhibitor (Dölen et al., 2007; Feng et al., 1997; Khandjian et al., 1996). It is widely believed that excessive protein synthesis is a major contributor to the pathophysiology in FXS.

FMRP can regulate the translation of its target mRNAs through multiple mechanisms: it can directly bind RNAs, regulate the translation initiation, bind polyribosomes and interact with RNA-Induced silencing complex (RISC).

Translation involves three broad steps: initiation, elongation and termination (Groppo and Richter, 2009). Initiation of translation begins with the eukaryotic initiation factor 4F (eIF4F), a multiprotein complex formed by eIF4E, eIF4G and eIF4A: eIF4E, also known as cap-binding protein, is responsible for binding the 5′-terminal 7-methyl-GTP (m^{7}GTP) cap found on all eukaryotic mRNAs; eIF4A is a subunit of an RNA helicase that unwinds secondary structure in the mRNA and eIF4G is the scaffolding subunit to which the other subunits bind and has a binding site for eIF3, which links the eIF4F-mRNA complex to the 40S ribosomal subunit. The 40S subunit scans the 5′ untranslated region

until the initiation codon, after which the 40S subunit is joined by the 60S ribosomal subunit to form an 80S ribosome that can elongate the polypeptide chain. Finally, the termination of translation occurs when the 80S ribosome dissociates from the mRNA at the termination codon, releasing the completed polypeptide. The inhibition of the translation process occurs when eIF4E-binding proteins (4E-BPs) bind to eIF4E. If 4E-BPs are phosphorylated by mammalian target of rapamycin complex 1 (mTORC1), the translation can start through the association of eIF4E with eIF4G (Gingras et al., 2001). In addition to this, eIF4E can be phosphorylated by MAP kinase-interacting serine/threonine-protein kinase 1 and 2 (MNK1-2), increasing the affinity of eIF4E for capped mRNA and for an associated scaffolding protein, eIF4G. This process leads to an enhancement of mRNA translation (Waskiewicz et al., 1999).

FMRP-mediated repression of translation requires an interaction with Cytoplasmic FMRP Interacting Protein CYFIP1 (Schenck et al., 2003; Schenck et al., 2001), which is known to be a non-canonical 4E-BP (Napoli et al., 2008). In the brain, FMRP helps recruit and stabilize CYFIP1 on the 5′ end of specific mRNAs to repress translation. This interaction is modulated by the activation of MNK1-2, which have a regulatory effect on long lasting synaptic plasticity (Panja et al., 2014). In *Fmr1*-KO mice, interactions between eIF4E and eIF4G are increased (Ronesi et al., 2012; Sharma et al., 2010), as well as eIF4E phosphorylation (Gkogkas et al., 2014). Thus, FMRP can directly and indirectly regulate translation initiation.

FMRP has a role in regulation of protein elongation through an interaction with polyribosomes: different studies have shown that FMRP co-sediments with polyribosomes during a sucrose gradient ultracentrifugation, suggesting a direct interaction with the translational apparatus (Corbin et al., 1997; Khandjian et al., 2004; Stefani et al., 2004). FMRP might inhibit translation at the level of polypeptide elongation slowing or stalling ribosome transit, thereby reducing the rate of the protein synthesis (Stefani et al., 2004). FMRP-regulated translation on polypeptide elongation might occur through the direct binding of the protein to the RNA, acting as a simple roadblock. In addition to this, FMRP can blocks tRNA association with the ribosome and regulate RNA degradation by optimal codon recognition (Shu et al., 2020). The use of 3-letter codons in mRNA leads to 64 codons that encode for 20 amino acids and translation stop signals (Hanson and Coller, 2018). This has caused the degeneracy in the genetic code, where different codons code for a single amino acid. These codons are recognized by the ribosome, which is characterized by a property called *codon optimality*, which refers to the non-uniform decoding rate of the ribosome. A codon can be defined as optimal or non-optimal depending on how efficiently the appropriate tRNA can be selected from

the cytoplasmic pool of tRNAs by the ribosomes. Codon bias is the propensity for some codons to be disproportionately represented in the transcriptome for codifying an aminoacidic and it is partially defined by codon optimality. This phenomenon affects ribosome translocation and RNA stability (Ascano et al., 2012; Hanson and Coller, 2018). Ribosome stalling has been shown to occur on RNAs with nonoptimal codons. The role of FMRP is to modulate the relationship between ribosome stalling and codon optimability (Shu et al., 2020); in particular, FMRP associates with optimal codons on the RNAs. Moreover, FMRP also prevents RNA degradation by inhibition of not yet known nucleases. FMRP does not associate with the translational machinery on RNAs with nonoptimal codons. On these RNAs, ribosome translocation is normal, but the mRNAs tend to be unstable because there is no FMRP to block nuclease attack. In FMRP-deficient cortex, RNAs with optimal codons are associated with normally translocating ribosomes, but the RNAs are unstable because there is no FMRP to block nucleases. Therefore, FMRP deficiency can lead to either increased or decreased RNA stability depending on their codon optimality status, which impacts the gene network controlling cellular functions (Shah et al., 2020b).

FMRP also regulates translation through its interactions with micro RNAs (miRNAs), the Argonaute Ago, also called Eif2c (Sasaki et al., 2003), **p**rotein of the RNA-induced silencing complex (RISC) (Edbauer et al., 2010; Jin et al., 2004; Muddashetty et al., 2011), Dicer and miRNA precursors (Cheever and Ceman, 2009).

In mice, FMRP is associated with the RISC and/or miRNAs such as miR-125a, miR-125b and miR-132 that cooperate to regulate protein synthesis involved in dendritic spine morphology (Edbauer et al., 2010; Muddashetty et al., 2011). FMRP regulates the accessibility of miRNA target sequence that are involved in the secondary structure of mRNA (Stefanovic et al., 2015). In absence of FMRP, dysregulation of miRNAs was demonstrated in Fmr1-KO mice (Liu et al., 2015) and in human FXS induced pluripotent stem cell derived neurons (Halevy et al., 2015).

FMRP also has deep effects on nuclear events such as DNA damage response, transcription, and splicing. Different studies point out that FMRP controls RNA synthesis through regulated translation of critical transcriptional factors or chromatin modulators (Korb et al., 2017; Shah et al., 2020a). Most of the RNA identified by Darnell and colleagues (Darnell et al., 2011) code for proteins with nuclear functions (110 out of 842), many of which modify chromatin, particularly histone acetylation

and methylation (Korb et al., 2017). Indeed, Fmr1-KO neurons have an enhancement of methylation on the lysin in position 4 of the Histon H3 (H3K4me3) and an increment of acetylation on the lysin in position 8 and 19 of Histone H4 (H4K8ac and H4K19ac). FMRP regulates chromatin-modifying proteins in addition to synaptic proteins. In particular, Brd4 is overexpressed in *Fmr1* KO mice and JQ1, a molecule that is able to inhibit Brd4, reduced gene expression of critical genes in *Fmr1* KO neurons. JQ1 also reversed behavioural phenotypes of *Fmr1* KO mice and mitigated aberrant dendritic spine density. Another chromatin modifier SETD2 is regulated by FMRP (Shah et al., 2020b). This protein induces trimethylation of lysine 36 on histone H3 (H3K36me3), mediating alternative splicing. When FMRP is absent, translation of SETD2 mRNA is elevated with an enhancement of H3K36me3 in *Fmr1* KO hippocampus, leading to mis-splicing events, which were also observed in human postmortem autistic brain (Corley et al., 2019).

Not only does FMRP modify chromatin through the regulation of mRNAs encoding for epigenetic and transcription factors, it also acts directly in the nucleus to bind and alter chromatin structure 85. By way of its Agenet domain, FMRP regulates the levels and positioning of gammaH2A.x, a histone H2 subtype associated with cell death, in response to replicative stress in mouse embryo fibroblasts and mammalian spermatocytes. Without FMRP, spermatocytes are unable to undergo DNA repair and resolve single stranded chromatin intermediates at the pachytene stage, a necessary event for meiotic progression. Whether FMRP directly regulates chromatin in neurons and if so whether it plays a role in activity and/or stress-induced cellular responses has not been assessed.

1.5 Animal models of Fragile X Syndrome

A better understanding of Fragile X Syndrome has been achieved thanks to the development of animal models, providing an increased knowledge about molecular, cellular and behavioural mechanisms underlying the pathology.

Animal models of FXS have been developed in various species, such as the Drosophila fruit fly, zebrafish, mouse, and rat (Bakker et al., 1994; Hamilton et al., 2014; McBride et al., 2012; Tucker et al., 2004). They show several symptoms in common with human patients such as defects in neuronal development, dendritic spine morphology, synaptic plasticity, and behaviour.

Much effort has focused on the characterization of mouse models of FXS, in particular the *Fmr1* knockout (KO) mouse. Mice and men share almost 99% of their genes (Waterston et al., 2002) as well as most physiological functions and pathogenic mechanisms (Eilam, 2014; Tecott,

2003). Since mice are also easy to keep, they became the most widely used model organism in life sciences.

The first *Fmr1* KO mouse was created and characterized by the Dutch-Belgian Fragile X Consortium (Bakker et al., 1994). The model was generated using a homologous recombination targeting vector, pMG5, containing a disrupted *Fmr1* DNA: exon 5 was interrupted by the positive selection marker gene neomycin (neo) while the negative selection marker inserted was the thymidine kinase gene. The vector pMG5 was introduced into the embryonic stem cells (ES). A clone was injected into C57BL/6J blastocysts and transferred to pseudo-pregnant females. This *Fmr1* KO mice harbouring this mutation did not produce FMRP protein but did possess detectable levels of *Fmr1* mRNA because of the presence of Fmr1 promoter (Yan et al., 2004). To remove the Fmr1 mRNA, a second generation model was created known as Fmr1-KO2 (Mientjes et al., 2006), where the first exon was modified to remove the promoter region. This second-generation model has been largely used for brain studies, focusing on understanding the neurobiological underpinnings of FXS.

Fmr1 KO2 mice share different features with FXS patients such as having significantly heavier testes than wildtype controls, but normal structural morphology (Mientjes et al., 2006), probably due to an increase in the proliferative activity of Sertoli cells in the seminiferous tubules, which increases the number of germs cells in the testicles, and therefore, their weight.

In spite of patients suffering from FXS, *Fmr1* KO mice have not been reported to display spontaneous seizures, but are more susceptible to audiogenic seizures, induced by exposure to a 125 decibel, high-intensity siren (Musumeci et al., 2000). This audiogenic seizure vulnerability in *Fmr1* KO mice is a readout of seizure susceptibly in FXS patients. Electrophysiological recordings from auditory cortex of *Fmr1* KO mice revealed an enhancement of responses to auditory tones, demonstrating that neurons of *Fmr1* KO mice are hyper-responsive to stimuli (Rotschafer and Razak, 2013). These data are consistent with the increased responses to pure tones seen in individuals with FXS (Rojas et al., 2001).

In line with the clinical features of FXS patients, attention and impulsivity were evaluated in *Fmr1* KO mice through the five choice serial reaction time test (Winstanley et al., 2006). This test assesses attentional performance by the detection of a brief visual stimulus presented randomly across several spatial locations, in five nose-poke holes box: the animal is required to perform a nose-poke response to obtain a food response in one of five response apertures only when a stimulus light located there is illuminated. After beginning a trial and prior to illumination of a stimulus light, there is a 5-s inter-trial interval during which the animal must withhold from responding in the apertures.

Any responses made during this time are described as premature responses and are punished. These premature responses provide another way of measuring motor impulsivity. At the end, mice were subjected to two final tests, one to measure sustained attention and one to measure inhibitory control. *Fmr1* KO mice were impaired in the acquisition of a visuospatial discrimination task but did not display deficits in sustained attention or inhibitory control compared to wild-type mice. In addition to this, *Fmr1* KO mice demonstrated heightened perseveration and responding during novel rule acquisition, which normalized with training (Kramvis et al., 2013).

Nevertheless, in other attention tests, *Fmr1* KO mice displayed altered inhibitory control, having a higher rate of premature responses than wildtype mice (Moon et al., 2006). This was associated with changes in task contingencies, suggesting that inhibitory control in *Fmr1* KO mice may be affected by stress or novelty. In addition to this, *Fmr1* KO mice are characterized by an enhancement of locomotor activity compared to wild-type controls in the open field test (Bakker et al., 1994; Dahlhaus and El-Husseini, 2010; Ding et al., 2014; Mineur et al., 2002; Peier et al., 2000; Pietropaolo et al., 2011; Restivo et al., 2005; Spencer et al., 2005).

Fmr1 KO mice also exhibited higher levels of self-grooming, a repetitive behaviour, than wild-type controls (McNaughton et al., 2008; Pietropaolo et al., 2011). These behavioural features are consistent with perseveration and repetitive behaviour found in FXS patients (Hagerman et al., 2017b). Additionally, as a ratio of repetitive behaviour (Thomas et al., 2009), in the marble burying test *Fmr1* KO mice buried more marbles (Gholizadeh et al., 2014; Spencer et al., 2011).

Anxiety is a main trait of FXS in young and adult patients. Evaluation of anxiety levels in *Fmr1* KO mice leads to contrasting results depending on the different protocols, genetic background and on tested age. To evaluate the level of anxiety it is possible to use the elevated plus-maze: this test uses an elevated, plus-shaped (+) apparatus with two open and two enclosed arms: the animals prefers to spend more time in darker enclosed arms rather than stay in the lighted open arms (Lister, 1987) and exploration in open area is associated with decreased anxiety. Some studies show that *Fmr1* KO mice spent significantly more time in the open arms and less time in the closed arms compared to wild-type littermates (Heulens et al., 2012; Liu et al., 2011; Peier et al., 2000; Yuskaitis et al., 2010), suggesting reduced anxiety which is contrary to human findings (Cordeiro et al., 2011; Ezell et al., 2019). It has been suggested that increased open arm exploration is potentially indicative of increased locomotor activity or hyperactivity rather than decreased anxiety (Heulens et al., 2012). Otherwise in some studies, no behavioural differences were detected in *Fmr1* KO mice as compared to wild-type littermates in the elevated plus-maze (Mineur et al., 2002; Nielsen et al., 2002; Yan et

al., 2004). Using the zero-maze test, a modification of the plus-maze with the advantage of lacking the ambiguous central area of the elevated plus-maze, *Fmr1* KO mice spent more time in the open area (Liu et al., 2011; Liu and Smith, 2009). Results obtained using the open field test are contrasting. In this test, wild-type mice display a natural aversion to brightly lit open areas, thereby the time spent in the centre of the open arena is considered as an indicator for low anxiety. In some studies, *Fmr1* KO mice spent more time in the centre of the open field (Peier et al., 2000; Spencer et al., 2005; Yan et al., 2004; Yuskaitis et al., 2010), but in others *Fmr1* KO mice avoid to stay in the centre of the open field (Restivo et al., 2005) and in others no differences were noticed between *Fmr1* KO and wild-type (Veeraragavan et al., 2011a; Veeraragavan et al., 2011b; Veeraragavan et al., 2012).

Individuals affected by FXS suffer from social phobia and avoidance (Cohen et al., 1988; Cordeiro et al., 2011; Hagerman et al., 2017b; Hall et al., 2009). Studying the social behaviour of FXS murine model, data show contradictory results. In the three-chamber sociability test, which aims to assess cognition in the form of general sociability and interest in social novelty, rodents normally prefer to spend more time with another rodent (sociability) and will investigate a novel intruder more often than a novel object stimulus (Yang et al., 2011). In some studies, *Fmr1* KO mice have normal social behaviour, preferring to spend more time exploring the novel mouse (Liu et al., 2011; Liu and Smith, 2009; McNaughton et al., 2008; Pietropaolo et al., 2011). On the other hand, other studies show that *Fmr1* KO mice do not display a preference for the novel mouse over the novel object (Dahlhaus and El-Husseini, 2010) and spend less time to sniff the novel mouse during social interactions (McNaughton et al., 2008; Pietropaolo et al., 2011). Another readout of social interactions in mice is the pup ultrasonic vocalization (Ehret, 2005; Fischer and Hammerschmidt, 2011): *Fmr1* KO pups show a decrease in terms of emitted vocalization (Gholizadeh et al., 2014; Rotschafer et al., 2012; Roy et al., 2012)

Several cognitive tests were performed to characterize the intellectual ability of the murine FXS model. Passive avoidance is an associative learning task depending on hippocampus (Lorenzini et al., 1996) and amygdala (Slotnick, 1973), in which the animal makes an active choice to avoid entering in a dark compartment associated with an aversive event like a foot shock. Passive avoidance appears to be disrupted in *Fmr1* KO mice (Ding et al., 2014; Michalon et al., 2014; Michalon et al., 2012; Veeraragavan et al., 2011a; Yuskaitis et al., 2010). In addition to this, passive avoidance extinction happens faster in *Fmr1* KO mice than in wild-type (Dölen et al., 2007; Michalon et al., 2014). Fear conditioning is another behavioural test used to characterize emotional aspects of cognition in rodents. It could be contextual or delay-cued: the first requires the amygdala and the

hippocampus, while the second solely depends on the amygdala (Fanselow et al., 1994; Logue et al., 1997; Phillips and LeDoux, 1992). In delay-cued fear conditioning, an altered behaviour was reported in *Fmr1* KO mice (Ding et al., 2014; Paradee et al., 1999), but other studies did not observe any difference between wild-type and *Fmr1* KO mice (Dobkin et al., 2000; Uutela et al., 2012; Van Dam et al., 2000). In contextual fear conditioning, results are also contrasting: some studies have shown a deficit in contextual discrimination (Auerbach et al., 2011) but in other studies no differences were detected (Dobkin et al., 2000; Peier et al., 2000; Van Dam et al., 2000). In the water maze test, a hippocampus-mediated task where visual spatial abilities are tested, mice must learn to find a hidden platform in a pool of opaque water. A learning deficit for *Fmr1* KO mice was observed in the reversal phase of the test where the position of the hidden platform is suddenly changed (Bakker et al., 1994; Boda et al., 2014; Kooy et al., 1996; Nolan and Lugo, 2018). To test the cortex- and hippocampus-dependent novelty detection ability, novel object recognition test was performed (Broadbent et al., 2010). This test is based on the spontaneous tendency of mice to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the efficiency of learning and recognition memory, which are impaired in *Fmr1* KO mice (Costa et al., 2018; Franklin et al., 2014; Gomis-González et al., 2016; King and Jope, 2013; Ventura et al., 2004), since less time is spent exploring the novel object. This data is consistent with human studies demonstrating alterations of novelty preferences in autism spectrum disorder (Hagerman et al., 2017b).

1.6 Synaptic plasticity in the hippocampus

The hippocampus is a brain region playing a crucial role in the formation and storage of episodic and semantic declarative memories (Scoville and Milner, 1957; Squire et al., 2004). A famous study conducted by Dr. Brenda Milner on H.M. patient confirmed that two different kinds of memory exist: the declarative memory, allowing the formation of memories about experiences, and the procedural memory, which controls behaviour without awareness of learning. H.M. suffered from epilepsy not pharmacologically treatable, therefore his hippocampus was surgically removed in both brain hemispheres. After the surgery, the epilepsy improved but an anterograde amnesia for declarative memory was manifested, while procedural memory remained intact. This study revealed that the hippocampus plays a critical role in formation and retrieval of declarative memory.

The hippocampus consists of dentate gyrus (DG), cornu ammonis (CA) 1, CA2, CA3 and CA4 (Amaral and Witter, 1989; Swanson et al., 1978). Input from the entorhinal cortex (EC) is transmitted to the DG, CA1 and CA3 regions via perforant path fibers; DG neurons project to CA3 pyramidal neurons via mossy fibers, CA3 neurons send fibers to CA1 pyramidal neurons via Schaffer collaterals), and CA1 neurons in turn project back to the cortex unidirectionally forming the "tri-synaptic hippocampal circuit". Each pathway contributes to synaptic transmission and plasticity in the hippocampus, by forming synaptic circuits for storage, consolidation and retrieval of declarative, spatial, and associative long-term memory (Burgess et al., 2002; Gold and Kesner, 2005; Nakazawa et al., 2001; Squire et al., 2004). The main source of excitatory glutamatergic signals to the hippocampus come from the EC. The EC directs spatial and non-spatial information to the hippocampus (Van Strien et al., 2009), in particular layer 3 cells project to CA1 as the temporoammonic pathway, while layer 2 cells of EC project excitatory axons through the perforant path (PP) to granule cells in the DG (Kerr et al., 2007). The hippocampal CA1 region provides an output from the hippocampus, sending signals to several parts of the brain, such as the subiculum, lateral septum, ventral striatum, amygdala, prefrontal cortex and retrosplenial cortex (McNaughton et al., 1996; Squire et al., 2004; Van Groen and Wyss, 1990; Wyass and Van Groen, 1992). All together this studies show that the hippocampus recruits several brain regions to form learning and memory circuits (Duncan et al., 2012; Lisman and Grace, 2005).

Memory formation and learning require a plastic arrangement of synaptic connectivity based on modifications in the strength and number of synapses (Kessels and Malinow, 2009; Middei et al., 2014). By modifying the synaptic connection network and the structural and morphological organization of neurons, the nervous system can either strengthen or weaken the efficacy of a specific neuronal circuit, based on functional requests. Synaptic plasticity in the hippocampus assists with consolidation and storage of long-lasting memories.

Synaptic strengthening and weakening depend on exocytosis and endocytosis of glutamatergic a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) for glutamate (Malinow and Malenka, 2002). In excitatory synapses, AMPARs go through translocation into or removal from synapses (Lu et al., 2009). These receptors are made of tetramers of GluA1-4 subunits. In the hippocampus, the main subunits are GluA1 and GluA2 and in the CA1 region GluA1/2 heterodimer is predominant (Clem and Barth, 2006; Plant et al., 2006). The presence of GluA2 leads to lack of permeability to Ca^{2+} flux through AMPARs. Sensory experience or long-term potentiation plasticity

cause an expression of AMPARs without GluA2 in hippocampal region. Exocytosis and endocytosis of AMPARs are influenced by the activity of kinase and phosphatase: phosphorylation of GluA1 induces the exposure of AMPARs to synapses, whereas GluA1 de-phosphorylation is associated with AMPARs endocytosis and related synaptic weakening. Increased expression and phosphorylation of GluA1 subunit in AMPARs in brain regions involved in long-term plasticity support the persistent of memory. For example, inhibitory avoidance (IA) learning (a hippocampus-dependent task during which rodents learn to avoid the dark compartment of a two-chamber apparatus after administration of an electric foot-shock delivered during the IA training) induced an increase of AMPARs trafficking into hippocampal synapses (Whitlock et al., 2006). In particular, a fast and transient increase of GluA1 and GluA2 subunits in synaptosomal fractions were observed with an enhancement of phosphorylation of GluA1 at Ser831, a reaction that is associated with AMPARs delivery to synapses (Hayashi et al., 2000; Heynen et al., 2000). Fear memory also leads to synaptic trafficking of GluA1-containing AMPARs into dendritic spines, which are going to grow to support the formation of a new memory circuit. In different fear conditioning protocols, rodents learn to associate an electric foot-shock with a sound (tone fear conditioning, TFC), or with a context (contextual fear conditioning, CFC) in which the shock takes place. 24 hours after TFC, newly synthesized GluA1 are recruited in dendritic spines of hippocampal CA1 neurons due to memory formation, going back to the control condition 72 h after TFC training (Matsuo et al., 2008). The same results were obtained during the CFC, with an enhancement of dendritic spines density (Restivo et al., 2008), GluA1 levels and phosphorylation of Ser845 and Ser831 in isolated CA1 hippocampal Post Synaptic Densities (PSDs), a dense ore of dendritic spines containing receptors and scaffolding proteins (Middei et al., 2012).

After the acquisition, a memory can be consolidated, becoming a medium- or a long-term memory: following re-exposition to reminders of the original episode, a stored memory can be recalled. Thereby memory can either stabilize to persist, a process known as reconsolidation, or be extinguished. The expression of GluA1 is also involved in consolidation, since the level of GluA1 in the hippocampus follows a two wave fluctuation: a peak happens 1 hour after the FC training in mice, followed by a decrease 3 days after and back to an enhancement 28 days later (Thoeringer et al., 2012).

Reconsolidation and extinction can be tested in fear conditioning or in inhibitory avoidance protocols. During reconsolidation, a fluctuation is observed in the expression of GluA2, which are

removed from hippocampal synapses 1 h after protocol re-exposure. GluA2 internalization is associated to a synaptic weakening, measured by recordings of glutamate-mediated post synaptic current in the CA3-CA1 synapse. During the maintenance of reconsolidation phase, the level of GluA2 become stable (Rao-Ruiz et al., 2011).

Both potentiation and depression of synaptic plasticity lead to structural changes such as modulation of the number, size and shape of dendritic spines (Matsuzaki et al., 2004; Okamoto et al., 2004), which are specialized for synaptic transmission.

Other forms of plasticity occurring in hippocampus are short-term plasticity phenomena, which can either potentiate or weaken a circuit from milliseconds to several minutes, being respectively a short-term facilitation and short-term depression. These forms of plasticity are considered to be important for short-term memory (Citri and Malenka, 2008; Zucker and Regehr, 2002).

Metaplasticity, an additional kind of synaptic plasticity, was also discovered in the hippocampus (Abraham and Bear, 1996) and is also called "the plasticity of synaptic plasticity", because it involves activity-dependent changes in neuronal function that modulate synaptic plasticity. However, several reports have indicated that it may also serve to stabilize synapses (Baione et al., 2020; Crestani et al., 2019; Gebhardt et al., 2019; Hegemann and Abraham, 2019; Lutzu and Castillo, 2021; Yang et al., 2014).

A further type of plasticity that controls the total synaptic strength of a neuron is homeostatic plasticity (Turrigiano and Nelson, 2004). It can either increase or decrease the strength of all synaptic inputs of a neuron to keep homeostasis over a wide range of spatial and temporal scales. Homeostatic plasticity regulates synaptic scaling and is thought to stabilize synaptic strength at the level of a single neuron.

Synaptic strengthening and weakening persisting for several hours are respectively defined as long term potentiation (LTP) or long term depression (LTD) (Kessels and Malinow, 2009). LTP and LTD are two well-known cellular events for synaptic changes thought to occur during mnesic processes.

LTP and LTD can be elicited by activation of *N*-methyl-D-aspartate receptor (NMDAR) (Collingridge et al., 1983). NMDARs are ionotropic receptors permeable to sodium, potassium and calcium: at negative membrane potentials close to the resting membrane potential, magnesium ions enter the pore of the NMDAR, blocking the passage for all other ions (Lüscher and Malenka, 2012). Upon depolarization the magnesium is expelled from the pore, allowing sodium, potassium, and, importantly, calcium ions to pass. The activation mode of NMDA and the amount of Ca^{2+} influx are the discriminating factors to induce LTP or LTD. For example, in acute hippocampal slices, high

frequency stimulation (100 Hz) of Schaffer collateral fibers causes a large depolarization of the postsynaptic cell that is sufficient to remove the Mg^{2+} block of NMDARs and allow increases of cytosolic Ca^{2+} up to 5 µM in CA1 neurons, inducing the activation of protein kinases which are responsible for induction and maintenance of LTP. Conversely, stimulation at 1 Hz causes a Ca^{2+} influx across NMDARs up to 1 µM, leading to activation of protein phosphatases which dephosphorylate AMPA receptors, increasing their endocytosis.

Moreover, LTD or LTP can be induced by activation of different types of G protein-coupled receptors, such as M1 and M3 muscarinic receptors or I group metabotropic glutamate receptors (mGluR1 and mGluR5). Acetylcholine (ACh) muscarinic receptors (mAChRs) can induce plasticity at excitatory and inhibitory synapses and are essential in learning and memory processes (Fernández de Sevilla et al., 2021). M1 and M3 mAChRs are coupled to phospholipase C (PLC) via G-proteins (Gq/11). The activation of PLC catalyzes the phosphatidylinositol 4,5-bisphosphate hydrolysis and inositol 1,4,5-trisphosphate (IP3) and diacylglycerol are produced. IP3 receptor (IP3R) activation induces Ca2+ release from endoplasmic reticulum (ER) stores (Rose and Konnerth, 2001). It has been shown that M1 and M3 mAChRs activation triggers IP3 production and Ca2+ release from the ER in CA1 pyramidal neurons, resulting in LTP at Schaffer's collaterals synapses. This LTP is NMDAR independent and is expressed postsynaptically by an increase of AMPARs in spines and an enhanced NMDA response. In addition, activation of mAChRs can also induce LTD (mAChR-LTD) of excitatory synaptic transmission in various brain regions, such as visual cortex (McCoy et al., 2008), perirhinal cortex (Jo et al., 2006) and hippocampus (Volk et al., 2007). The activation of M1 receptors results in an LTD that is dependent on the activity of protein tyrosine phosphatases (PTPs), but is independent of Ca^{2+}, PKC, serine/threonine protein phosphatases and protein synthesis (Dickinson et al., 2009).

As well as muscarinic receptor, group I metabotropic glutamate receptors (mGluRs) can induce LTP and LTD. The mGluR-LTP activation of Type I mGluRs and depends crucially on protein synthesis controlled by Fragile X Mental Retardation Protein and on Arc signaling (Wang et al., 2016a). Although group I of mGluRs modulate the induction of NMDAR-LTP in different synapses (Abraham, 2008), LTP that exclusively needs the mGluRs but not NMDARs has only been identified in the subiculum (Fidzinski et al., 2008).

1.7 Metabotropic Glutamate receptor-induced long-term depression (mGluR-LTD)

A particular form of LTD can be triggered by the activation of group I metabotropic glutamate receptors (mGluRs), which includes mGluR1 and mGluR5 (Palmer et al., 1997). mGluR1 expression is high in Purkinje cells in the cerebellum, in mitral and tufted cells in the olfactory bulb and in the cell body of hippocampal stratum radiatum neurons (Ferraguti and Shigemoto, 2006). In addition, they are expressed in cell body neurolateral septum, pallidum and in the thalamus. mGluR5 is present in the cerebral cortex, subiculum, olfactory bulb, striatum, nucleus accumbens, lateral septal nucleus and in dendrites of stratum radiatum of hippocampus. Both receptors are mainly expressed in postsynaptic neurons in an area surrounding the ionotropic receptors (Lujan et al., 1996).

mGluR-LTD was first described at the granule cell parallel fiber (PF) synapses onto Purkinje cells (PC) in the cerebellum and was later observed in diverse brain regions such as the hippocampus, neocortex, dorsal and ventral striatum and spinal cord (Bellone et al., 2008; Gladding et al., 2009).

Hippocampal long-term depression has an important role in hippocampal-dependent learning: administration of group I mGluR antagonists or even deletion of group I mGluRs in animal models alters the acquisition and extinction of hippocampus-dependent learning tasks, such as radial arm maze or Morris water maze (Manahan-Vaughan and Braunewell, 2005; Naie and Manahan-Vaughan, 2004; Xu et al., 2009). Hippocampal localization of LTD induction can change based on the nature of the novel cues: small novel features induce LTD in the CA1 region, suggesting that LTD in CA1 play a role to encode spatial arrangement of novel objects; on the other side, large novel orientation cues facilitate LTD in the dentate gyrus (Kemp and Manahan-Vaughan, 2008).
Long-term depression mediated by group I mGluRs can be induced either pharmacologically or through synaptic stimulation. In hippocampal CA1 pyramidal neurons, mGluR-LTD takes place when CA3 Schaffer axons are stimulated either at low frequency (between 1–3 Hz for 5–15 min) or by pharmacologic application of R,S-dihydroxyphenylglycine (DHPG), a selective group I mGluR agonist (Bolshakov and Siegelbaum, 1994; Huber et al., 2000; Kemp and Bashir, 1999; Manahan-Vaughan, 1997; Naie and Manahan-Vaughan, 2005; Palmer et al., 1997; Volk et al., 2007).
mGluR1 and mGluR5 are coupled to a heterometric Gαq/11 protein (Ferraguti and Shigemoto, 2006), which activates phospholipase C (PLC), inducing the production of inositol trisphosphate (IP_3).

This event leads to the release of Ca^{2+} from intracellular stores and subsequent Protein Kinase C (PKC) activation. Hippocampal mGluR-LTD occurs independently of postsynaptic Ca^{2+} increases, IP_3 sensitive Ca^{2+} stores, PLC or PKC activity (Fitzjohn et al., 2001; Moult et al., 2006). Nevertheless, the presence of the endoplasmic reticulum is essential in dendrites to induce synaptic functional changing: dendritic spines with endoplasmic reticulum are susceptible to mGluR-LTD, have a greater volume, respond to glutamate with bigger post-synaptic currents and show larger mGluR-mediated Ca^{2+} increases with respect to dendritic spines that do not have endoplasmic reticulum proteins. This suggests that Ca^{2+} in the intracellular endoplasmic reticulum plays a role in hippocampal mGluR-LTD (Holbro et al., 2009).

The expression mechanism of mGluR-LTD in CA1 neurons consists of an increase in the endocytosis of AMPA receptors containing GluA1 and GluA2 subunits, which are removed from the post-synaptic membrane (Nakamoto et al., 2007). This event relies on tyrosine dephosphorylation of the GluA2 subunit. In addition to this, mGluRs activation stimulates the matrix metalloproteinase (MMP) TACE (tumor necrosis factor-α-converting enzyme): TACE cleaves the intramembrane protein NPR (neuronal pentraxin receptor), releasing the extracellular pentraxin domain, which in turns stimulates the endocytosis of AMPARs through an extracellular interaction (Cho et al., 2008).

The cellular mechanism of hippocampal mGluR-LTD crucially relies on rapid (in minutes) protein synthesis that occurs in dendrites (Huber et al., 2000; Waung and Huber, 2009): mGluRs activation stimulates the rapid synthesis of new proteins, known as LTD proteins, that participate in the regulation of AMPARs endocytosis (Volk et al., 2007). However, it is important to note that protein translation dependence of mGluR-LTD was reported to change with age: inhibition of protein translation did not affect mGluR-LTD induction in neonatal rats (Nosyreva and Huber, 2005) and in hippocampal slices obtained from 10 to 15 week rats mGluR-LTD occurred independently of protein translation, using either synaptic induction protocols or DHPG (Moult et al., 2008).

Group I mGluRs regulate translation during initiation and elongation processes (Costa-Mattioli et al., 2009; Waung and Huber, 2009). Translation initiation is stimulated by mGluRs through ERK-MAPK and PI3K–Akt-mTOR pathways (Gallagher et al., 2004; Hou and Klann, 2004). mGluRs induce phosphorylation of eukaryotic initiation factor 4E (eIF4E) and eIF4E binding protein (4EBP), stimulating the association of translation initiation (eIF4F) complex and increasing protein synthesis (Banko et al., 2006; Ronesi and Huber, 2008). Activation of Akt and ERK pathways also induces

phosphorylation of ribosomal S6 kinase (RSK); RSK in turn increases translation of a subset of mRNAs that encode ribosomes and translation factors (Antion et al., 2008; Ronesi and Huber, 2008).

One of the LTD proteins synthetized in response to mGluR-mediated pathways is Arc (Activity-regulated cytoskeletal associated protein) (Park et al., 2008; Waung et al., 2008). Arc associates with dynamin 2 and endophilin, inducing AMPAR endocytosis from the post—synaptic membrane (Chowdhury et al., 2006; Shepherd et al., 2006; Verde et al., 2006). Activation of group I mGluRs leads to the rapid translation of Arc in dendrites and this rapid synthesis is required to maintain decreases in surface AMPARs (Link et al., 1995; Steward et al., 1998; Steward and Worley, 2001). Consistent with its role in the induction of mGluR-LTD, Arc levels remain elevated for the duration of LTD (Park et al., 2008).

Another LTD protein is microtubule-associated protein 1B (MAP1B) and its mRNA is a FMRP target (Waung and Huber, 2009). DHPG treatment of hippocampal neurons increases MAP1B levels in dendrites (Davidkova and Carroll, 2007). MAP1B interacts with GluR2 with the scaffold GRIP1, a protein that stabilizes surface GluRs. The synthesis of MAP1B serves to sequester GRIP1 away from the synapse and destabilize GluR surface expression.

PSD-95 is a scaffold protein which regulates the trafficking of AMPARs at the synapse (Opazo et al., 2012; Won et al., 2017). AMPARs interact with the PSD-95 through transmembrane AMPAR regulatory proteins (TARPs), (Chen et al., 2000). The TARP—PSD-95 interaction reduces the mobility of AMPARs at the synapse, and disrupting this interaction allows AMPARs to diffuse away from the synapse, still bound to TARPs (Bats et al., 2007).

FMRP contributes to group I mGluR-induced translational activation of specific mRNAs (Ronesi and Huber, 2008; Waung and Huber, 2009) and regulates mGluR-dependent protein synthesis and plasticity acting predominantly as translational suppressor. Indeed many of the mRNAs that are translated in response to group I mGluRs interact with FMRP, including PSD-*95* (Todd et al., 2003), amyloid precursor protein (*APP*) (Westmark and Malter, 2007), elongation factor 1a (*Ef1a*) (Huang et al., 2005), MAP1b (Davidkova and Carroll, 2007; Hou et al., 2006) and Arc (Park et al., 2008; Waung et al., 2008).

In *Fmr1* KO mice, the absence of FMRP causes an overproduction of LTD proteins, which in turn induce excessive AMPARs internalization. As a result of this process, *Fmr1* KO mice show an increase in hippocampal mGluR-LTD (Huber et al., 2002), which in turn affects learning and memory (Malenka and Bear, 2004).

1.8 Alterations of dendritic morphology in Fragile X Syndrome

Dendritic spines, small protrusions along neuronal dendrites, are the sites receiving excitatory synaptic input: they contain receptors and signalling molecules that are essential for synaptic neurotransmission (Nimchinsky et al., 2002). Dendritic spines undergo shrinkage following endocytosis of AMPARs and decreases in AMPAR-mediated synaptic transmission in mGluR-LTD. At dendritic spines, actin is in monomeric globular (G)-actin form and filamentous (F)-actin form, and the shift between these two arrangements leads to changes in spine morphology (Cingolani et al., 2008). Dendritic spine morphology is strictly associated with synaptic plasticity: indeed, molecules that inhibit both polymerization and depolymerization of actin have been shown to block mGluR-LTD (Morishita et al., 2005; Moult et al., 2006; Xiao et al., 2001). Moreover, AMPAR endocytosis after the induction of mGluR-LTD and actin reorganization are correlated (Eales et al., 2014; Vanderklish and Edelman, 2002; Zhou et al., 2004).

An important regulator of cytoskeleton structure during mGluR-LTD is cofilin1. Preventing the activation of cofilin1 blocks mGluR-LTD (Asrar and Jia, 2013; Zhou et al., 2011), showing a role of cofilin1 in actin remodelling for synaptic plasticity (Hotulainen and Hoogenraad, 2010; Mizuno, 2013). Some upstream regulators of cofilin1 during mGluR-LTD have been identified, among which Ras-related C3 botulinum toxin substrate 1 (Rac), p-21-activated kinase, and LIM kinase (Chevy et al., 2015). In addition, p38-MK2 cascade is required to regulate cofilin1 activity in hippocampal neurons (Eales et al., 2014). These results are consistent with the idea that mGluR-LTD is associated with cytoskeleton reorganization resulting in spine morphological changes.

Essential for mGluR-LTD is the interaction of AMPAR subunit GluA2 with N-cadherin, a cell adhesion element (Zhou et al., 2011). This interaction is important to stimulate the cofilin1-dependent actin reorganization during the mGluR-LTD. Moreover, the activation of the ERK1/2 pathway could also induce changes in actin reorganization via the STEP-βcatenin-Rac-p-21-activated kinase pathway to regulate cofilin1 activity (Asrar and Jia, 2013).

In post-mortem analysis of human cortical tissue, individuals who suffered from Fragile X Syndrome had an increased density of dendritic spines with elongated and immature shape (Galvez and Greenough, 2005; Greenough et al., 2001; Hinton et al., 1991; McKinney et al., 2005; Rudelli et al., 1985; Wisniewski et al., 1991). Similar altered dendritic spine density and morphology have been

found in *Fmr1* KO mice (Comery et al., 1997; Grossman et al., 2006; Irwin et al., 2002; Nimchinsky et al., 2001).

Developmental studies revealed an increase in spine density and length in brain cortex of *Fmr1* KO mice compared to controls (Nimchinsky et al., 2001). In addition, a hippocampal CA1-specific altered protrusion phenotype was observed, which was absent in the CA3 region of the hippocampus (Levenga et al., 2011), indicating that the lack of FMRP differently influences dendritic spine morphology in distinct brain areas.

To summarize, as a negative regulator of mRNA translation, FMRP influences protein synthesis and therefore affects the synaptic components located in dendritic spines. Given the importance of FMRP for the regulation of synaptic proteins, it is unsurprising that loss of FMRP results in abnormalities in the structure and functionality of neuronal synapses.

1.9 Mitochondrial alterations in Fragile X Syndrome

Mitochondria are present in axons and dendrites of neurons and are play a prominent role in synaptic plasticity (Mattson, 2007). Indeed, synaptic activation and LTP induce changes in mitochondria (Mattson and Liu, 2003), such as an enhancement of energy production (Wieraszko, 1982), of calcium pump activity (Stanton and Schanne, 1986) and of gene expression (Williams et al., 1998).

Mitochondria are ubiquitous dynamically motile organelles with their own DNA and independent mitochondrial translation system (Protasoni and Zeviani, 2021; Trigo et al., 2022). They are involved in energy metabolism as main cellular ATP producers and regulate cellular functions such as Ca^{2+} homeostasis in cooperation with the endoplasmic reticulum (Rowland and Voeltz, 2012) and reactive oxygen species (ROS) signalling, which modulates immune responses (Singer and Chandel, 2019).

Mitochondria have two phospholipidic membranes, the outer and the inner mitochondrial membrane, which divide the organelle into two spaces, the matrix and the intermembrane space (Kühlbrandt, 2015). The two membranes show a different lipid composition: the outer membrane is more similar to eukaryotic cell membranes, whereas the inner membrane is characterized by a higher protein/lipid ratio and forms highly packed invaginations in the matrix, called cristae (Ernster and Schatz, 1981). Anchored in the cristae, respiratory chain complexes perform oxidative

phosphorylation (OXPHOS) (Trigo et al., 2022). During this process, oxygen is metabolized to generate energy in form of ATP through a series of reductive steps at the inner mitochondrial membrane via the electron transport chain, composed by the respiratory chain complexes I to IV, associated with transport of protons across the mitochondrial membrane (van der Bliek et al., 2017).

The first complex, NADH dehydrogenase, catalyses the oxidation of nicotinamide adenine dinucleotide (NADH) into NAD+ by ubiquinone, also called as coenzyme Q10, conserving the free energy of the reaction as a transmembrane proton gradient (Hirst, 2009). Complex II, or succinate dehydrogenase, has a role in the tricarboxylic acid cycle and in the electron transport chain, linking the two essential energy-producing processes of the cell (Ackrell, 2000; Cecchini, 2003; Saraste, 1999). In tricarboxylic acid cycle, it oxidises the succinate to fumarate, while as a component of the respiratory complex, it transfers electrons from succinate to ubiquinone, through flavin adenine dinucleotide (FAD) (Tomitsuka et al., 2009). Ubiquinone provides electrons from complexes I and II to complex III (ubiquinone-cytochrome c oxidoreductase), which in turn brings electrons to cytochrome c, a mobile protein that transfers them to complex IV (cytochrome c oxidase) (Solmaz and Hunte, 2008). Finally complex IV enables the terminal reduction of O_2 to H_2O. Complexes I, III, and IV pump H^+ into the mitochondrial intermembrane space, creating a strong proton gradients that drives ATP synthesis by complex V or ATP synthase complex (Payne and Chinnery, 2015).

The by-products of oxidative phosphorylation are ROS, generating from a premature electron leak along the electron transport chain from complex I, II and III (Liu et al., 2002; Zhao et al., 2019). These electrons are transferred to O_2, producing superoxide (O_2^-). This is an extremely reactive free radical which is turned into H_2O_2 by the mitochondrial or cytosolic superoxide dismutase (SOD) (Cadenas and Davies, 2000; Chance et al., 1979). O_2^- and H_2O_2 are kept at low concentrations (from 10^{-11} to 10^{-8} M) (Chance et al., 1979; Giorgio et al., 2007; Sies et al., 2017), but when they reach high levels, ROS cause oxidative damage of proteins, lipids and nucleic acids (Sies, Berndt, & Jones, 2017).

FMRP specifically binds *Superoxide Dismutase 1* (*Sod1*) mRNA via a motif called SoSLIP, composed of three stem loops separated by a short sequence (Bechara et al., 2009). The absence of FMRP results in decreased expression of Sod1 in polyribosomes, leading to a reduced expression in the brain of *Fmr1* KO mice. The decreased expression of Sod1 leads to a more sensitive mitochondrial oxidative stress in neurons.

The human brain necessitates of 20% of the whole organism metabolic production (Attwell and Laughlin, 2001), using glucose that undergoes glycolysis and oxidative phosphorylation to produce ATP and to assist synaptic transmission (Yin et al., 2016).

Thanks to generating energy, mitochondria rule important processes in neuron such as neuroplasticity, neurotransmitter release, axonal polarity and outgrowth (Cheng et al., 2010; Lee and Peng, 2008; Mattson, 2007; Verstreken et al., 2005). Dendritic, axonal, and presynaptic regions have different energy requests, which mean an adaptation of ATP production due to a strict connection between neuronal and mitochondrial activity (Kann and Kovács, 2007). Mitochondria are present along the length of axons and in presynaptic terminals; they are located mainly in the dendritic shafts and occasionally associated with spines (Popov et al., 2005). To adapt to variable energy requests, mitochondria move within and between neural regions involved in neuroplasticity.

During neurogenesis, a process in which neuronal stem cells differentiate into neurons, there is an involvement of mitochondria in regulating an adaptive response to environmental energy demand (Kempermann et al., 2004; Kitamura et al., 2009). In neurogenesis, neurons start to make axons, dendrites and synapses and mitochondria bunch up at the active growing cone of the developing neurites (Mattson and Partin, 1999). As soon as the axon is made, mitochondria migrate into the new neurite, following an anterograde movement in growing axons and retrograde movement in non-growing axons (Ruthel and Hollenbeck, 2003).

When ATP production is altered in mitochondria, axogenesis is abolished although the growth of dendrites remains unaffected (Mattson and Partin, 1999). The axonal and dendritic behaviour of mitochondria are also different in hippocampal neuron cultures, where mitochondria are more motile but less active in axons, whereas in dendrites they are less motile but more metabolically active (Overly et al., 1996). An altered mitochondrial ATP production and an enhancement of free radicals due to a leak of electrons from the mitochondrial chain complexes are key aspects in a large amount of neurological diseases (Breuer et al., 2013; Sai et al., 2012) characterized by developmental delay (Gibson et al., 2010). Defective mitochondria especially affect tissues that are more sensitive to oxidative stress, particularly the brain (Wallace and Fan, 2010). Alterations in dendritic spine densities due to dysfunctional mitochondria or impaired ROS homeostasis are indicated to be culprits in neurodevelopment diseases such as Down syndrome, Rett syndrome, Fragile X Syndrome (Valenti et al., 2014).

In the last years increasing attention was paid to mitochondrial dysfunctions. Growing evidence suggests that mitochondrial dysfunctions and defects in oxidative phosphorylation play a central role in Fragile X syndrome. FMRP regulates microtubules formation in neurites (De Diego Otero et al., 2002) and recently it has been reported that drosophila FMRP regulates microtubule network formation and axonal transport of mitochondria (Yao et al., 2011). Moreover, it has been recently demonstrated an altered expression of mitochondrial genes and increased oxidative stress that contribute to deficits in dendritic maturation and behaviour in *Fmr1* KO mice (Shen et al., 2019). Consistent with the latter result, an increased oxidative stress has been described in *Drosophila* lacking FMRP (Weisz et al., 2018). An alteration in the balance between fission and fusion was also shown in *Fmr1* KO mice, leading to structural and functional abnormalities in mitochondria (Shen et al., 2019) which might compromise mitochondrial bioenergetic efficiency. This hypothesis was confirmed by a significant reduction in the rate of mitochondrial ATP production in the brain cortex of *Fmr1* KO mice (D'Antoni et al., 2020). Analysing the activity of mitochondrial respiratory chain complexes, there was an increasing activity of all five complexes in the range between 40% and 50% in the cortex of post-natal day 21- and 12-month-old *Fmr1* KO mice. In line with these results, an enhancement in the activity of mitochondrial complexes was observed in the striatum and in the cerebellum of 12-month-old *Fmr1* KO mice (D'Antoni et al., 2020). These data are consistent with the evidence of mitochondrial hyperactivity and greater susceptibility to oxidative stress reported in ASD (Rose et al., 2017). The hyperactivation of mitochondrial complexes could be caused by FMRP absence, since FMRP is able to bind mRNAs encoding for some components of mitochondria respiratory chain complexes (Ascano et al., 2012; Maurin et al., 2018a). One of FMRP targets is the mRNA coding for mitochondrial glycerol-3-phosphate dehydrogenase (mG3P-DH) (Ascano et al., 2012; Maurin et al., 2018a), an enzyme of glycerophosphate shuttle which links lipid and glucose catabolism to OXPHOS (Mráček et al., 2013). In the brain cortex of *Fmr1* KO mice, increased activity and expression of mG3P-DH have been observed, that likely lead to glycerophosphate shuttle potentiation (D'Antoni et al., 2020), with possible metabolic implications. Indeed, glycerol-3-phosphate dehydrogenase competes with glycerol-3-phosphate acyltransferase, which is implicated in lipid synthesis, leading to a defect in lipid production and storage in FXS (Weisz et al., 2018). In addition, the mitochondrial respiratory chain and mitochondrial glycerol-3-phosphate dehydrogenase are producer of ROS, and their hyperactivation induced an increased oxidative stress (Bechara et al., 2009; Davidovic et al., 2011; de Diego-Otero et al., 2009; El Bekay et al., 2007).

Beyond the increased activity of the mitochondrial respiratory chain complexes, fragile X neurons show an enhancement in some glycolytic enzymes including hexokinase II, pyruvate kinase M2 variant and lactate dehydrogenase and also in enzymes required for tri-carboxylic acids cycle and NAD^+/NADH metabolism, including enzymes of the malate/aspartate shunt and isocitrate dehydrogenase (Licznerski et al., 2020). High glycolytic activity and lactate production, but also increases in TCA cycle enzymes are hallmark features of immature and developing cells (Fame et al., 2019), suggesting that mitochondrial abnormalities could be emblematic of neuronal immaturity (Licznerski et al., 2020). It was recently shown that forebrain mitochondria from the *Fmr1* knock out mice brains have inefficient thermogenic respiration due to a coenzyme Q-regulated proton leak, leading to synaptic spine and behavioral abnormalities (Griffiths et al., 2020). *Fmr1* KO forebrain mitochondria show an increased Complex II and Complex V kinetic activity compared to control, whereas the activities of Complex I + III and Complex II + III within forebrain mitochondria were significantly decreased than control, suggesting CoQ deficiency. Consistent with these results, levels of CoQ via HPLC were quantified, showing a decreased level of this CoQ in *Fmr1* KO mitochondria. A readout of the appropriate function of the electron transport chain is the mitochondrial inner membrane potential (Licznerski et al., 2020). The mitochondrial membrane potential is generated by proton pumps (Complexes I, III and IV) and it serves as an intermediate form of energy storage which is used by ATP synthase to make ATP. (Zorova et al., 2018). FXS mitochondria has less than half of the membrane potential in WT mitochondria (Licznerski et al., 2020).

To produce ATP, H^+ ions move across the mitochondrial ATP synthase (complex V) and cause a conformational change in the enzyme, making ATP. ATP synthase (F_0F_1) is a large protein complex located in the inner membrane, where it catalyzes ATP synthesis from ADP, P_i, and Mg^{2+} at the expense of an electrochemical gradient of protons generated by the electron transport chain (Pedersen et al., 2000). The mammalian ATP synthase has 15 subunit types (BUCHANAN and WALKER, 1996; Catterall and Pedersen, 1971; Ko et al., 2000), forming the F_1 catalytic unit (Catterall & Pedersen, 1971), an ATP hydrolysis-driven motor and F_0. F_0, containing subunits a and c, is anchored in the inner membrane to form a proton-driven motor, and a second part composed of subunits b and F_6 (Collinson et al., 1994; Golden and Pedersen, 1998; Ko et al., 2000). Pathological opening of the channel may occur upon conformational change of the ATP synthase (Gerle, 2016; Gu et al., 2019; Mnatsakanyan and Jonas, 2020; Vlasov et al., 2019), separation of the F_1 from the F_O (Alavian et al., 2014) or loss of F_1 (Chen et al., 2019).

In *Fmr1* KO neurons, the level of ATP synthase, b-subunit and c-subunit levels were highly elevated compared to those measured in control mitochondria (Licznerski et al., 2020). The mRNA of b-subunit is a target of FMRP (Darnell et al., 2011). Through RT-PCR experiment, the mRNAs codifying for b-subunit and c-subunit were increased in Fmr1 KO synapses compared to those of WT synapses (Licznerski et al., 2020). These results elucidate the influence of the lack of FMRP on the transcription of ATP synthase subunits: only b-subunit, but not c-subunit, translation is to be regulated by FMRP. The abnormal levels of ATP synthase c-subunit in FXS mitochondria lead to a mitochondrial inner membrane leak.

1.10 5-HT7 receptors

Serotonin (5-hydroxytryptamine, 5-HT) acts as a monoamine neuro-hormone and neurotransmitter in the central nervous system (CNS), with a role in regulation of mood, perception, circadian rhythm, nociception, hormone secretion, aggression, anxiety, appetite and sexual behaviour, (Abela et al., 2020; Cervantes-Durán et al., 2013; Cummings and Leiter, 2020; Hannon and Hoyer, 2008; Nichols and Nichols, 2008; Paulus and Mintz, 2016), and in peripheral nervous system (PNS), where it controls intestinal motility (Foxx-Orenstein et al., 1996) and immune/inflammatory response (Ahern, 2011). 5-HT has also been linked to cognition, memory, learning, and attention (Pourhamzeh et al., 2021).

During neuronal development, 5-HT influences synapse formation and has a modulatory role in proliferation, migration, differentiation, maturation of postmitotic neurons (Daubert and Condron, 2010). Notably, 5-HT also regulates cell adhesion molecules, which influences neuronal plasticity in both developing and adult brains (Dalva et al., 2007) and controls adult hippocampal neurogenesis (Duman and Monteggia, 2006).

In the CNS, serotonergic neurons are located in two groups of nuclei of dorsal and median raphe (DRN and MRN), and in part of the reticular formation in the brain stem (Abela et al., 2020) and project their axons to cortical, limbic, midbrain, and hindbrain regions (Huang et al., 2019).

In the PNS, 5-HT is synthesized by both gut neurons and enterochromaffin cells, located in the gastrointestinal (GI) system, and serves several roles as a hormone, autocrine, or paracrine factor. Because 5-HT cannot cross blood–brain barrier (BBB), these two central and peripheral 5-HT systems are entirely independent (Sahu et al., 2018).

5-HT exerts a large number of effects by activation of seven subtypes of transmembrane receptors ($5\text{-}HT_{1–7}$). $5\text{-}HT_3$ receptors are ligand-gated ion channels mediating fast depolarization (Sugita et al., 1992). All the other 5-HT receptors are G protein-coupled metabotropic receptors: $5\text{-}HT_1$ and $5\text{-}HT_5$ receptors inhibit adenylate cyclase, $5\text{-}HT_4$, $5\text{-}HT_6$ and $5\text{-}HT_7$ receptors instead stimulate adenylate cyclase, whereas the $5\text{-}HT_2$ receptor family is positively linked to phospholipase C (Hannon and Hoyer, 2008; Millan et al., 2008; Pytliak et al., 2011). Autoreceptors are present presynaptically on the soma ($5\text{-}HT_{1A}$Rs) or on axon terminals ($5\text{-}HT_{1B}$ and $5\text{-}HT_{1D}$ receptors) of serotonergic neurons and control 5-HT release via regulation of neuronal firing rate and negative feedback in concordance with the function of 5-HT transporters. Moreover, the activity of serotonergic neurons is regulated by $5\text{-}HT_{2B}$Rs (Belmer et al., 2018) and $5\text{-}HT_7$Rs (Martín-Cora and Pazos, 2004).

$5\text{-}HT_7$R belongs to the family of G protein-coupled receptors (GPCRs) (Hoyer et al., 2002). It is expressed in different area of mice and rat brain, among which thalamus, hypothalamus, hippocampus, prefrontal cortex, amygdala, raphe nuclei, suprachiasmatic nucleus, and spinal cord (Dogrul and Seyrek, 2006; Hedlund and Sutcliffe, 2004; Thomas and Hagan, 2004). $5\text{-}HT_7$R expression in the human brain is similar to that found in mice (Hagan et al., 2000; Martín-Cora and Pazos, 2004; Varnäs et al., 2004). However the human $5\text{-}HT_7$R is also expressed in caudate nucleus, putamen and substantia nigra (Martín-Cora and Pazos, 2004), where mice have no expression of this receptor. $5\text{-}HT_7$R brain expression level is age-related: in mice, the amount of $5\text{-}HT_7$R in neurons is high at birth and then decreases progressively during development (García-Alcocer et al., 2006; Kobe et al., 2012; Muneoka and Takigawa, 2003). However $5\text{-}HT_7$ receptors exert important functions also in the adult: it has been implicated in the regulation of sleep, circadian rhythm, body temperature control, learning, memory and cognition (Gellynck et al., 2013; Matthys et al., 2011). The role of $5\text{-}HT_7$ receptors has been studied using the $5\text{-}HT_7$ receptor knock-out ($5\text{-}HT_7$ KO) mice (Guscott et al., 2005; Roberts et al., 2004; Sarkisyan and Hedlund, 2009; Witkin et al., 2007).

Behavioural studies on $5\text{-}HT_7$ KO mice (Roberts et al., 2004; Sarkisyan and Hedlund, 2009) and on wild-type animals (Eriksson et al., 2012; Manuel-Apolinar and Meneses, 2004; Perez-García and Meneses, 2005) point out a pro-cognitive action exerted by activation of $5\text{-}HT_7$ receptors. $5\text{-}HT_7$ KO mice have no memory alteration in operant food conditioning tests, a kind of hippocampus-independent memory, and Barnes maze, which in contrast is a hippocampus-dependent spatial learning. However, these mice displayed a memory deficit in the fear conditioning test, which involves hippocampus-dependent contextual learning with an emotional component. These studies suggest that $5\text{-}HT_7$ receptors do not influence hippocampus-independent memory, whereas they

have a specific role in hippocampus-dependent learning with a strong emotional part. The specific contextual learning impairment of 5-HT_7 KO mice was consistent with a decrease of CA1 hippocampal LTP. However, 5-HT_7 KO mice show normal recognition of novel objects (Sarkisyan and Hedlund, 2009), which is a cortex – dependent memory based on visual stimuli and that correspond to human declarative episodic memory. A study on allocentric spatial memory (a hippocampus-dependent memory which encodes information about the location of an object respect to other objects in the space and independent from the observer), showed that either 5-HT_7 KO mice or wild type mice treated with the 5-HT_7 antagonist SB-269970 have an impairment in the recognition of a novel location, whereas no alteration was found about the egocentric memory, which is a striatum-dependent memory in which the location of an object is related to the observer (Sarkisyan and Hedlund, 2009). During the passive avoidance test, a contextual learning, in vivo administration of a 5-HT_7 agonist induced a pro-cognitive effect; this effect was abolished and replaced with a learning impairment when 5-HT_1 receptors were activated (Eriksson et al., 2008; Eriksson et al., 2012). 5-HT_7 receptors are able to influence learning based on Pavlonian and instrumental tasks (Perez-García and Meneses, 2005). During an instrumental learning task, a rodents pressed a lever and a food reward was rapidly delivered, while a food reward was delivered with a short delay following a light signal during conditioned learning. The activation of 5-HT_7 receptors by a subcutaneous injection of agonist AS-19 increased memory formation in adult rats.

The 5-HT_7 receptor gene contains several introns in the coding region (Ruat et al., 1993) that cause a significant number of functional splice variants. Three 5-HT_7 isoforms were identified in human tissues which possess different C-terminal tails: 5-HT_{7a} (445-aa), 5-HT_{7b} (432-aa) and 5-HT_{7d} (479-aa) (Heidmann et al., 1997). All the isoforms are coupled to G_s protein and also interact with G_{12} protein (Kvachnina et al., 2005; Riobo and Manning, 2005; Strathmann and Simon, 1991).

The activation of G_s protein, leads to the stimulation of Adenylate Cyclase (AC), which in turn causes an increase in intracellular cAMP level (Shen et al., 1993). The 5-HT_7R can also stimulate ACs without the activation of G_s proteins: the 5-HT_7(a) isoform stimulates ACI and ACVIII, which are present exclusively in the brain and are G_s-insensitive (Wirth et al., 2017); their activation depends on intracellular calcium concentration and on Ca2+/calmodulin-dependent signalling pathways. The activation of all types of AC produces cAMP, which stimulates protein kinase A (PKA), triggering the activation of the kinases ERK and Akt, both depending on the activation of Ras and involved in morphogenic changes. In particular, Akt activation necessitates an enhancement of both [cAMP] and intracellular [Ca2+], while ERK is inhibited by Ca^{2+} increasing level and can be induced in a PKA-

independent pathway or by EPAC exchange protein that is directly activated by cAMP (Grimes et al., 2015; Lin et al., 2003). Another kinase linked to ERK is Cdk5: this kinase activates ERK by phosphorylation at threonine 202 and tyrosine 204, leading to neurite outgrowth in cortical and striatal neurons isolated from embryonic rats, as well as in cortical, striatal, and hippocampal neurons from embryonic mice (Speranza et al., 2013). Stimulation of 5-HT_7Rs can also activate mTOR-mediated pathway; this mechanism was shown to influence the expressions of proteins involved in synaptogenesis such as CamKII and Shank3 (Bhattacharya et al., 2012) and to modulate synaptic plasticity and memory formation (Odajima et al., 2011).

As already mentioned, in addition to Gs the 5-HT_7R is coupled to the Ga12 subunit of the G12/13 protein family (Wirth et al., 2017). The main downstream effectors of the G12/13 proteins are Rho small G proteins (Chen et al., 2005; Fukuhara et al., 1999). The Rho family of GTPases belongs to a subfamily of the Ras superfamily (Boureux et al., 2007). The major members of the Rho family are Cdc42, Rac1, and RhoA (Fukuhara et al., 2001). These Rho GTPases modulate cell morphology and in particular actin cytoskeleton organization, influencing the neural branch dynamics, dendritic development, and neurite outgrowth through the cell rounding and filopodia formation in the neurons (Li et al., 2000; Ruchhoeft et al., 1999; Sit and Manser, 2011; Zipkin et al., 1997). Rac1 and Cdc42 activities promote neurite extension and branching, while RhoA causes neurite retraction and growth cone collapse (Ponimaskin et al., 2007).

Conditions determining a preferential activation of either G_s or G_{12} by 5-HT_7Rs are not clear, but some indication exists. Palmitoylation of 5-HT_7 receptors can influence the G_s-mediated constitutive activity but has no effect on G_{12}-mediated stimulation (Gorinski and Ponimaskin, 2013; Kvachnina et al., 2009), suggesting that post-translational modifications of 5-HT_7 receptors are able to influence the intracellular pathway activated, thus changing their final effect. In addition, there is a different expression of G proteins coupled to 5-HT_7R during neuro-development: the amount of G_{12} is higher at early post-natal age, whereas the expression of G_s remains constant during development (Kobe et al., 2012).

5-HT_7 receptors play an important role in actin cytosketon remodelling: 5-HT_7R activation in cultured hippocampal neurons enhanced neurite length, promoted dendritic spine formation, enhanced the number of structurally intact synapses, and increases both the general level of AMPA receptor expression as well as the number of synaptic AMPA receptors, increasing the amplitude of excitatory postsynaptic potentials (Kobe et al., 2012; Kvachnina et al., 2005). In addition, the number of

dendritic protrusions and synapse density in $G_{\alpha12}$ knockout (KO) neurons were reduced compared to wild type neurons, showing that morphogenic synaptic changes are mediated by 5-HT_7R/G_{12} (Kobe et al., 2012).

Activation of 5-HT_7R increased neurite length, the number of dendritic protrusions, and the number of synaptic contacts in cultured striatal and cortical neurons (Speranza et al., 2017), consistent with results on primary hippocampal neurons (Kobe et al., 2012; Kvachnina et al., 2005). Moreover, Speranza and colleagues observed that cyclin-dependent kinase 5 (Cdk5) and Cdc42 are required to maintain 5-HT_7R-mediated spine formation, acting as downstream effectors of 5-HT_7R of striatal neurons (Speranza et al., 2017).

5-HT_7-mediated effects on synapse morphology also involve extracellular matrix remodelling: a very interesting study shows that 5-HT_7 receptors increase neuronal outgrowth and promote elongation of dendritic spines by activation of matrix metalloproteinase 9 (MMP-9), leading to cleavage of CD44 followed by Cdc42 activation (Bijata et al., 2017).

5-HT_7R/G_{12} signalling influences neuronal morphology especially during early development (Herlenius and Lagercrantz, 2001). As already mentioned, the expression of 5-HT_7R and G_{12} are downregulated during later development (Kobe et al., 2012). Therefore, 5-HT_7R/G_{12} signalling influences dendrite morphogenesis, synaptogenesis, and functional plasticity of hippocampal networks during early stages of development and a disruption of serotonergic transmission participates to the onset of neurodevelopmental disorders.

Nevertheless, 5-HT_7R-mediated modulation of neural plasticity is not restricted to embryonic and early postnatal development but also occurs in adulthood (Ciranna and Catania, 2014).

1.11 The cAMP theory in Fragile X syndrome

Several studies have reported an aberrant cAMP pathway in patients suffering from FXS (Berry-Kravis and Huttenlocher, 1992; Kelley et al., 2007). Blood platelets from FXS patients have a reduced basal level of cAMP (Berry-Kravis and Huttenlocher, 1992; Berry-Kravis and Sklena, 1993) and a reduced cAMP production induced by forskolin (Kelley et al., 2007). Importantly, as already mentioned, in absence of FMRP, PDE2A, a cAMP degradative enzyme and FMRP target, is over-expressed in cortical and hippocampal FXS neurons leading to low cAMP levels (Maurin et al.,

2018a). Consistent with the cAMP theory for FXS, exaggerated mGluR LTD in *Fmr1* KO mice was corrected by blockade of mGluR2 (Choi et al., 2016; Choi et al., 2011), by inhibition of PDE4 (Choi et al., 2016; Choi et al., 2015) and by inhibition of PDE2A (Maurin et al., 2018b), all increasing intracellular cAMP level.

In agreement with the studies above indicated, results from our laboratories show that excessive mGluR-LTD in *Fmr1* KO mice was corrected by activation of 5-HT_7R and PACAP receptors, both stimulating adenylate cyclase (Costa et al., 2018). In the same work, we show that in WT neurons, following blockade of adenylate cyclase the amount of mGluR-LTD became comparable to that observed in *Fmr1* KO slices, suggesting that exaggerated mGluR-LTD in *Fmr1* KO mice might be related to reduced cAMP production.

Taken together these results suggest that Gs-coupled receptors might correct the cAMP deficit in FXS and represent a new pharmacological strategy for FXS therapy.

1.12 Cyclin-dependent kinase 5 (Cdk5)

Cyclin-dependent kinase 5 (Cdk5) belongs to a large family of cyclin-dependent kinases and is involved in 5-HT_7 receptor-mediated effects on axonal and dendritic growth (Speranza et al., 2013; Speranza et al., 2015; Speranza et al., 2017). Cdk5 is a proline-directed serine/threonine protein kinase, which was first discovered thanks to its close sequence homology to the human cell division cycle protein 2 (Cdc2, also known as Cdk1), a regulator protein of cell cycle (Hellmich et al., 1992; Lew et al., 1992; Meyerson et al., 1992). Unlike the other cyclin-dependent kinases, Cdk5 is not involved in the cell cycle, being mostly expressed in post-mitotic neurons, and plays a crucial role in the brain controlling neuronal differentiation and migration during development, cytoskeletal and microtubule regulation and synaptic plasticity (Kawauchi, 2014; Shah and Rossie, 2018; Ximerakis et al., 2019). Unlike other Cdks, which are expressed at high levels during development, Cdk5 is expressed not only during development (Pao and Tsai, 2021) but also in adult mouse brain (Tsai et al., 1993). As a Cdk family member, Cdk5 activity relies on the association with specific partners to become active. Cdk5 activators present only in neurons are the intracellular membrane-bound peptides p35 and p39 (Ko et al., 2001). The expression of Cdk5 and p35 match during the same period in the developing mouse neocortex, and p35 is primarily expressed in the post-mitotic neurons like Cdk5 (Tsai et al., 1994). The other regulatory subunit, p39, was discovered thanks to its high sequence identity to p35 (Tang et al., 1995). P39 is highly expressed in the brain (Humbert et

al., 2000; Ko et al., 2001; Tang et al., 1995), but shows differences from p35: during neural development, the expression of p35 is high from embryonic stage to postnatal stage, whereas p39 is more expressed postnatally (Takahashi et al., 2003). In addition, p35 and p39 are differently located in the brain: p35 is most present in the cerebral cortex and cerebellum, whereas p39 is predominantly localized in the cerebellum, brain stem, and spinal cord. Moreover, p39 protein is more stable than p35 but has lower binding affinity for Cdk5 (Minegishi et al., 2010; Yamada et al., 2007). The lack of p39 or Cdk5 in cultured neurons causes impairment in dendritic morphogenesis whereas no alteration was observed in cultured neurons lacking p35 expression (Ouyang et al., 2020). Cdk5/p39 also plays an important role in Rac1-induced remodelling of cytoskeleton (Ito et al., 2014).

Cdk5 has different roles in neuronal migration, neurite outgrowth, axonal guidance, and synaptic plasticity (Pao and Tsai, 2021). In particular, this kinase influences microtubule and cytoskeleton-related function (Xie et al., 2003), promoting axon formation (Fang et al., 2011) (Nikolic et al., 1998) (Duhr et al., 2014) (Furusawa et al., 2017), and regulating neural migration (Nikolic et al., 1998; Nishimura et al., 2014; Perlini et al., 2015; Xie et al., 2003; Ye et al., 2014). Moreover Cdk5 was shown to affect synaptic functions increasing clathrin-mediated endocytosis (Floyd et al., 2001; Tomizawa et al., 2003), increasing vesicle release (Shuang et al., 1998), regulating synaptic plasticity (Huang et al., 2017; Lai et al., 2012; Li et al., 2001; Morabito et al., 2004; Seeburg et al., 2008; Wang et al., 2003) and calcium influx (Su et al., 2012; Tomizawa et al., 2002). p35-null mice show impairment in axonal and dendritic organization (Chae et al., 1997) and in long-term depression and display a depotentiation of long-term potentiation (Ohshima et al., 2005), showing a role for the Cdk5/p35 complex in synaptic plasticity.

Cdk5 is also involved in BDNF-TrkB signalling phosphorylation of TrkB on Ser 478 by Cdk5 increases activity-dependent structural plasticity and spatial memory (Lai et al., 2012).

In some pathological conditions, the cleavage of p35 to a shorter activator peptide p25 causes an aberrant Cdk5 activity. Moreover, p25 lacks the myristoylation signal that normally anchors Cdk5 to the membrane. Neurotoxic insults cause calcium influx and trigger the activation of a cysteine protease named calpain (Lee et al., 2000). p35 is cleaved by calpain at Phe^{98}/Ala^{99} sequence producing an accumulation of p25 into cytoplasm and nucleus, causing an constitutive activation and a mislocalization of Cdk5 (Allnutt et al., 2020) due also to a 5- to 10-fold longer protein half-life compared to p35 (Patrick et al., 1999). Aberrant p25/Cdk5 signalling is involved in neurotoxicity,

neuroinflammation (Sundaram et al., 2012), neurodegeneration (Cheung and Ip, 2004), Alzheimer's disease (Patrick et al., 1999; Shukla et al., 2012; Tseng et al., 2002) and Parkinson's disease (He et al., 2020).

Moreover, Cdk5 has an influence in regulation of mitochondrial fission. Mitochondria are dynamically interconnected, allowing them to share membranes, solutes, metabolites and proteins (Liu et al., 2020). Mitochondria separate and merge using fission and fusion processes to respond to changes in energy and stress status: fusion happens when two adjacent mitochondria join, while fission separates one mitochondria into two, facilitating the removal of damaged components through mitophagy (Giacomello et al., 2020). Cdk5 hyperactivity leads to abnormal mitochondrial fission in pathological conditions, such as neurotoxic insults and neurodegenerative diseases (Cherubini et al., 2015; Jahani-Asl et al., 2015; Meuer et al., 2007; Park et al., 2019; Park et al., 2020; Rong et al., 2020; Sun et al., 2008; Yang et al., 2020). Excessive mitochondrial fission is associated with mitochondrial defects and neuronal death.

CHAPTER 2: Aim of the study

FXS is classified as a synaptopathy, since the lack of FMRP, an mRNA binding protein regulating translation of a large amount of synaptic protein(Pfeiffer and Huber, 2009), leads to alterations of synaptic morphology and function. The murine model of the disease, the *Fmr1* KO mouse, shows abnormal synaptic plasticity, aberrant maturation of dendritic spines and altered mitochondrial functions. At present, no specific therapy is available for FXS patients: the failure of numerous clinical trials underlines the urgency to identify new therapeutic targets.

Our research group has demonstrated that activation of 5-HT_7 receptors is able to reverse mGluR-LTD in wild type mouse hippocampal neurons and to reduce excessive mGluR-LTD in *Fmr1* KO mouse neurons, thus correcting a typical synaptic malfunction in a FXS mouse model. Moreover, *in vivo* administration of a selective agonist for 5-HT_7 receptors, LP-211, can rescue learning and behaviour in *Fmr1* KO mice, suggesting that 5-HT_7 receptor agonists might became pharmacological tools for a possible therapy of Fragile X syndrome.

In this perspective, the aim of my PhD studies was to investigate the intracellular molecular pathways involved in 5-HT_7 receptor-mediated reversal of mGluR-LTD. On this purpose, I used the patch clamp technique on hippocampal slices from wild-type and *Fmr1* KO mice to record mGluR-LTD in the synapse between CA3 and CA1 pyramidal neurons. In particular, I focused on two main points: 1) a possible involvement of Cdk5 and Akt kinases, which were shown to be involved in 5-HT_7 receptor-mediated effects on maturation of dendritic spines; 2) a possible role of protein translation in 5-HT_7-mediated reversal of mGluR-LTD.

In addition, *Fmr1* KO neurons show an enhancement of oxidative stress, an aberrant mitochondrial respiratory chain activity and an alteration in the ATP production (D'Antoni et al., 2020). Therefore, I have investigated whether the activation of 5-HT_7 receptors could influence mitochondrial activity. On this purpose, I have studied 5-HT_7 receptor expression and effects in a neuroblastoma cell line, a widely used *in-vitro* cellular model to study neuro-pathologies.

During my abroad period at the IPMC (Institute Pharmacology Moléculaire Et Cellulaire) in Valbonne, I have characterized a new murine model of intellectual disability. The Dr. Bardoni's research group identified a spontaneous R857G mutation in the *Kcc2* gene (unpublished data). The new variant causes the onset of seizures only in 4 months old mice with just the movement of the cage. Therefore, I investigated if this spontaneous mutation in the Kcc2 gene could affect the

expression of KCC2 protein in different brain region and influence neural activity and dendritic spine shape in the brain of *Kcc2* mutated mice.

CHAPTER 3: Materials and methods

3.1 Electrophysiology

Experiments were performed on mice *Fmr1* KO mice from C57BL/6J strain from a breeding colony kept at the University of Catania. Mice were maintained with a controlled temperature (21◦C ± 1◦C) and humidity (50%) on a 12 h light/dark cycle, with ad libitum food and water. Acute hippocampal slices were prepared from wild type and *Fmr1* KO mice (postnatal age 14 – 23 days). The brains were removed, placed in oxygenated ice-cold artificial cerebrospinal fluid (ACSF; in mM NaCl 124; KCl 3.0; NaH2PO4 1.2; MgSO4 1.2; CaCl2 2.0; NaHCO3 26; D-glucose 10, pH 7.3) and cut into 300 µm slices with a vibratome. Slices were continually perfused with oxygenated ACSF and viewed with infrared microscopy. Schaffer collaterals were stimulated with negative current pulses (duration 0.3 ms, delivered every 15 s). Evoked excitatory post synaptic currents (EPSCs) were recorded under whole-cell configuration from CA1 pyramidal neurons (holding potential −70 mV). Data were acquired and analysed using Signal software. The recording micropipette was filled with intracellular solution (in mM: K-gluconate 140; HEPES 10; NaCl 10; MgCl2 2; EGTA 0.2; Mg-ATP 3.5; Na-GTP 1; pH 7.3). To isolate AMPA receptor-mediated EPSCs, bath solution contained (-)-bicuculline methiodide (5 µM) and D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 µM). (S)-3,5-dihydroxyphenylglycine (DHPG; 100 µM) and LP-211 (10 nM) were dissolved in ACSF and applied by bath perfusion, whereas anisomycin (10 µM), Akt inhibitor III (1 µM) or roscovitine (1.6 µM) were included in the intracellular solution in different sets of experiments.

Experiments of spiking activity were performed in brain slices from mice obtained from a breeding colony kept at the IPMC (Institute Pharmacology Moléculaire Et Cellulaire) in Valbonne. We used *Kcc2* mutant and WT mice from C57BL/6J strain. Mice were maintained with a controlled temperature (21◦C ± 1◦C) and humidity (50%) on a 12 h light/dark cycle, with ad libitum food and water. Acute hippocampal slices were prepared from wild type and *Kcc2* mutant mice on a C57BL/6J background (postnatal age 20 –30 days). The brains were removed, placed in oxygenated ice-cold cutting solution (cutting solution; in mM; Sucrose 195 KCl 5.0; NaH2PO4 1.25; MgCl2 1.0; CaCl2 2.0; NaHCO3 25; D-glucose 25, Sucrose pH 7.3) and cut into 300 µm slices with a vibratome. Individual slices were transferred into store chamber with oxygenated artificial cerebrospinal fluid (ACSF, in mM NaCl 125; KCl 5.0; NaH2PO4 1.25; MgCl 1; CaCl2 2.0; NaHCO3 25; D-glucose 15, pH 7.3) at 37°C. Cell-attached recording was performed on CA3 neurons using long-shank borosilicate micropipettes (5–10 MΩ), that were pulled with a P-97 puller (Sutter) and filled with ACSF. Micropipettes were installed on a MultiClamp 700B headstage (Molecular Devices). Minimal seal resistance was 20 MΩ.

Data were acquired under 'I = 0' mode (zero current injection) with a Multiclamp 700B. CA3 neurons were recorded for 5 minutes to obtain a stable baseline, isoguvacine (10 uM) was bath applied for 3 minutes and washed out for at least 10 minutes.

3.2 Cell Culture

SH-SY5Y neuroblastoma cells were cultured in a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's F12 Medium. This medium was supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum, 1% (v/v) Glutamine and 1% (v/v) Penicillin – Streptomycin. Cells were cultivated in T75 flasks at 37°C with 5% CO_2 at saturated humidity and kept below 25 passage to avoid senescence.

3.3 Mitochondrial Enriched Fraction

The medium was removed from T75 flasks and collected in a 50 ml polypropylene tube. Cells were washed once with DPBS and detached using 0.05% (wt/v) trypsin – EDTA. After cell detachment, trypsin was blocked adding medium and the cell suspension was transferred in a 50 ml polypropylene tube. Then the medium and cell suspension were centrifugated at 125 g for 5 minutes, the supernatant was discarded and cells were resuspended in Ringer NaCl buffer (NaCl 135 mM, HEPES 20 mM, $MgSO_4$ 0.8 mM, KCl 3 mM, $CaCl_2$ 1.8 mM, D-Glucose 11 mM, pH=7.5) (Palacino et al., 2004). Afterward cells were centrifuged at 125g for 5 minutes, suspended in A buffer (Sucrose 320 mM, Tris-HCl 5 mM, EGTA 2 mM, pH=7.4) and homogenized with a glass-teflon grinder kept in ice. The homogenate was centrifuged at 4°C for 6 minutes at 2000 g to removed nuclei and tissue particles, while the supernatant was collected and centrifuged at 4°C for 15 minutes at 12000 g to pellet mitochondria. Finally, the pellet was washed with A Buffer to reduce the cytosolic contamination.

3.4 Western Blot analysis

The mitochondrial enriched fraction, obtained as above described, was treated with RIPA buffer and protease inhibitor cocktail. The mitochondrial lysate was centrifugated at 4°C for 15 minutes at 12000 g and protein concentration in the supernatant was dosed with DC protein Assay. Denatured proteins were separated through SDS-PAGE using Mini protean TGX stain free gels at 10% of polyacrylamide and transferred in a 0.2 um PVDF membrane using Trans Turbo Blot Transfer System. The membranes were blocked with 5% non-fat milk in TBS-Tween 20 0.1% for 1 hour at room temperature and incubated overnight with an anti-5-HT_7, anti-β-tubulin and anti-β-ATP synthase antibody. The membranes were rinsed three times in TBS-Tween 20 0.1% and incubated with anti-

mouse or anti-rabbit antibody. Blots were revealed using Clarity Western ECL Substrate through UVITEC Cambridge Chemiluminescence imaging system.

Denaturated protein gel electrophoresis on hippocampal and cortex of Kcc2 mutant and WT mice was performed with NuPAGE Bis-Tris Mini Gel. Samples were combined with NuPAGE LDS Sample Buffer (4x) and NuPAGE Reducing Agent (10x) and incubated at 95°C for 10 minutes. Samples were run for about 1,5 h (150V; 1x NuPAGE MOPS SDS Running Buffer). After gel electrophoresis, proteins were transferred on a NC-membrane for 1.5 h (at 0,25A). Subsequently, membranes were saturated in 5% milk for 1 h and incubated with primary antibodies overnight anti-GAPDH (calbiochem, 1:5000); anti-KCC2 (Invitrogen, 1:1000). Membranes were washed 3 times in PBS-0.1 % Tween and incubated with secondary antibodies (1:5000) for 1h. After 3 washes in PBS-0.1% Tween, membranes were revealed with Immobilon Western (Millipore Ref. P90720).

3.5 Complex IV activity measurements

To estimated cytochrome *c* oxidase (complex IV) activity, we performed spectrophotometric assays with and without administration of 5-HT_7 agonist LP-211 (1 µM) and 5-HT_7 antagonist SB-269970 (1µM) using a standard method (Spinazzi et al., 2012) with some modifications. Isolated mitochondria, obtained as described above, were subjected to three cycles of freeze and thaw in hypotonic potassium phosphate buffer (20 mM, pH = 7,4) to maximize the enzymatic rates. Then mitochondria were added to 250 µl of potassium phosphate buffer (0.1 M, pH= 7.5), 5 µl of n-dodecyl-β-D-maltoside 150 mM and distilled water in a 1 ml cuvette. The reactions started with the addition of 50 µl of reduced cytochrome *c* (1 mM) and it was followed by a decrease in absorbance at 550 nm due to oxidation of cytochrome *c*. Complex IV specific activity was checked by adding 20 µl of KCN 60 mM. LP-211 and SB-269970 at 1 µM in ethanol 10% were incubated with mitochondria for 3 minutes before to start reaction by adding reduced cytochrome *c*.

3.6 SH-SY5Y Membrane Preparation for Saturation-Binding Assay

The membrane preparation was carried out as described by Colabufo et al. with minor modifications (Colabufo et al., 2004). SH-SY5Y cells were cultured to 80% confluence; then, the medium was removed, and cells were rinsed in PBS. After detaching, cells were suspended in ice-cold 10 mM Tris-HCl (pH 7.4), containing 0.32 M of sucrose and homogenized in a Potter-Elvehjem homogenizer (Teflon pestle). The homogenate was centrifuged at 31,000 g for 15 min at 4 ◦C, and the supernatant was discarded. The final pellet was resuspended in ice-cold 10 mM Tris-HCl (pH 7.4) and stored at −80 ◦C until use. 4.7. Saturation-Binding Assay Saturation experiments were carried out as

previously reported with minor modification (Lacivita et al., 2020). 5-HT7Rs were radiolabeled using [3H]-SB269970 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) at concentrations in the range of 0.1–20 nM. Samples containing 100 µg of SH-SY5Y cells membranes or 70 µg of SH-SY5Y cells mitochondrial-enriched fraction, radioligand, and 10 µM SB-269970 (Tocris Bioscience, Bristol, UK) to determine nonspecific binding were incubated in a final volume of 0.5 mL (50 mM Tris-HCl, pH 7.4, 4 mM MgCl2, 0.1% ascorbic acid, 10 µM pargyline hydrochloride) for 20 min at 37 ◦C. The suspension was filtered through a Whatman GF/C glass microfiber filter (presoaked in 0.3% polyethylenimine for at least 20 min prior to use). Filters were washed 3 times with 1 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4). Scatchard parameters (Kd and Bmax) and Hill slope (nH) were determined by nonlinear curve fitting, using Prism version 5.0 GraphPad software.

3.7 Saturation-Binding Assay

Saturation experiments were carried out as previously reported with minor modification (Lacivita et al., 2020). 5-HT7Rs were radiolabeled using [3H]-SB269970 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) at concentrations in the range of 0.1–20 nM. Samples containing 100 µg of SH-SY5Y cells membranes or 70 µg of SH-SY5Y cells mitochondrial-enriched fraction, radioligand, and 10 µM SB-269970 (Tocris Bioscience, Bristol, UK) to determine nonspecific binding were incubated in a final volume of 0.5 mL (50 mM Tris-HCl, pH 7.4, 4 mM MgCl2, 0.1% ascorbic acid, 10 µM pargyline hydrochloride) for 20 min at 37 ◦C. The suspension was filtered through a Whatman GF/C glass microfiber filter (presoaked in 0.3% polyethylenimine for at least 20 min prior to use). Filters were washed 3 times with 1 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4). Scatchard parameters (Kd and Bmax) and Hill slope (nH) were determined by nonlinear curve fitting, using Prism version 5.0 GraphPad software.

3.8 Rapid Golgi staining

Golgi Staining was perfomed as described in (Du, 2019). Kcc2 mutant and WT mice brains were removed, rinsed twice in Milli-Q water and immersed in impregnation solution, prepared by mixing equal volume of solution A and B. The samples were stored at room temperature for two weeks. Then, samples were transferred into Solution C and stores at room temperature in the dark. After 3 days, the brains were cut into 100 µm sections using a vibratome (Leica VT 1000 S). Slices were rinsed twice in Milli-Q water and placed in the staining solution, composed by solution D and E, for 10 minutes. Then slices were rinsed in Milli-Q water 2 times for 4 min each rinse and dehydrated in sequential rinses of 50%, 75%, 95% and 100% ethanol, 4 min each rinse. Afterward, the sections

were cleared in xylene 3 times for 4 min each rinse. The slices were analysed through a bright-field microscope. Spine density and length were quantified using ImageJ as software.

3.9 Genotyping

Fragments of mouse tails were incubated overnight at 55 °C in lysis buffer (Tris pH=8 0,1M, EDTA 10mM; 0,1% SDS; 0,5% NP40) with addition of proteinase K. After inactivation of proteinase K for 10 min at 96 °C, DNA was diluted 10x and used directly for PCR reaction with primers (Seq-mSlc12a5-Rev = 5'-TCATCCACTGACGGCTATGG; Seq-mSlc12a5-For = 5'-ACGGGACCTTTCTTTTGGGA). PCR products were purified with QIAGEN MinElute PCR Purification Kit (Cat. No. 28004) and subjected to Sanger sequencing. Chromatograms were analyzed with SnapGene Viewer.

3.10 Statistical analysis

For electrophysiology experiments, peak amplitude values of EPSCs were averaged over 1 min and expressed as % of baseline EPSC amplitude (calculated from EPSCs recorded during at least 15 min before DHPG application). % EPSC values from groups of neurons were pooled (mean ± standard error of mean, SEM) and graphically represented as a function of time (GraphPad Prism 7). One-way ANOVA and Tukey's multiple comparisons test were used to compare three groups of data, whereas unpaired Student's t test was used to compare two groups of data. Statistical significance was accepted at $p < 0.05$ (*$p < 0.05$; ***$p < 0.001$).

Spiking activity measured by current clamp (I=0) recordings in loose patch configuration were normalized and analysed through the the one-sample Wilcoxon signed rank test and Mann-Whitney test (*$p < 0.05$).

Scatchard analysis data were analysed by applying one-way repeated-measures analysis of variance (ANOVA test), and unpaired t test followed as a post hoc test. Results were reported as mean ± SEM (standard error of the mean) of at least two to three independent experiments, performed in triplicate. Statistical significance was accepted at $p < 0.05$.

Cytochrome c oxidase activity data, represent mean rates (nmol/min/mg) ± SEM obtained from at least four independent experiments. *, $p < 0.05$, nonparametric Wilcoxon test between mitochondria administered with LP-211 and SB-269970, and nontreated mitochondria.

CHAPTER 4: Results

4.1 Blockade of Cyclin-dependent Kinase 5 (Cdk5) in WT neurons enhanced mGluR-LTD and abolished 5-HT7 receptor-mediated reversal of mGluR-LTD

Excitatory post synaptic currents (EPSCs) mediated byAMPA receptors were recorded from CA1 pyramidal neurons in whole-cell patch clamp.In wild-type hippocampal slices, application of DHPG (100 µM, 5 min), an agonist of group I metabotropic glutamate receptors (mGluRs), induced a long-term depression(mGluR-LTD) of AMPA receptor-mediated EPSCs (EPSC amplitude 40 min after DHPG: 79 ± 10% with respect to baseline EPSC amplitude prior to DHPG application, $n = 11$; Figure 1 A). In another group of recordings, the Cdk5 inhibitor roscovitine (1.6 µM) was included in the intracellular pipettesolution. In this condition, the amount of mGluR-LTD induced by DHPGwas significantly enhanced respect to control conditions (EPSC amplitude: 51 ± 9%, $n = 7$, versus 79 ± 10%, $n = 11$,wild-type DHPG + roscovitine versus wild-type DHPG, $p = 0.04$, $t = 1.821$, $df = 16$; unpaired t test; Figure 1 A and B). We have previously shown that activation of 5-HT7 receptors reverses mGluR-LTD in wild-type and in *Fmr1*KO neurons (Costa et al., 2018; Costa et al., 2015; Costa et al., 2012). In the presence of intracellular roscovitine (1.6 µM), application of the 5-HT7 receptor agonist LP-211 (10 nM, 5 min) was unable to reverse mGluR-LTD in wild-type slices (EPSC amplitude: 51 ± 9%,$n = 7$, versus 49 ± 9%, $n = 6$; wild-type DHPG + roscovitine versus wild-type DHPG + roscovitine + LP-211, $p = 0.42$,$t = 0.1895$, $df = 11$, Figure 1 A and B).

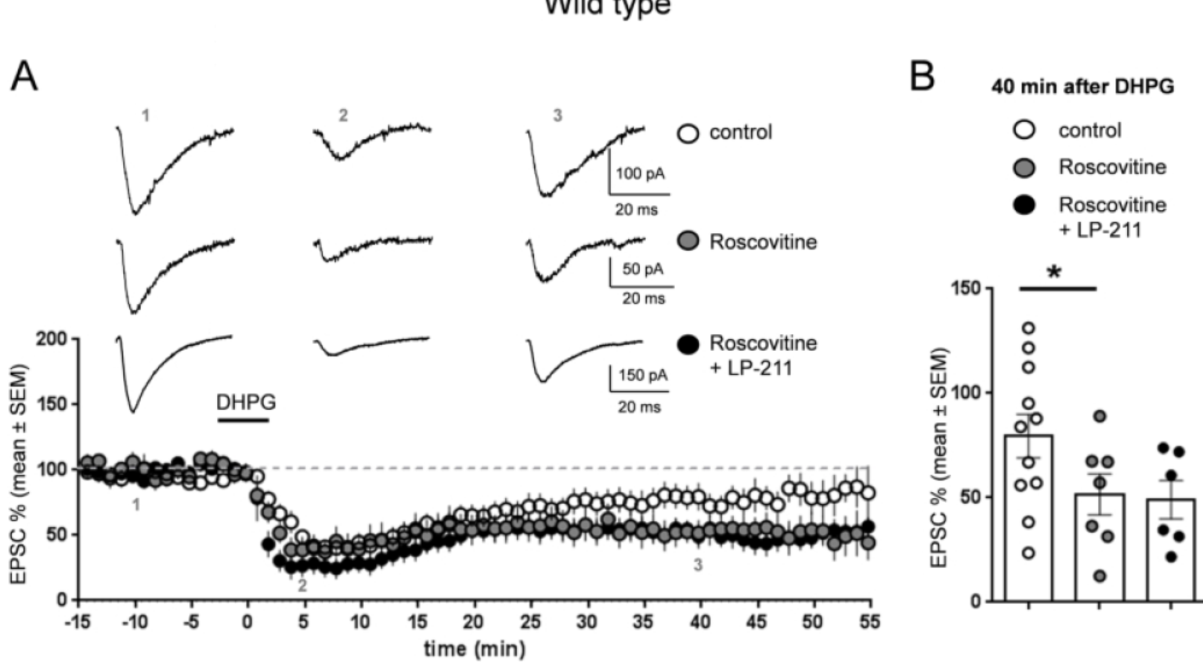

Figure 1 Blockade of Cdk5 enhanced mGluR-LTD in CA1 neurons from wild-type mice and abolished 5-HT7 receptor-mediated reversal on mGluR-LTD. AMPA receptor-mediated excitatory post-synaptic currents (EPSCs) were recorded in the presence of D-AP5 (50 µM) and bicuculline (5 µM) under whole-cell patch clamp in the CA3-CA1 synapses in hippocampal slices from wild-type mice. (A) Bath application of the group I mGluR agonist DHPG (100 µM, 5 min) induced a long-term depression (mGluR-LTD) of EPSC amplitude (white dots, n = 11). When theCdk5 inhibitor roscovitine (1.6 µM) was added to intracellular solution, DHPG-induced mGluR-LTD was enhanced (light grey dots, n = 7) respect to control. In the presence of intracellular roscovitine (1.6 µM), application of LP-211 did not modify the amount of mGluR-LTD (black dots, n = 6). (B) The bar graph shows that the amount of mGluR-LTD measured 40 min after DHPG application (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude; EPSC values of single neurons are displayed for each bar) in the three different experimental conditions (One-way ANOVA followed by Tukey's multiple comparisons test; *p < 0.05; ***p < 0.001).

4.2 Blockade of Cyclin-dependent Kinase 5 (Cdk5) abolished 5-HT7 receptor-mediated reversal of mGluR-LTD also in Fmr1 KO neurons

In *Fmr1* KO slices, the amount of mGluR-LTD induced by application of DHPG (100 µM, 5 min)in control conditions and in the presence of intracellular roscovitine (1.6 µM) was similar (EPSC amplitude: 53 ± 10%, *n* = 8 versus 50 ± 3%, *n* = 6; *Fmr1* KO DHPG versus *Fmr1* KO DHPG + roscovitine; *p* = 0.39,*t* = 0.2670, *df* = 12; Figure 2 A and B). The intracellular presence of roscovitine induced a comparable amount of mGluR-LTD in *Fmr1* KO and WT neurons (EPSC amplitude 51 ± 9%, *n* = 7 versus 50 ± 3%, *n* = 6; wild-type DHPG + roscovitine versus *Fmr1* KO DHPG + roscovitine; *p* = 0.78, *t* = 0.2817, *df* = 11; compare the grey dots columns in Figure 2 B and Figure 2 B). In *Fmr1* KO neurons, application of LP-211 (10 nM,5 min) significantly reversed mGluR-LTD (Costa et al., 2018; Costa et al., 2015; Costa et al., 2012) but had no effect in the presence of roscovitine, (EPSC amplitude: 51 ± 12%, *n* = 7, versus 50 ± 3, *n* = 6; *Fmr1* KO DHPG + roscovitine + LP-211 versus *Fmr1* KO DHPG +

roscovitine; p = 0.47, t = 0.07344, df = 11; Figure 2 A and B). 5-HT$_7$-mediated reversal of mGluR-LTD was completely abolished by roscovitine in wild-type and in *Fmr1* KO to a comparable extent (EPSC amplitude: 49 ± 9%, n = 6, versus 51 ± 12%, n = 7, wild-typeDHPG + LP-211 + roscovitine versus *Fmr1* KO DHPG + LP-211 + roscovitine, p = 0.896, t = 0.1336, df = 11; un- paired t test; compare Figures 1 B and 2 B).

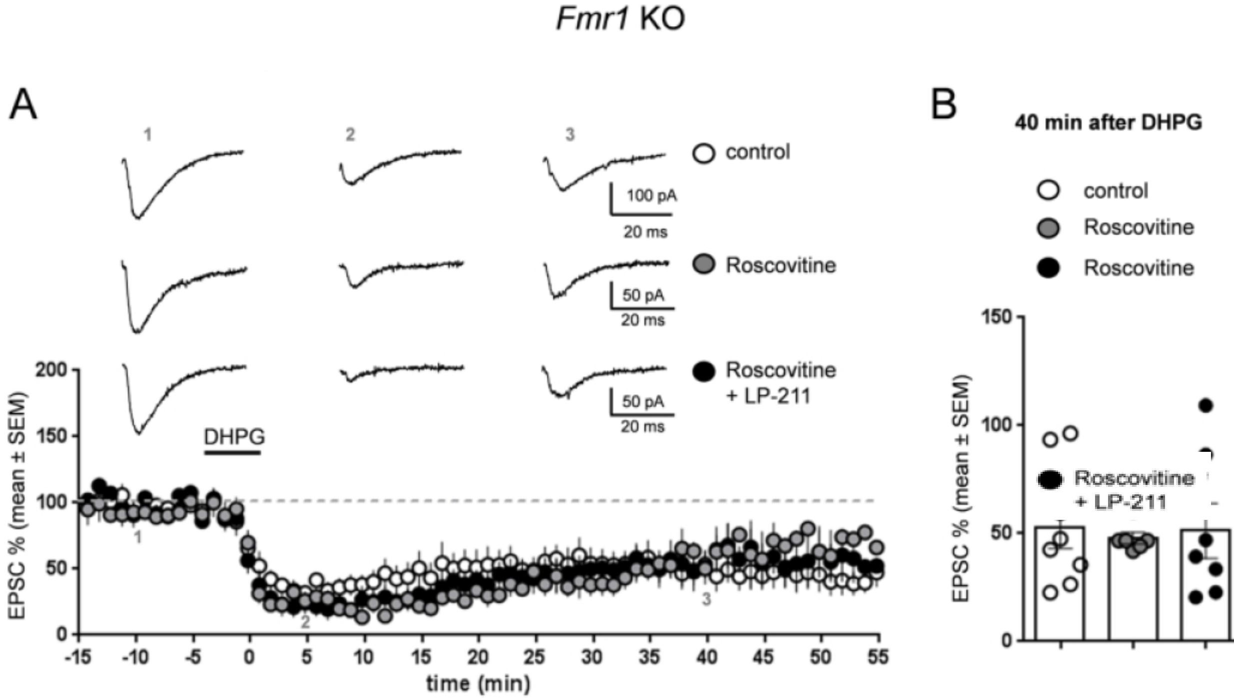

Figure 2 Blockade of Cdk5 did not modify mGluR-LTD in CA1 neurons from *Fmr1* KO mice and abolished 5-HT7 receptor-mediated reversal on mGluR-LTD. AMPA receptor-mediated excitatory post-synaptic currents (EPSCs) were recorded from CA1 neurons in the presence of D-AP5 (50 µM) and bicuculline (5 µM) in hippocampal slices from *Fmr1* KO mice. (A) Bath application of DHPG (100 µM, 5 min) induced mGluR- LTD (white dots; n = 8). In the presence of intracellular roscovitine (1.6 µM) the amount of mGluR-LTD was not modified (grey dots, n = 6) respect to control conditions. The application of LP-211 (10 nM, 5 min) had no effect on mGluR-LTD in the presence of intracellular roscovitine (black dots, n = 7). (B) The bar graph shows the amount of mGluR-LTD measured 40 min after DHPG application (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude; EPSC values of single neurons are displayed for each bar) in the three different experimental conditions (One-way ANOVA followed by Tukey's multiple comparisons test; *p < 0.05; ***p < 0.001).

4.3 Inhibition of Akt abolished mGluR-LTD in wild-type but not in Fmr1 KO neurons.

To study the role of Akt kinase in the mGluR-LTD pathway, we measured the amount of mGluR-LTD in *Fmr1* KO and WT slices in presence of intracellular Akt inhibitor III (1µM). When Akt inhibitor III was present in the intracellular solution, mGluR-LTD was inhibited in WT (Figure 3 A) but not in *Fmr1* KO neurons (Figure 3 B), indicating that Akt activation is necessary for mGluR-LTD only in WT slices (EPSC amplitude after 40 min from application of DHPG: 106.5 ± 35.19%, *n* = 6, versus 43.66 ± 14.09%, *n* = 6; WT DHPG + Akt inhibitor III versus *Fmr1* KO DHPG + Akt inhibitor III; *p* = 0,029; t=2.641; df=8; Figure 3 C).

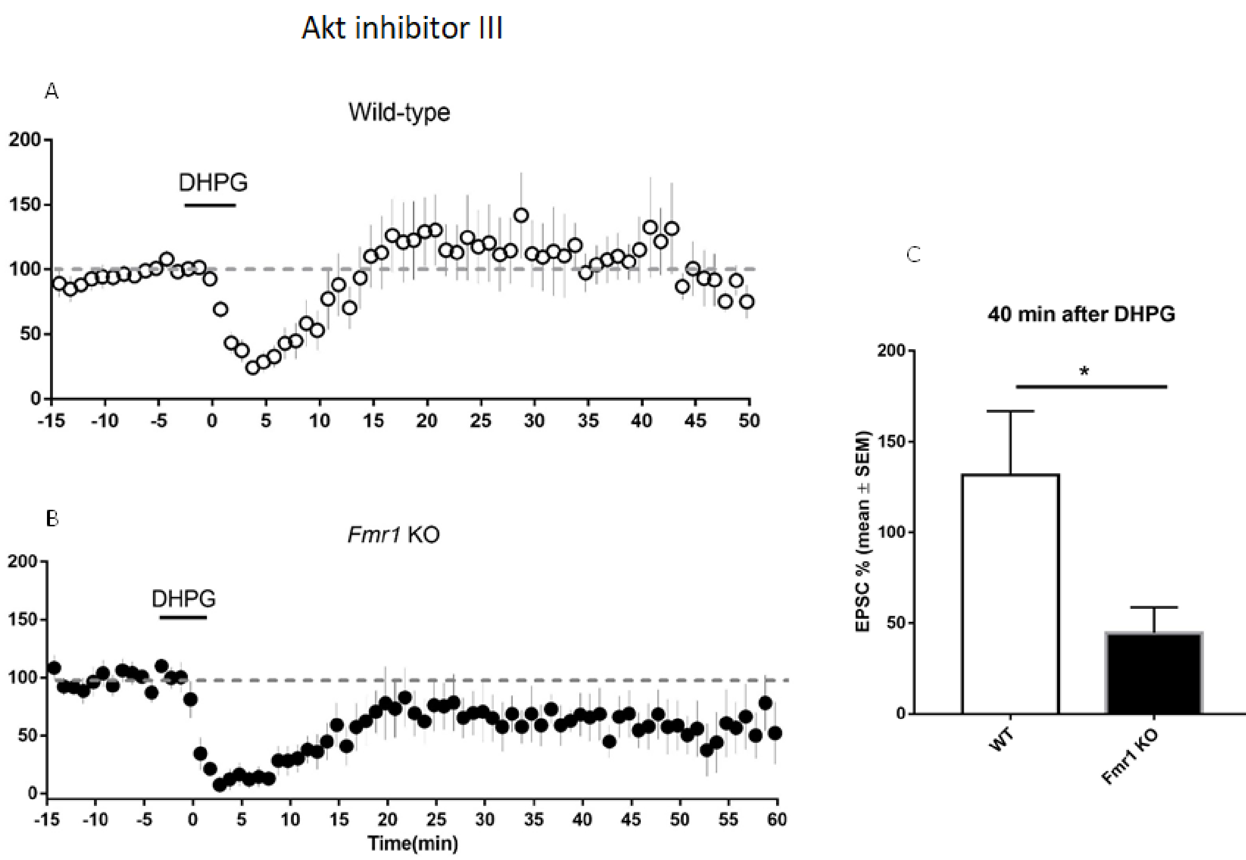

Figure 3 Blockade of Akt abolished mGluR-LTD in WT but not in Fmr1 Knockout (KO) slices. AMPAR-mediated excitatory post-synaptic currents (EPSCs) were recorded in the presence of D-AP5 (50 µM) and bicuculline (5 µM) under whole-cell configuration in the CA3–CA1 synapses in hippocampal slices from Fmr1 KO and WT mice in presence of intracellular Akt inhibitor III (1µM). (A) Bath application of DHPG (100 µM, 5 min) induced mGluR- LTD. In the presence of intracellular Akt III inhibitor, mGluR-LTD was abolished in wild-type neurons (white dots, n = 6). (B) DHPG-mediated mGluR-LTD was still observed in *Fmr1* KO slices in the presence of intracellular Akt III inhibitor (black dots, n=6). (C) The bar graph shows the amount of mGluR-LTD in WT and *Fmr1* KO neurons in the presence of Akt inhibitor III (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude) (Unpaired t test; *p < 0.05; ***p < 0.001).

4.4 5-HT7 receptor-mediated reversal of mGluR-LTD in Fmr1 KO neurons did not require activation of Akt

We studied a possible involvement of Akt in 5-HT_7 receptor-mediated reversal of mGluR-LTD. Bath application of LP-211, was still able to reverse mGluR-LTD in presence of intracellular Akt inhibitor III (1 µM) in *Fmr1* KO neurons (EPSC amplitude after 40 min from application of DHPG: 44,71 ± 14,09%, n=6, versus 85,44 ± 9,138%, n=6; *Fmr1* KO DHPG + Akt inhibitor versus *Fmr1* KO DHPG + Akt inhibitor + LP-211; p = 0,0359; t=2,425 df=10; Figure 4 B). This result indicates that 5-HT_7 receptor-mediated reversal of mGluR-LTD in *Fmr1* KO neurons does not require Akt.

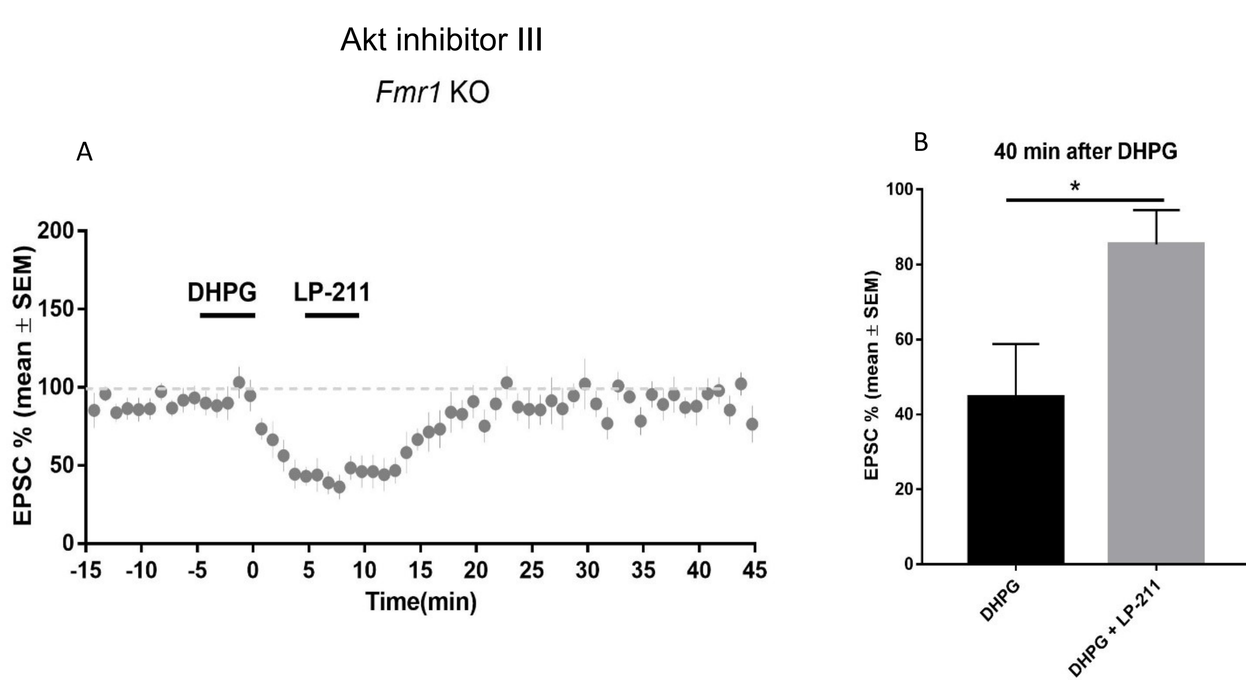

Figure 4 Blockade of Akt did not influence the 5-HT_7 receptor-mediated reversal of mGluR-LTD in CA1 neurons from *Fmr1* KO mice. AMPA receptor-mediated excitatory post-synaptic currents (EPSCs) were recorded from CA1 neurons in the presence of D-AP5 (50 µM) and bicuculline (5 µM) in hippocampal slices from *Fmr1* KO mice. Bath application of DHPG (100 µM, 5 min) induced mGluR-LTD. The application of LP-211 (10 nM, 5 min) reversed mGluR-LTD in the presence of intracellular Akt inhibitor III (grey dots, n = 8). (B) The bar graph shows the amount of mGluR-LTD measured 40 min after DHPG application in *Fmr1* KO neurons (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude), without and with the application of LP-211 in the presence of Akt inhibitor III (Unpaired t test; *p < 0.05; ***p < 0.001).

4.5 mGluR-LTD requires protein translation in wild-type but not in Fmr1 KO neurons

We measured the amount of mGluR-LTD in hippocampal *Fmr1* KO and WT neurons in the presence of intracellular anisomycin (10 µM), a protein translation inhibitor. mGluR-LTD was inhibited in WT (Figure 5 A) but not in *Fmr1* KO neurons (Figure 5 B) when anisomycin was present in the intracellular solution (EPSC amplitude after 40 min from application of DHPG: 102 ± 10.69%, n = 4, versus 60.58 ± 7.96%, n = 5; WT DHPG + anisomycin versus *Fmr1* KO DHPG + anisomycin; p = 0,0214; t=2 ; df=9; Figure 5 C). This result confirms previous data (Nosyreva and Huber, 2005) showing that protein translation is necessary for mGluR-LTD in WT but not in *Fmr1* KO slices.

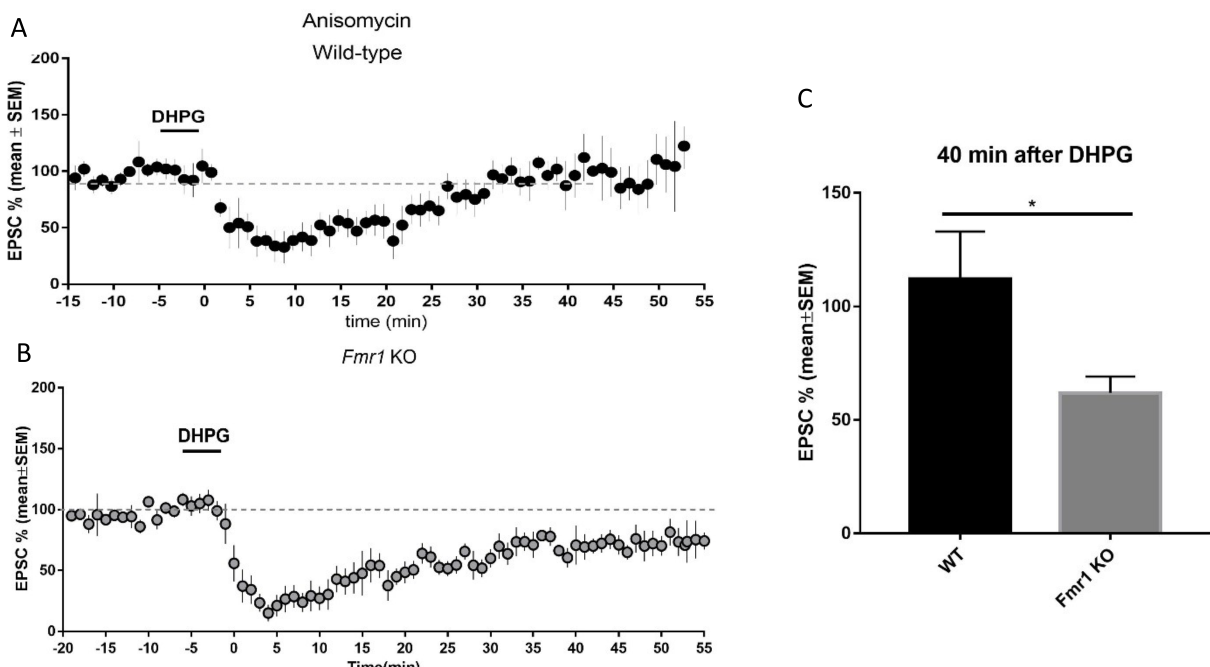

Figure 5 Inhibition of protein synthesis abolished mGluR-LTD in WT but not in Fmr1 Knockout (KO) slices. AMPAR-mediated excitatory post-synaptic currents (EPSCs) were recorded in the presence of D-AP5 (50 µM) and bicuculline (5 µM) under whole-cell configuration in the CA3–CA1 synapses in hippocampal slices from *Fmr1* KO and WT mice in the presence of intracellular anisomycin (10 µM). (A) Bath application of DHPG (100 µM, 5 min) induced mGluR- LTD. In the presence of intracellular anisomycin the mGluR-LTD was abolished (white dots, n = 4) in wild-type neurons. (B) The DHPG-mediated mGluR-LTD in hippocampal Fmr1 KO neurons was maintained in Fmr1 Knockout (KO) slices in presense of intracellular anisomycin (black dots, n=6). (C) The bar graph shows the amount of mGluR-LTD measured 40 min after DHPG application (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude) in the two different conditions (Unpaired t test; *p < 0.05; ***p < 0.001).

4.6 5-HT$_7$ receptor-mediated reversal of mGluR-LTD in *Fmr1* KO neurons required protein translation

We tested the effect of LP-211 on mGluR-LTD in presence of intracellular anisomycin (10 µM): in these conditions, activation of 5-HT$_7$ receptors was unable to reverse mGluR-LTD in *Fmr1* KO slices (Fig. 6 A and B), indicating that 5-HT$_7$ receptor-mediated effect required protein synthesis (EPSC amplitude 40 min after application of DHPG: 70.8 ± 15.89%, n = 6, versus 50.15 ± 10.25%, n = 6; Fmr1 KO DHPG + anisomycin versus *Fmr1* KO DHPG + anisomycin + LP-211; p = 0,29 t=1.13 ; df=9; Figure 6 B).

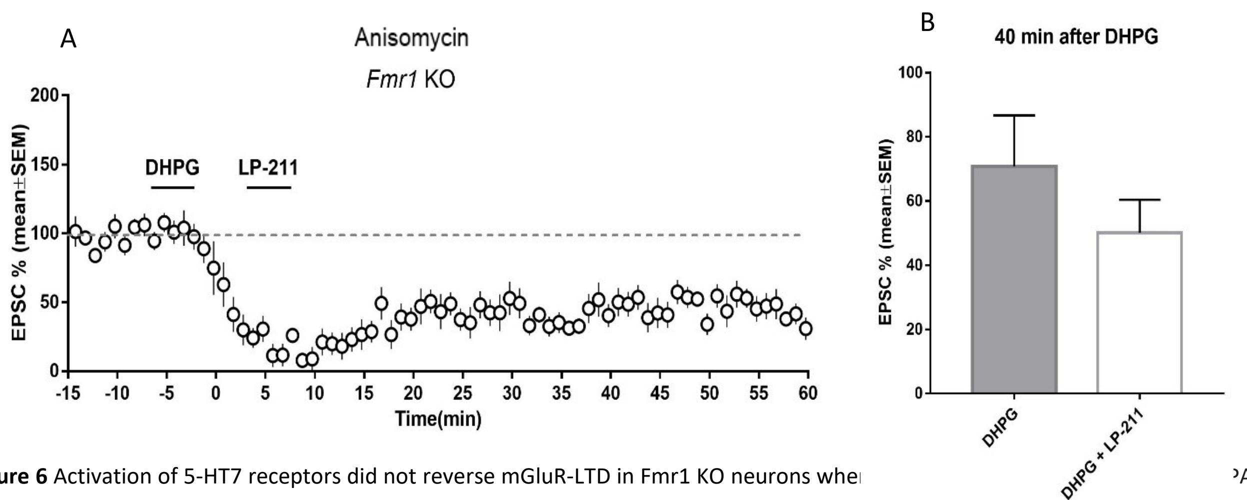

Figure 6 Activation of 5-HT7 receptors did not reverse mGluR-LTD in Fmr1 KO neurons whe ... A receptor-mediated excitatory post-synaptic currents (EPSCs) were recorded from CA1 neuro ... nd bicuculline (5 µM) in hippocampal slices from Fmr1 KO mice. Bath application of DHPG (100 µM, 5 min) induced mGluR- LTD. The application of LP-211 (10 nM, 5 min) had no effect on mGluR-LTD in the presence of intracellular anisomycin (grey dots, n = 6). (B) The bar graph shows the amount of mGluR-LTD measured 40 min after DHPG application (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude), without and with the application of LP-211 in presence of anisomycin (Unpaired t test; *p < 0.05; ***p < 0.001).

4.7 Two different isoforms of 5-HT$_7$ receptors are located in the cytosolic and mitochondrial fractions in SH-SY5Y

We first investigated 5-HT$_7$ receptor localization in SH-SY5Y cell line through an immunoblotting analysis of the cytosolic and the mitochondrial enriched fractions using a rabbit polyclonal antibody against a sequence identical for all human receptor splice variants. To ensure that there were no issues in our western blotting protocol, as positive control we used membranes obtained from HEK 293 cells, stably transfected with cDNA for 5-HT$_7$ receptor. These membranes were used in radioligand binding assay. The Western Blot analysis revealed that 5-HT$_7$ receptor was present in both cytosolic and mitochondrial fractions (Fig. 7 A). Two bands, with molecular masses of 40 and 50 KDa, were detected: the 50 KDa isoform in mitochondrial enriched fraction and the 40 KDa

isoform localizing in the cytosol. This pattern of data was observed in at least three independent experiments. Therefore, the results show that two protein forms of the 5-HT_7 receptor are expressed in human neuroblastoma cells. In order to rule out cytosolic contamination in mitochondrial fraction and *vice-versa*, we performed western blot analysis on the different fractions using an anti β-ATP synthase and an anti β-tubulin antibodies (Fig. 7 B). β ATP synthase is a mitochondrial protein while β- tubulin is mainly expressed in the cytosol. Our results show that the β ATP synthase band was absent in the cytosolic fraction (Fig. 7 B) and the β- tubulin band was not detected in the mitochondrial fraction (Fig. 7 B), indicating that there was no contamination in the analysed fractions.

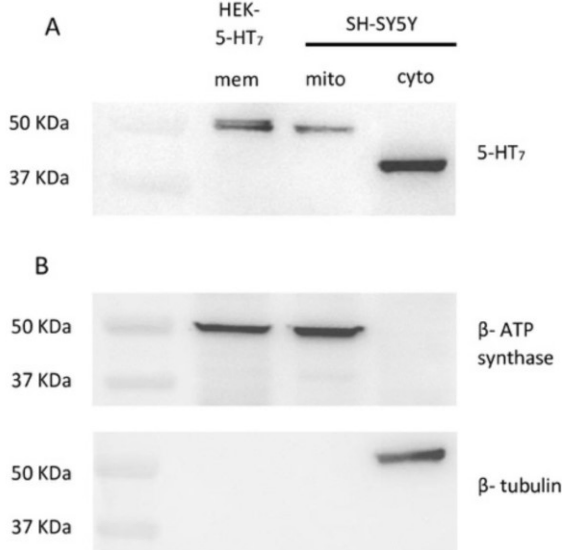

Figure 7 (A) Expression of 5-HT7R in cytosolic (cyto) and mitochondrial (mito) enriched fractions obtained from SH-SY5Y cell line. Positive control represented by membranes (mem) obtained from 5-HT7R-stably transfected HEK 293 cells. (B) Same fractions of SH-SY5Y analyzed to detect β-ATP synthase (mitochondria marker) and β-tubulin (cytosol marker) expression by sequential reprobing on same blot. Molecular mass markers (KDa) indicated on the left.

4.8 Saturation-Binding Assay confirms the presence of 5-HT7Rs in mitochondria

The presence of 5-HT7R in the SH-SY5Y cell line was investigated with saturation-binding analysis. The assay was performed on both whole SH-SY5Y cell membranes and SH-SY5Y cell mitochondrial fractions. Results demonstrated the presence of 5-HT7R in both preparations, albeit with different expressions. SH-SY5Y cell membrane Bmax was 0.51 pmol/mg of protein (Fig. 8 A), whereas SH-SY5Y cell mitochondrial fraction Bmax was 0.081 pmol/mg of protein (Fig. 8 B). Furthermore, experiments gave different Kd values for [^{3}H]SB-269970 in whole SH-SY5Y cells (Kd = 6.55 nM) and

SH-SY5Y cells mitochondrial-enriched fraction (Kd = 1.90 nM). For comparative purposes, saturation-binding analysis, performed with membranes obtained from HEK 293 cells stably transfected with cDNA for 5-HT7R, is reported in Figure 8 C. Schild regression analysis indicated the presence of a single binding site in the SH-SY5Y cells' mitochondrial-enriched fraction and the presence of an additional binding site in whole SH-SY5Y cell membranes.

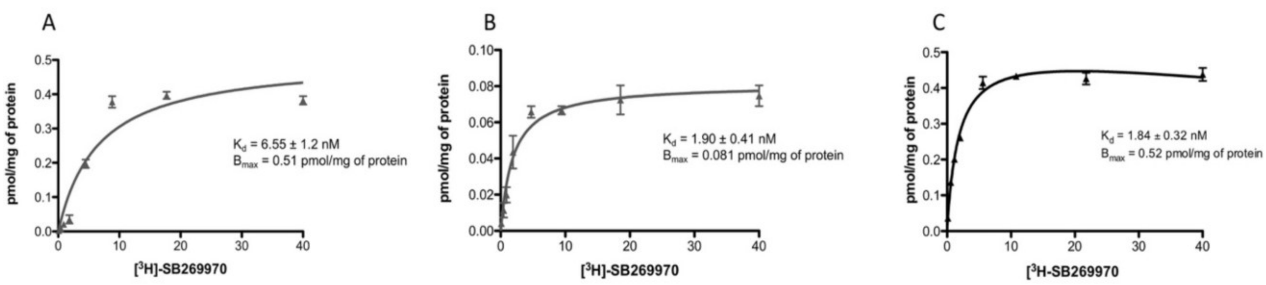

Figure 8 Scatchard analysis with selective 5-HT7R radioligand [^{3}H]SB-269970 on (A) whole SH-SY5Y cell membranes, (B) mitochondrial-enriched fractions obtained from SH-SY5Y cell line, and (C) membranes of 5-HT7R-transfected HEK 293 cells.

4.9 Administration of SB-269970 (but not LP-211) to mitochondria weakly influences Mitochondrial Respiratory Chain (MRC) Cytochrome c Oxidase activity

To investigate whether mitochondrial functions are influenced by activation of 5-HT$_7$ receptors located on mitochondria in human neuroblastoma cell line SH-SY5Y, we measured the MRC complex IV activity of mitochondria after incubation with selective 5-HT$_7$ agonist LP-211 and the 5-HT7 antagonist SB-269970 (Fig. 9). Cytochrome c oxidase activity was 258.6 ± 4.28 nmol/min/mg in H_2O and 286.9 ± 29.41 nmol/min/mg in 10% ethanol in H_2O. Lastly, we studied the effect of the selective 5-HT$_7$ antagonist SB-269970 on the mitochondrial enriched fraction. The incubation of mitochondria with SB-269970 resulted in a weak increase in cytochrome c oxidase activity compared to control. Upon treatment with SB-269970, cytochrome c oxidase activity was 303.63 ± 30.48 nmol/min/mg (Fig. 9).

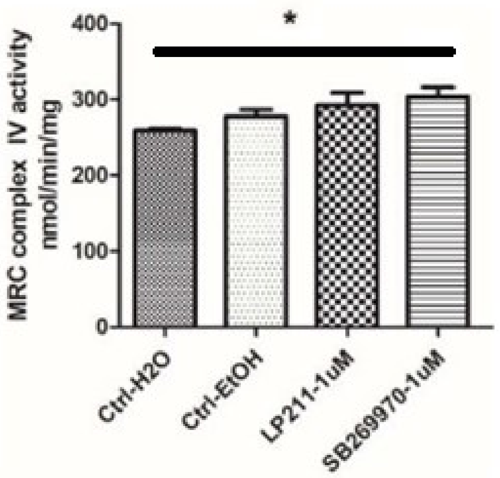

Figure 9. SB-269970 weakly stimulate cytochrome c oxidase activity, which was spectrophotometrically measured in mitochondrial fractions from SH-SY5Y cells incubated with LP-211 and SB-269970 3 min before measurements. Values represent mean rates (nmol/min/mg) ± SEM obtained from at least four independent experiments. * $p < 0.05$, nonparametric Wilcoxon test between mitochondria administered with SB-269970 and nontreated mitochondria in two controls. Ctrl-EtOH, 10% EtOH in H_2O.

4.10 Activation of $GABA_A$ receptors induced comparable inhibitory effects in WT and *Kcc2* mutant hippocampal neurons

To evaluate whether the spontaneous mutation R857G detected in the *Kcc2* gene *Slc12a5* can influence the neural activity of hippocampus, we analysed the effect of this mutation on spiking activity of CA3 hippocampal neurons in acute slices prepared from WT and *Kcc2* mutant mice. The effect of a brief application of the $GABA_A$ agonist isoguvacine on neuronal firing recorded in cell-attached mode inhibited neuronal activity in WT neurons with respect to the baseline (Wilcoxon matched-pairs signed rank test; p<0.0001; two-tailed; sum of positive - negative ranks 0, -153; sum of signed ranks -153; n=17; Fig. 10 A). The selective activation of $GABA_A$ receptor also induced a reduction in the spiking activity of pyramidal CA3 neurons recorded in *Kcc2* mutant hippocampal slices (Wilcoxon matched-pairs signed rank test; p=0.0039; two-tailed; sum of positive - negative ranks 0, -45; sum of signed ranks -45; n=10; Fig. 10 B). To determine if there was any difference in terms of inhibition of action potential frequency between WT and *Kcc2* mutant neurons, we performed a Mann-Whitney test: spiking activity of CA3 neurons under isoguvacine was not significantly different between WT and *Kcc2* mutant neurons (Mann Whitney test; p=09803; Two-

tailed; Mann-Whitney U 84; sum of rank in Kcc2 mutant neurons, WT neurons 139,239; median of WT neurons 0.209 n=17; median of Kcc2 neurons 0.1246 n=10; Fig 10 C).

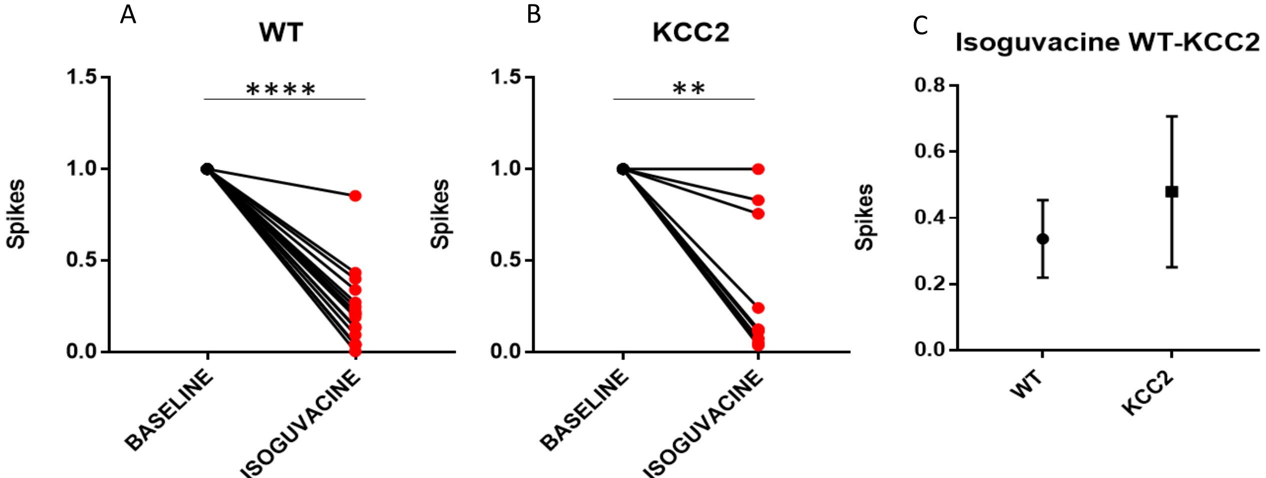

Figure 10 The activation of GABA A receptors has an inhibitory effect on WT and *Kcc2* CA3 neurons. Spiking activity measured by current clamp (I=0) recordings in loose patch configuration in Wild-type (A) and Kcc2 (B) mutant mice. Cells were recorded for 5 minutes to obtain a stable baseline, isoguvacine (10 uM) was bath applied for 3 minutes and washed out for at least 10 minutes. The graphs show the analysis of spiking frequency (s^{-1}) normalized on baseline of WT (n=17) (A) and *Kcc2* mutant mice (n=10) (B) CA3 pyramidal neurons upon isoguvacine application. The one-sample Wilcoxon signed rank test, *p<0,05; Mann-Whitney test, *p<0,05.

4.11 R857G mutation in *Kcc2* gene does not influence the KCC2 protein expression in hippocampus and in cortex

To investigate if the mutation R857G in the *Kcc2* gene affects the KCC2 protein level in hippocampus and cortex, we performed a Western blot analysis in wild type and *Kcc2* mutant mice at the age of 12 months.

Through the quantification of the intensity of KCC2 signal in hippocampus, we highlighted that gene mutation did not influence protein expression between WT and *Kcc2* mutant mice in hippocampus (Mann Whitney test; p= 0.4206; two-tailed; sum of ranks in WT and *Kcc2* mutant hippocampus 32, 23; Mann-Whitney U 8; median of WT hippocampus 1.032 n=5; median of *Kcc2* mutant hippocampus 0.7794 n=5; Fig. 11 A, B) or in cortex (Mann Whitney test; p= 0.4206; two-tailed; sum of ranks in WT and *Kcc2* mutant cortex 23, 32; Mann-Whitney U 8; median of WT cortex 0.9616 n=5; median of Kcc2 mutant cortex 1.268 n=5; Fig 11 C, D).

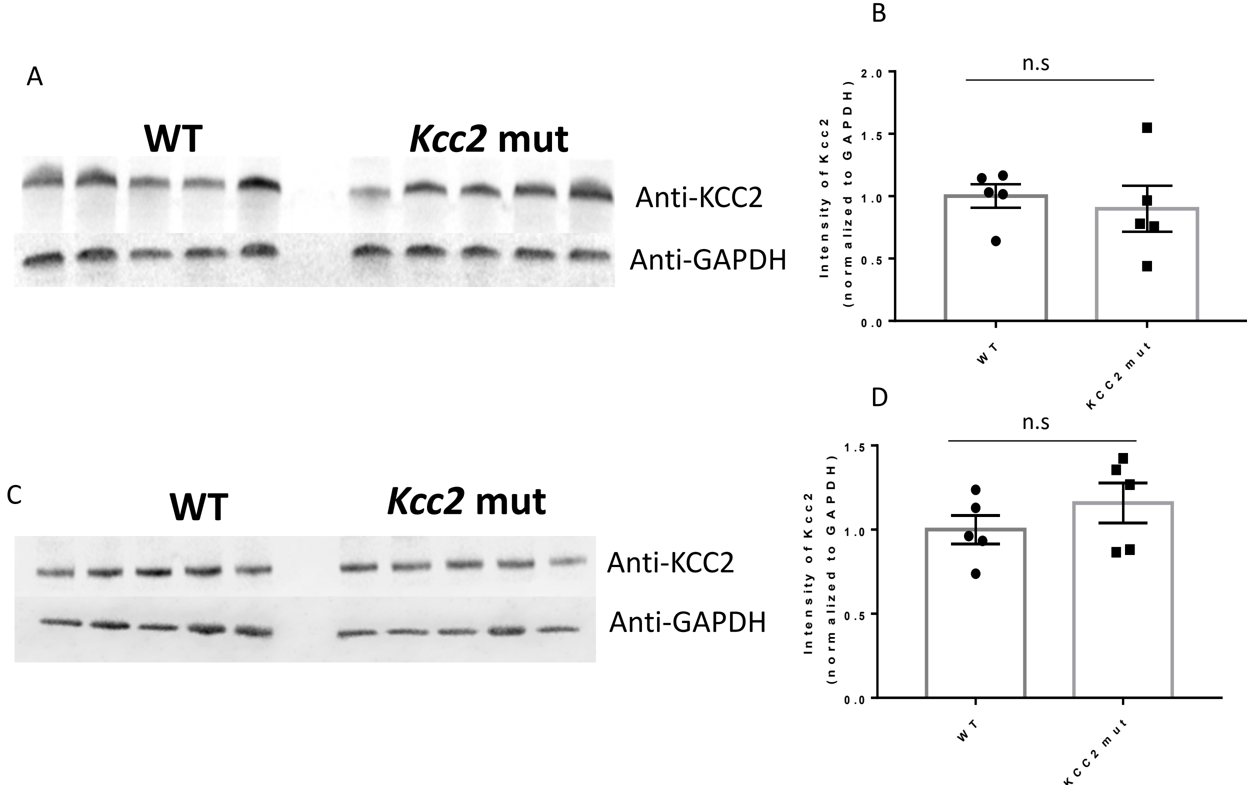

Figure 11. The mutation R857G in *Kcc2* gene does not affect the expression of the protein in hippocampal and cortical region between wild-type and *Kcc2* mutant (*Kcc2* mut) mice. (A) Western blot analysis of KCC2 protein in hippocampus of wild-type and *Kcc2* mutant mice at the age of 12 months. (B) Quantification of western blot analysis of KCC2 protein in hippocampus of wild-type (n=5) and *Kcc2* mutant mice (n=5) at the age of 12 months. (C) Western blot analysis of KCC2 protein in cortex of wild-type and *Kcc2* mutant mice at the age of 12 months. (D) Quantification of western blot analysis of KCC2 protein in cortex of wild-type (n=5) and *Kcc2* mutant mice (n=5) at the age of 12 months. Mann-Whitney test, *p<0,05.

4.12 R857G mutation in Kcc2 gene does not influence the morphology of dendritic spines in terms of density and length in hippocampus and cortex between wild type and Kcc2 mutant mice.

Lastly, we studied a possible influence of the mutation R857G in *Kcc2* gene on dendritic spine morphology. Using the Golgi staining technique, we highlight the structure of dendritic spines in wild type and in *Kcc2* mutant mice (Fig. 12 A, D, G, L). Quantifying the density and the length of the dendritic spines through the software ImageJ, we discovered that the presence of the mutation does not affect the dendritic spine morphology in cortex (Fig 12 M, N) and neither in the three regions of hippocampus DG, CA1 and CA3 (Fig. 12 B, C, E, F, H, I) in *Kcc2* mutant mice compared to wild-type.

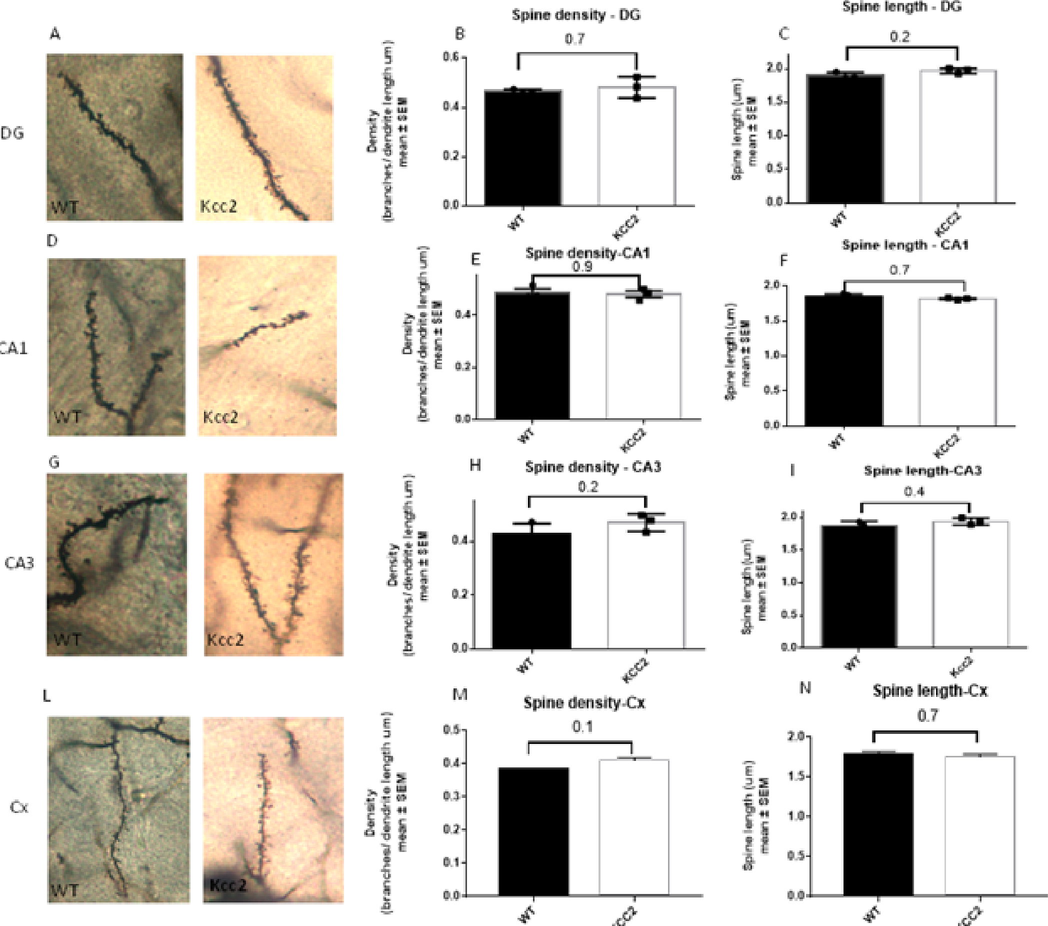

Figure 12 The mutation R857G in *Kcc2* gene does not influence the morphology of dendritic spines in cortical and hippocampal region in wild-type and *Kcc2* mutant mice. Pictures of wild type (n=3) and *Kcc2* mutant (n=3) dendritic spine morphology in the dentate gyrus DG (A), cornu ammonis 1 CA1 (B), cornu ammonis 3 CA3 (B) and cortex Cx (C). Spine density and length were quantified in the dentate gyrus DG (B, C), cornu ammonis 1 CA1 (E, F), cornu ammonis 3 CA3 (H, I) and cortex Cx (M, N). Mann-Whitney test, *p<0,05.

CHAPTER 5: Discussion

Fmr1 KO mice, a murine model of Fragile X Syndrome, display a large number of malfunctions in synaptic transmission and plasticity, among which exaggerated mGluR-LTD in the hippocampus (Huber et al., 2002). The abnormal enhancement of mGluR-LTD in *Fmr1* KO neurons is considered as a readout of synaptic malfunction and is believed to account for learning and behavioural impairment (Sanderson et al., 2016).

Our research group has previously shown that mGluR-LTD in *Fmr1* KO mice can be rescued by activation of serotonin 5-HT_7 receptors, which activate adenylate cyclase leading to stimulation of protein kinase A (Costa et al., 2018; Costa et al., 2015; Costa et al., 2012). The rescue effect of 5-HT_7 receptors is in line with with the "cAMP theory" of Fragile X syndrome (Kelley et al., 2007) and with the important finding that phosphodiesterase 2 (PDE) is a major FMRP target and is overexpressed in *Fmr1* KO neurons, leading to reduced cAMP levels (Maurin et al., 2018b).
In my PhD experimental work, I have studied additional intracellular mechanisms involved in 5-HT_7R-mediated reversal of mGluR-LTD, focusing on the role of the kinases Cdk5 and Akt. Cdk5 is related to synaptic plasticity and to the development of dendritic spines and was found to be involved in several effects mediated by 5-HT_7 receptors. As a matter of fact, Cdk5 is involved in 5-HT_7 receptor-induced axonal outgrowth and dendritic spine formation in cultured neurons from rodent brain cortex, hippocampus and striatum (Speranza et al., 2013; Speranza et al., 2015; Speranza et al., 2017). Interestingly, Cdk5 activation might be related to the cAMP pathway, since cAMP elevation induced by 5-HT_7 receptors was shown to stimulate p35 expression and Cdk5 activity in rat cultured neurons (He et al., 2016).

Therefore, I investigated the role of Cdk5 on mGluR-LTD and on 5-HT_7R-mediated reversal of mGluR-LTD. Our results show that in physiological conditions Cdk5 exerts a negative modulation on mGluR-LTD, since the Cdk5 inhibitor roscovitine increased mGluR-LTD in WT neurons to a level similar to exaggerated mGluR-LTD measured in *Fmr1* KO slices. Our results also suggest that either the expression or the function of Cdk5 in *Fmr1* KO neurons might be reduced compared to wild-type and that reduced Cdk5 function might account for enhanced mGluR-LTD. Consistent with our hypothesis, the expression of Cdk5 in the hippocampus of *Fmr1* KO mice was found to be reduced (Zhang et al., 2020).

Then we tested if application of LP-211, a selective agonist for 5-HT_7 receptors, was able to rescue mGluR-LTD in presence of the Cdk5 inhibitor roscovitine. Following Cdk5 blockade, 5-HT_7R activation

did not reverse mGluR-LTD either in wild type or in *Fmr1* KO neurons, leading to the conclusion that Cdk5 activation is involved in 5-HT_7 receptor mediated reversal of mGluR-LTD.

We next studied a possible involvement of Akt in 5-HT_7 receptor-mediated reversal of mGluR-LTD in wild-type and in *Fmr1* KO mice. Akt is a serine/threonine kinase with three isoforms (Akt I,II,III) encoded by different genes, although the proteins share a high degree of structural homology (Kumar and Madison, 2005). The kinase regulates cell growth, proliferation and metabolism; in neurons, the Akt pathway has a significant impact on stress responses, neurotransmission and synaptic plasticity (O'Neill, 2013). Akt is involved in the mammalian target of rapamycin (mTOR) pathway controlling protein synthesis. In addition, Akt activation has been correlated with different forms of LTP and LTD (Horwood et al., 2006; Hou and Klann, 2004), including mGluR-LTD (Levenga et al., 2017). Our results show that Akt inhibition abolished mGluR-LTD in WT but not in *Fmr1* KO neurons, leading to the conclusion that Akt is necessary for mGluR-LTD only in WT slices. As a possible explanation, it was shown that in FMRP-deficient neural cells *de novo* protein synthesis is elevated and this increase is associated with elevated ERK1/2 and Akt signalling (Utami et al., 2020). In line with these findings, we might speculate that inhibition of Akt that we induced in *Fmr1* KO neurons might have been unable to compensate the aberrant and hyperactivated of Akt signalling, resulting in the persistence of mGluR-LTD in *Fmr1* KO slices.

We next tested if Akt plays a role in 5-HT_7 receptor-mediated reversal of mGluR-LTD, since Akt activation is involved in other effects mediated by 5-HT_7 receptors, among which actin filament remodelling (Guseva et al., 2014). On this purpose, we used *Fmr1* KO slices because, in the presence of Akt inhibitor III, mGluR-LTD was present only in *Fmr1* KO: in these conditions, 5-HT_7 receptor activation was still able to reverse mGluR-LTD, thus did not require Akt activation.

Another important aim of our study was to investigate a possible role of 5-HT_7 receptors on neuronal protein syntesis. FMRP is a RNA binding protein with a predominant inhibitory effect on mRNA translation; as a matter of fact, impaired local dendritic translation was recognized as a major mechanism of pathogenesis in FXS, where FMRP is absent (Osterweil et al., 2010). In our study, we tested the hypothesis that dendritic mRNA translation is required for 5-HT_7 receptor-mediated effect on mGluR-LTD. In the presence of intracellular anisomycin, a protein translation inhibitor, mGluR-LTD was inhibited in WT but not in *Fmr1* KO neurons, indicating that protein translation is necessary for mGluR-LTD only in WT slices. This result is consistent with previous data, showing that mGluR-LTD was abolished by protein synthesis inhibitors in WT neurons but persisted in *Fmr1* KO neurons (Nosyreva and Huber, 2005). Therefore, we confirm data from Huber and colleagues,

suggesting that mGluR-LTD in *Fmr1* KO neurons does not need new protein synthesis because an excess of "LTD proteins" is already present in dendrites. In the presence of intracellular anisomycin, activation of 5-HT_7 receptors was unable to reverse mGluR-LTD in *Fmr1* KO slices, indicating that 5-HT_7 receptor-mediated effect required protein synthesis. This result indicates that 5-HT_7 receptor activation stimulates the synthesis of one or several proteins, which ultimately reverse mGluR-LTD; the protein(s) involved in 5-HT_7R-mediated effect remain to be investigated.
The 5-HT_7 receptor is a G-protein coupled receptor, positively linked to adenylate cyclase through the stimulatory Gs protein and additionally linked to G12 (Kvachnina et al., 2005). Some GPCRs are associated with mitochondria: for example purinergic receptors were shown to influence the regulation of mitochondrial Ca^{2+} uptake (Belous et al., 2004) and serotonin 5-HT_3 and 5-HT_4 receptors, both present on cardiac mitochondria, regulate mitochondrial activities and cellular functions (Wang et al., 2016b). Interestingly, the 5-HT_7 receptor agonist LP-211 is able to rescue the mitochondrial respiratory chain dysfunction and the oxidative phosphorylation deficiency in murine models of Rett syndrome (Valenti et al., 2017) and CDKL5 deficiency (Vigli et al., 2019); the mechanism of action remains unclear. We demonstrate for the first time that 5-HT_7 receptors are present in both cytosol and mitochondria of a SH-SY5Y cell line. In our results, two bands with molecular masses of approximately 40 and 50 KDa were detected, the former present in the cytosolic fraction and the latter in the mitochondrial fraction. As a possible explanation, 5-HT_7R undergoes alternative splicing at the second intron, located in the carboxyl terminus, giving rise to three splice variants in humans (a,b,d) (Heidmann et al., 1997). The 45–50 KDa range that we detected was consistent with the expected molecular mass of 5-HT7R. It should also be considered that 5-HT_7 receptors undergo different post-translational modifications, having two consensus sequences for N-linked glycosylation sites in the extracellular N-terminal region (Lovenberg et al., 1993) and for attachment of saturated fatty acids (i.e., palmitate) to cysteine residues within the protein via thioesterification (S-palmitoylation) (Gorinski and Ponimaskin, 2013). The 40 KDa cytosolic form that we detected might be explained by the presence in SH-SY5Y cells of a form of the receptor not subjected to post-translational modifications (Mahé et al., 2004).

To our knowledge, this is the first demonstration that 5-HT7Rs are expressed in the mitochondrial membrane of SH-SY5Y cells.

Subsequently, we tested if the 5-HT_7R agonist LP-211 or the 5-HT_7R antagonist (inverse agonist) SB-269970 influenced the activity of cytochrome c oxidase, which is a critical regulator of oxidative phosphorylation and is used as a marker of neural functional activity (Hevner and Wong-Riley, 1989;

Hüttemann et al., 2012). Recently, it has been demonstrated that activation of mitochondrial cannabinoid receptor 1 in mouse hippocampus, which is coupled to an intracellular Gi protein, reduced the mitochondrial level of cAMP, causing a decrease of oxidative phosphorylation and thereby of ATP production (Hebert-Chatelain et al., 2016). Our result demonstrated that 5-HT_7R antagonist (inverse agonist) SB-269970 weakly increased cytochrome c oxidase activity, as estimated on mitochondria isolated and purified from the investigated cells. The weak increase in cytochrome c oxidase activity elicited by 5-HT_7R inverse agonist SB-269970 might be linked to a reduction in the intramitochondrial levels of cAMP, consistent with previous findings in which variations of intramitochondrial cAMP levels may upregulate or downregulate cytochrome c oxidase activity (Valsecchi et al., 2013).

Mitochondrial impairments are also present in a murine model of Fragile X syndrome. The murine model of the pathology shows an increased oxidative stress in neurons (Shen et al., 2019), impairments in mitochondrial respiratory chain and altered ATP production (D'Antoni et al., 2020). FMRP binds mRNAs of the mitochondrial respiratory chain components and its absence causes an enhancement of the mitochondrial complex activity (Ascano et al., 2012; Maurin et al., 2018a). In future studies, it would be interesting to test whether LP-211 can rescue the mitochondrial impairment in a mouse model of Fragile X syndrome.

During the period that I spent abroad at the IPMC (Institute Pharmacology Moléculaire Et Cellulaire), I was involved into a project based on the characterization of a new spontaneous mutation in the *Slc12a5* gene codifying for the K^+- Cl^- cotransporter KCC2, affecting the C-terminal region of the protein (Bardoni et al., unpublished). The same KCC2 variant was also found in a human patient affected by epilepsy and intellectual disability (Saito et al., 2017). Other variants in the C-terminal region have been identified and most of them are related to the ASD and neurodevelopmental phenotype in patients, suggesting that alteration in the KCC2 functions contributes to the pathogenesis of ASD. ASD show well-established strong associations with other neuropsychiatric disorders, such as epilepsy (Keller, Basta, Salerno, & Elia, 2017). Both epilepsy and autism have as major hallmark altered synaptic structure and function and for this reason are often named as synaptopathies (Bagni and Zukin, 2019). KCC2 is a cotransporter implicated in brain excitation/inhibition balance; thus mutations of Kcc2 gene might impair the transporter function. In other to investigate the influence of the Kcc2 mutation on the function of KCC2 transporter, I recorded the spiking activity of CA3 neurons in acute hippocampal slices from adult wild-type and Kcc2 mutant mice. After isoguvacine application (a selective agonist of $GABA_A$ receptors), the firing

activity of wild-type and KCC2 mutated CA3 neurons decreased, but there was no significant difference between the action potential frequency in WT respect to KCC2 mutated hippocampal neurons. This absence of difference in the firing frequency could depend on the same amount of KCC2 expressed in the two strains. Our hypothesis was confirmed through a western blot analysis of the hippocampal region: the mutation in *Kcc2* gene does not influence the expression of protein in cortex and hippocampus. KCC2 regulates a number of processes that are crucial for development, such as maturation of dendritic spines (Fiumelli et al., 2013; Gulyás et al., 2001; Li et al., 2007), remodelling the actin filament through the interaction between its C-terminal domain and the synaptic protein, independently from its role as cotransporter (Llano et al., 2015). For this reason, I studied the influence of KCC2 mutated protein on dendritic spine morphology; however, the mutation did not influence dendritic spine morphology in WT and Kcc2 mutant mouse brain, neither in hippocampus nor in cortex. Further studies should be performed to study how the mutations in Kcc2 gene might affect the activity of KCC2 transporter.

Conclusions

To date, no specific therapy is available for patients who suffer from Fragile X syndrome and the clinical treatment focuses on the symptomatic treatment of psychiatric problems and of comorbidities. Several clinical trials in FXS are currently being carried out, although many trials have failed (Berry-Kravis et al., 2016; Youssef et al., 2018). Sertraline, a selective serotonin reuptake inhibitor (SSRI), is widely used to treat anxiety in patients with FXS, in line with the finding that serotonin production is reduced in the brains of young children with autism (Chugani, 2002; Hanson and Hagerman, 2014) and metabolomic studies of lymphoblastoid lines of all types of ASD, including those with FXS, demonstrate down-regulation of the enzymes leading to serotonin production from tryptophan (Boccuto et al., 2013). Sertraline may therefore be considered a targeted treatment for FXS. Our results suggest that in addition to SSRIs, enhancing overall serotonergic transmission, a selective activation of 5-HT_7 receptors using specific agonists may represent a novel strategy for a possible therapy of Fragile X Syndrome.

In the next future it will be interesting to study the role of 5-HT_7 receptors in the mitochondrial respiratory chain of the murine model of FXS, investigating the possibility to rescue the mitochondrial impairments typical of the pathology using a 5-HT_7 agonist.

In addition, our data on the molecular mechanisms of 5-HT_7-mediated rescue of synaptic plasticity in *Fmr1* KO mice might be translated to human models, using iPSC-derived neurons obtained from FXS patients.

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PUBLICATIONS

Received: 8 October 2020 | Revised: 14 March 2021 | Accepted: 9 April 2021

DOI: 10.1111/ejn.15246

SHORT COMMUNICATION

WILEY

Serotonin 5-HT7 receptors require cyclin-dependent kinase 5 to rescue hippocampal synaptic plasticity in a mouse model of Fragile X Syndrome

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Funding information
Università di Catania; Fondazione Telethon, Grant/Award Number: GGP13145

Abstract

Fragile X Syndrome is a genetic form of intellectual disability associated with autism, epilepsy and mood disorders. Electrophysiology studies in *Fmr1* knockout (KO) mice, a murine model of Fragile X Syndrome, have demonstrated alterations of synaptic plasticity, with exaggerated long-term depression induced by activation of metabotropic glutamate receptors (mGluR-LTD) in *Fmr1* KO hippocampus. We have previously demonstrated that activation of serotonin 5-HT7 receptors reverses mGluR-LTD in the hippocampus of wild-type and *Fmr1* KO mice, thus correcting a synaptic dysfunction typically observed in this disease model. Here we show that pharmacological inhibition of cyclin-dependent kinase 5 (Cdk5, a signaling molecule recently shown to be a modulator of brain synaptic plasticity) enhanced mGluR-LTD in wild-type hippocampal neurons, which became comparable to exaggerated mGluR-LTD observed in *Fmr1* KO neurons. Furthermore, Cdk5 inhibition prevented 5-HT7 receptor-mediated reversal of mGluR-LTD both in wild-type and in *Fmr1* KO neurons. Our results show that Cdk5 modulates hippocampal synaptic plasticity. 5-HT7 receptors require Cdk5 to modulate synaptic plasticity in wild-type and rescue abnormal plasticity in *Fmr1* KO neurons, pointing out Cdk5 as a possible novel target in Fragile X Syndrome.

KEYWORDS

5-HT7 receptors, Cdk5, Fragile X Syndrome, hippocampus, mGluR-LTD, Serotonin

Abbreviations: 5-HT, 5-hydroxy-tryptamine; AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Cdk5, Cyclin-dependent kinase 5; D-AP5, D-(-)-2-amino-5-phosphonopentanoic acid; DHPG, dihydroxyphenylglycine; EPSC, excitatory post synaptic current; mGluR-LTD, long-term depression mediated by metabotropic glutamate receptors.

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Edited by: Clive R. Bramham

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1 | INTRODUCTION

Synaptic plasticity represents the cellular basis for activity-dependent establishment and refinement of nerve circuits underlying learning and memory. Among different forms of synaptic plasticity described in the hippocampus, long-term depression induced by activation of metabotropic glutamate receptors (mGluR-LTD) plays an important role in learning and behaviour (Luscher & Huber, 2010). Alterations of mGluR-LTD have been observed in several animal models of neurological diseases involving learning and behavioral deficits, including Fragile X Syndrome (Luscher & Huber, 2010; Sanderson et al., 2016). Fragile X Syndrome is a genetic form of intellectual disability associated with autistic features, epilepsy and mood disorders (Salcedo-Arellano et al., 2020). In *Fmr1* knockout (KO) mice, a murine model of this disease, metabotropic glutamate receptors (mGluRs) are abnormally coupled to their intracellular signaling machinery, leading to excessive activation of downstream pathways and exaggerated mGluR-LTD (Bear et al., 2004; Huber et al., 2002).

Our research group demonstrated that activation of serotonin 5-HT7 receptors is able to reduce excessive mGluR-LTD in *Fmr1* KO hippocampal neurons (Costa et al., 2012) and rescue learning and behavior in *Fmr1* KO mice in vivo (Costa et al., 2018). We have elucidated the first steps of this 5-HT7 receptor-mediated mechanism of action, which relies on cyclic adenosine monophosphate (cAMP) formation and PKA activation (Costa et al., 2018).

In the present work, we have investigated possible involvement of Cyclin-dependent kinase 5 (Cdk5), a kinase implicated in 5-HT7 receptor-mediated stimulation of axonal and dendritic growth in cortical, hippocampal and striatal neurons (Speranza et al., 2013, 2015, 2017). Cdk5 belongs to a large family of cyclin-dependent kinases, but differs from the other members in several ways: Cdk5 is not involved in the cell cycle, being mostly expressed in post-mitotic neurons, and plays a crucial role in the brain controlling neuronal differentiation and migration during development, cytoskeletal and microtubule regulation and synaptic plasticity (Kawauchi, 2014; Shah & Rossie, 2018). Two specific Cdk5 activators, the intracellular membrane-bound peptides p35 and p39, have been identified and localized exclusively in neurons (Ko et al., 2001). In pathological conditions, p35 is cleaved by calpain (a Ca^{2+}-activated protease) into a shorter activator peptide, p25, with a broad cytoplasmic and nuclear localization and a longer half-life, inducing hyperphosphorylation of Cdk5 physiological substrates and abnormal phosphorylation of cytoplasmic and nuclear proteins (Allnutt et al., 2020; Cheung & Ip, 2012; Shah & Rossie, 2018). Aberrant p25/Cdk5 signalling accounts for neuronal damage in mouse models of Alzheimer's disease (Giese, 2014; Liu et al., 2016), Parkinson's disease (He et al., 2020) and traumatic brain injury (Yousuf et al., 2016). Cdk5 downregulation has been associated with epilepsy (Liu et al., 2020), attention deficit and hyperactivity disorder (Drerup et al., 2010) and schizophrenia (Engmann et al., 2011). In the striatum of postmortem Huntington's disease patients and in a mouse model of this pathology, reduced expression of Cdk5 and p35 was observed (Luo et al., 2005; Paoletti et al., 2008) together with abnormal Cdk5 activation by p25 (Paoletti et al., 2008), indicating a complex dysregulation of Cdk5 signaling in Huntington's disease.

In the present work, we have tested a possible involvement of Cdk5 in 5-HT7 receptor-mediated reversal of mGluR-LTD in the hippocampus of wild-type mice and of the *Fmr1* KO mouse model of Fragile X Syndrome.

2 | METHODS

2.1 | Electrophysiology recordings

Experiments were performed using patch clamp recording in acute mouse hippocampal slices from wild-type and *Fmr1* KO mice on a C57BL/6J background, obtained from a breeding colony at the University of Catania (Italy). Mice were maintained with a controlled temperature (21°C ± 1°C) and humidity (50%) on a 12 hr light/dark cycle, with ad libitum food and water. All animal experimentation was conducted in accordance with the European Community Council guidelines (2010/63/EU) and was approved by the University Institutional Animal Care and Use Committee (Project # 250 – approval number: 352/2016-PR).

Acute hippocampal slices were prepared as described previously (Costa et al., 2012) from wild-type and *Fmr1* KO mice (postnatal PN age 14–23 days). Briefly, the brains were removed, placed in oxygenated ice-cold artificial cerebrospinal fluid (ACSF; in mM NaCl 124; KCl 3.0; NaH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 2.0; $NaHCO_3$ 26; D-glucose 10, pH 7.3) and cut into 300 μm slices with a vibratome (Leica VT 1200S). Slices were continually perfused with oxygenated ACSF and viewed with infrared microscopy (Leica DMLFS). Schaffer collaterals were stimulated with negative current pulses (duration 0.3 ms, delivered every 15 s by A310 Accupulser, WPI, USA). Evoked excitatory post synaptic currents (EPSCs) were recorded under whole-cell from CA1 pyramidal neurons (holding potential −70 mV; EPC7-plus amplifier HEKA, Germany). Stimulation intensity was set to induce half-maximal EPSC amplitude. Series resistance (Rs) was continuously monitored by 10 mV hyperpolarizing pulses; recordings were discarded from analysis if Rs changed by more than 20%. EPSC traces were filtered at 3 kHz and digitized at 10 kHz. Data were acquired and analysed using Signal software (CED, England). The recording micropipette (resistance 1.5–3 MΩ) was filled with intracellular solution (in mM: K-gluconate 140; HEPES 10; NaCl 10; $MgCl_2$ 2;

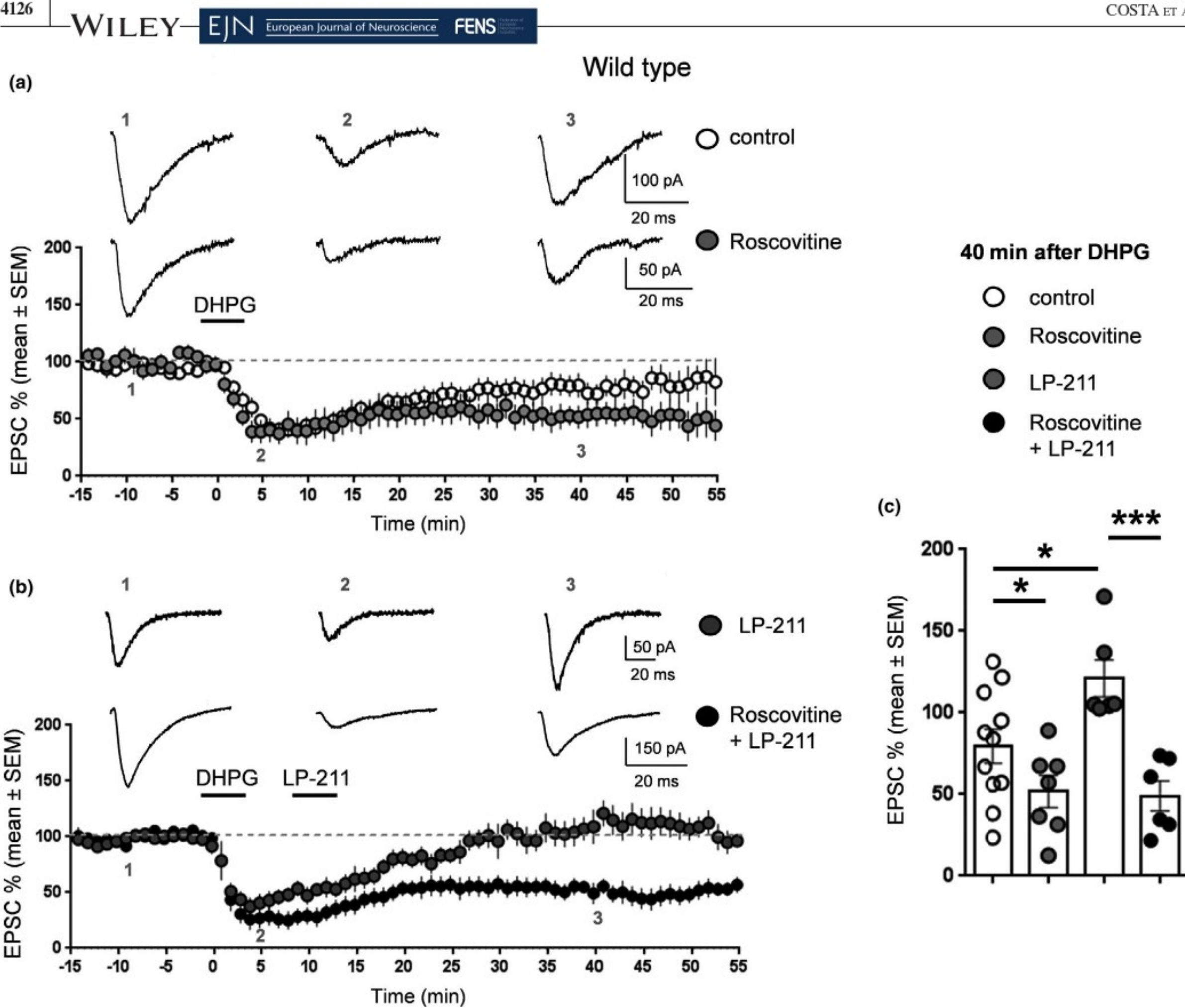

FIGURE 1 Inhibition of Cdk5 enhanced mGluR-LTD in CA1 neurons from wild-type mice and prevented 5-HT7 receptor-mediated effect on mGluR-LTD. AMPA receptor-mediated excitatory post-synaptic currents (EPSCs) were recorded in the presence of D-AP5 (50 μM) and bicuculline (5 μM) under whole-cell patch clamp in the CA3-CA1 synapse in hippocampal slices from wild-type mice. (a) Bath application of the group I mGluR agonist DHPG (100 μM, 5 min) induced a long-term depression (mGluR-LTD) of EPSC amplitude (white dots, $n = 11$). When the Cdk5 inhibitor roscovitine (1.6 μM) was added to intracellular medium, DHPG-induced mGluR-LTD was enhanced (light grey dots, $n = 7$) with respect to control. (b) When DHPG application was followed by application of the 5-HT7 receptor agonist LP-211 (10 nM, 5 min), mGluR-LTD was completely reversed (dark grey dots, $n = 6$). In the presence of intracellular roscovitine (1.6 μM), application of LP-211 did not modify the amount of mGluR-LTD (black dots, $n = 6$). (c) The bar graph shows that the amount of mGluR-LTD measured 40 min after DHPG application (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude; EPSC values of single neurons are displayed for each bar) in the four different experimental conditions (control; roscovitine; LP-211; LP-211 + roscovitine) was significantly different ($p = 0.0006$ by one-way ANOVA followed by Tukey's multiple comparisons test). $*p < 0.05$; $***p < 0.001$

EGTA 0.2; Mg-ATP 3.5; Na-GTP 1; pH 7.3). In a set of experiments, the intracellular solution contained roscovitine, a selective Cdk5 inhibitor, at a concentration (1.6 μM) 10-fold higher than the reported IC_{50} value (0.16 μM) of roscovitine on Cdk5/p35 (Meijer et al., 1997). Bath solution (ACSF) was continuously changed at a flow rate of 1.5 ml/min and routinely contained (-)-bicuculline methiodide (5 μM, Hello Bio) and D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5, 50 μM, Hello Bio) to isolate AMPA receptor-mediated EPSCs. S-3,5-dihydroxyphenylglycine (DHPG, 100 μM; Hello Bio), and LP-211 (10 nM) were dissolved in ACSF and applied by bath perfusion. LP-211 was synthesized and provided by the research group of Prof. Leopoldo (University of Bari, Italy).

2.2 | Data analysis

To compare the amount of DHPG-induced LTD in different groups of neurons, EPSC amplitude values were normalized

as follows: peak amplitude values of EPSCs were averaged over 1 min and expressed as % of baseline EPSC amplitude (calculated from EPSCs recorded during at least 15 min before DHPG application). Normalized % EPSC values from each group of neurons were pooled (mean ± *SEM*) and graphically represented as a function of time. The amount of mGluR-LTD was calculated 40 min after LTD induction by DHPG and was normalized as percentage of baseline (% EPSC amplitude; mean ± *SEM* from all tested neurons). Column graphs indicate normalized % EPSC amplitude (mean ± *SEM* from groups of neurons) 40 min after application of DHPG alone or DHPG with the 5-HT7 receptor agonist LP-211 under different experimental conditions. Single values from each recorded neuron are illustrated for each column. EPSC amplitude values from two groups of neurons were compared using unpaired Student's *t* test, with n indicating the number of neurons tested in each condition. Groups of data from four different experimental conditions (Figure 1c and Figure 2c)

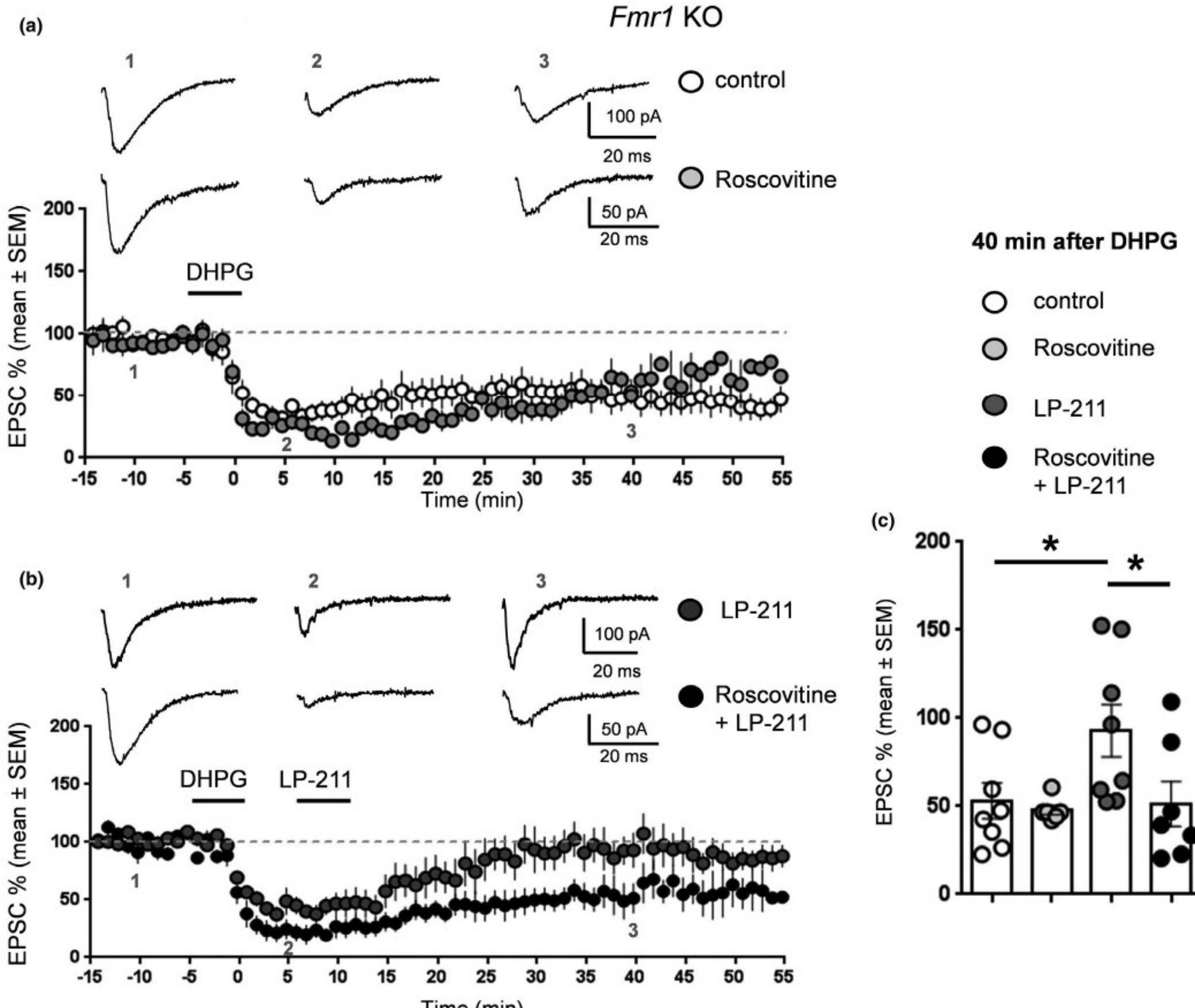

FIGURE 2 Inhibition of Cdk5 did not modify mGluR-LTD in CA1 neurons from *Fmr1* KO mice and prevented 5-HT7 receptor-mediated effect on mGluR-LTD. AMPA receptor-mediated excitatory post-synaptic currents (EPSCs) were recorded from CA1 neurons in the presence of D-AP5 (50 μM) and bicuculline (5 μM) in hippocampal slices from *Fmr1* KO mice. (a) Bath application of DHPG (100 μM, 5 min) induced mGluR-LTD (white dots; $n = 8$). In the presence of intracellular roscovitine (1.6 μM) the amount of mGluR-LTD was not modified (grey dots, $n = 6$) with respect to control conditions. (b) Application of LP-211 (10 nM, 5 min) completely reversed mGluR-LTD in control conditions (dark grey dots, $n = 8$) but had no effect on mGluR-LTD in the presence of intracellular roscovitine (black dots, $n = 7$). (c) The bar graph shows the amount of mGluR-LTD measured 40 min after DHPG application (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude; EPSC values of single neurons are displayed for each bar). The amount of mGluR-LTD in the four experimental conditions (control; roscovitine; LP-211; LP-211 + roscovitine) was significantly different (*$p = 0.0331$ by one-way ordinary ANOVA followed by Tukey's multiple comparisons test)

were compared by one-way ANOVA followed by Tukey's multiple comparisons test (GraphPad Prism 6, USA)

3 | RESULTS

Excitatory post synaptic currents (EPSCs) mediated by α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors for glutamate were evoked every 15 s by stimulation of Schaffer collaterals and were recorded from single CA1 pyramidal neurons under whole-cell patch clamp. In wild-type hippocampal slices, application of DHPG (100 μM, 5 min), an agonist of group I metabotropic glutamate receptors (mGluRs), induced a long-term depression (mGluR-LTD) of AMPA receptor-mediated EPSCs (EPSC amplitude 40 min after DHPG: 79 ± 10% with respect to baseline EPSC amplitude prior to DHPG application, $n = 11$; Figure 1a). In a series of experiments, the Cdk5 inhibitor roscovitine (1.6 μM) was included in the intracellular pipette solution, thus was present since the beginning of recording: in this condition the amount of DHPG-induced mGluR-LTD was significantly enhanced with respect to control conditions (EPSC amplitude: 51 ± 9%, $n = 7$, versus 79 ± 10%, $n = 11$, wild-type DHPG + roscovitine versus wild-type DHPG, $p = 0.04$, $t = 1.821$, $df = 16$; unpaired t test; Figure 1a and c).

We have previously demonstrated that activation of 5-HT7 receptors reverses mGluR-LTD in wild-type and in *Fmr1* KO hippocampal neurons (Costa et al., 2012, 2015, 2018). Confirming our previous data, application of the selective 5-HT7 receptor agonist LP-211 (10 nM, 5 min) 5 min after DHPG application significantly reversed mGluR-LTD (EPSC amplitude: 121 ± 1%, $n = 6$, versus 79 ± 10%, $n = 11$, wild-type DHPG + LP-211 versus wild-type DHPG, $p = 0.011$, $t = 2.513$, $df = 15$; unpaired t test; Figure 1b and c).

In the presence of intracellular roscovitine, (1.6 μM) application of LP-211(10 nM, 5 min) was unable to reverse mGluR-LTD in wild-type slices (EPSC amplitude: 51 ± 9%, $n = 7$, versus 49 ± 9%, $n = 6$; wild-type DHPG + roscovitine versus wild-type DHPG + roscovitine + LP-211, $p = 0.42$, $t = 0.1895$, $df = 11$, Figure 1b and c). LP-211 reversed mGluR-LTD in control conditions but not in the presence of roscovitine (EPSC amplitude: 121 ± 1%, $n = 6$, versus 49 ± 9%, $n = 6$, wild-type DHPG +LP-211 versus wild-type DHPG + LP-211 + roscovitine, $p = 0.0003$, $t = 4.912$, $df = 10$; unpaired t test; Figure 1b and c). Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the amount of mGluR-LTD in the four different conditions (control; roscovitine; LP-211; LP-211 + roscovitine, Figure 1c), confirming a highly significant difference (***$p = 0.0006$).

In *Fmr1* KO slices, application of DHPG (100 μM, 5 min) induced mGluR-LTD in control conditions and in the presence of intracellular roscovitine (1.6 μM) and the amount of mGluR-LTD was similar in the two conditions (EPSC amplitude: 53 ± 10%, $n = 8$ versus 50 ± 3%, $n = 6$; *Fmr1* KO DHPG versus *Fmr1* KO DHPG + roscovitine; $p = 0.39$, $t = 0.2670$, $df = 12$; Figure 2a and c). When comparing data obtained in the presence of intracellular roscovitine, the amount of mGluR-LTD in wild-type was not significantly different from *Fmr1* KO (EPSC amplitude 51 ± 9%, $n = 7$ versus 50 ± 3%, $n = 6$; wild-type DHPG + roscovitine versus *Fmr1* KO DHPG + roscovitine; $p = 0.78$, $t = 0.2817$, $df = 11$; compare the grey dots columns in Figure 1c and Figure 2c).

In *Fmr1* KO neurons, application of LP-211 (10 nM, 5 min) significantly reversed mGluR-LTD in control conditions (EPSC amplitude: 53 ± 10%, $n = 8$, versus 93 ± 14%, $n = 8$, *Fmr1* KO DHPG versus *Fmr1* KO DHPG + LP-211, $p = 0.0219$, $t = 2.216$, $df = 14$; unpaired t test; Figure 2b and c) but had no effect in the presence of roscovitine, (EPSC amplitude: 51 ± 12%, $n = 7$, versus 50 ± 3, $n = 6$; *Fmr1* KO DHPG + roscovitine + LP-211 versus *Fmr1* KO DHPG + roscovitine; $p = 0.47$, $t = 0.07344$, $df = 11$; Figure 2b and c). With intracellular roscovitine, the effect of LP-211 on mGluR-LTD was significantly reduced with respect to control (EPSC amplitude: 93 ± 14%, $n = 8$, versus 51 ± 12%, $n = 7$, *Fmr1* KO DHPG + LP-211 versus *Fmr1* KO DHPG + LP-211 + roscovitine, $p = 0.0286$, $t = 2.087$, $df = 13$; unpaired t test; Figure 2b and c). The amount of mGluR-LTD in the four different experimental conditions (control; roscovitine; LP-211; LP-211 + roscovitine, Figure 2c) was significantly different (*$p = 0.031$, one-way ANOVA followed by Tukey's multiple comparisons test). LP-211-mediated reversal of mGluR-LTD was completely abolished by roscovitine in wild-type and in *Fmr1* KO to a comparable extent (EPSC amplitude: 49 ± 9%, $n = 6$, versus 51 ± 12%, $n = 7$, wild-type DHPG + LP-211 + roscovitine versus *Fmr1* KO DHPG + LP-211 + roscovitine, $p = 0.896$, $t = 0.1336$, $df = 11$; unpaired t test; compare Figures 1c and 2c).

These results together show that Cdk5 inhibition prevented 5-HT7 receptor-mediated reversal of mGluR-LTD both in wild-type and in *Fmr1* KO neurons.

4 | DISCUSSION

Our data show that Cdk5 inhibition in wild-type hippocampal CA1 neurons enhanced mGluR-LTD to a level comparable to *Fmr1* KO neurons. This result differs from control conditions, in which the amount of mGluR-LTD in wild-type neurons is significantly lower than that observed in *Fmr1* KO neurons (Choi et al., 2011; Costa et al., 2012; Gomis-Gonzalez et al., 2016; Huber et al., 2002; Zhang et al., 2009). Enhancement of mGluR-LTD in wild-type neurons following Cdk5 inhibition suggests that, in physiological conditions, Cdk5 exerts a negative control on mGluR-LTD. Our results also suggest that either the expression or the function

of Cdk5 in *Fmr1* KO neurons might be reduced compared to wild-type and that reduced Cdk5 function might account for enhanced mGluR-LTD. In accordance with our hypothesis, a recent study shows a reduced expression of Cdk5 in the hippocampus of *Fmr1* KO mice (Zhang et al., 2020). In future studies, it might be interesting to measure the activation level of Cdk5 and of its physiological activators p35 and p39 in neurons from *Fmr1* KO mice and, possibly, in human neurons derived from Fragile X Syndrome patients using induced pluripotent stem cell (iPSC) differentiation strategies.

We further show that activation of 5-HT7 receptors was unable to reverse mGluR-LTD in both wild-type and *Fmr1* KO neurons following Cdk5 inhibition, showing that 5-HT7 receptors recruit Cdk5 to modulate mGluR-LTD.

Roscovitine has a similar affinity for Cdc2 (also known as Cdk1), Cdk2, Cdk5 and Cdk7, with reported IC_{50} values of 0.65, 0.7, 0.16 and 0.45 µM respectively (Meijer et al., 1997; Schang et al., 2002). However, published data suggest that in our experimental conditions roscovitine acted primarily on Cdk5. Indeed, Cdc2 and Cdk2 play a key role in the cell cycle and are expressed exclusively by dividing cells during embryonic development: their maximal expression in mouse forebrain was found between embryonic day 1 and 11 (E1-E11), was barely detectable by E16-17 and remained very low throughout adult life. Conversely, an opposite pattern of expression and activity was described for Cdk5, which is expressed in mouse forebrain and hippocampus exclusively in post-mitotic neurons, with a growing level of expression from embryonic to adult ages (Tsai et al., 1993). Another study showed a weak expression of Cdk1 and Cdk2 in mouse hippocampal pyramidal neurons, but at PN 11 (very close to the age of mice used in our study) they were detected at low levels only in the nucleus and not in the cytoplasm; cytoplasmic expression of Cdk1 and Cdk2 in hippocampal neurons was found only in adults (9 months PN) (Schmetsdorf et al., 2005). Very little information is presently available about Cdk7 expression in the brain. In mouse cortical neurons, Cdk7 levels were very low before PN 30 (He et al., 2017). In the present work, we have studied fully differentiated (non-dividing) mouse hippocampal pyramidal neurons at a post natal age (PN 14–23) when Cdk5 is highly expressed whereas Cdk1, Cdk2 and Cdk7 expression levels are very low. Therefore, we believe that in our experimental conditions roscovitine acted primarily through Cdk5 inhibition.

In our experiments, roscovitine was included in the intracellular pipette solution, thus Cdk5 inhibition was exclusively exerted in the CA1 neuron under recording, indicating a postsynaptic role of Cdk5 in 5-HT7 receptor-mediated effect.

In the last decade, interesting publications have indicated a connection between 5-HT7 receptors and Cdk5, showing that 5-HT7 receptors require Cdk5 to stimulate axonal elongation and dendrite formation in cultured neurons from rodent brain cortex, hippocampus and striatum (Speranza et al., 2013, 2015, 2017). The intracellular pathway linking 5-HT7 receptors to Cdk5 activation remains to be clarified. A plausible link might be the cAMP pathway, since increases in cAMP levels were shown to stimulate p35 expression and Cdk5 activity in rat cultured neurons (He et al., 2016). 5-HT7 receptors are coupled to Gs protein, stimulating adenylate cyclase and cAMP formation (Wirth et al., 2017), thus we might speculate that 5-HT7 receptor-induced cAMP increase might stimulate the p35/Cdk5 pathway in hippocampal neurons. This issue is particularly relevant to Fragile X Syndrome, since reduced levels of cAMP were measured in blood platelets of Fragile X patients (Berry-Kravis & Huttenlocher, 1992; Berry-Kravis & Sklena, 1993) and the cAMP signaling cascade is altered at different levels in neurons from *Fmr1* KO mice, originating a "cAMP hypothesis" of the disease (Kelley et al., 2008). In the brain of *Fmr1* KO mice, overexpression and increased activity of phosphodiesterase 2A (PDE2A), a cAMP degrading enzyme, leads to reduced cAMP formation and dysregulation of cAMP downstream signaling (Maurin et al., 2018, 2019). As above mentioned, cAMP can stimulate p35/Cdk5 expression and function in rodent neurons (He et al., 2016); thus reduced cAMP levels in mouse *Fmr1* KO hippocampal neurons might be related to the reduced Cdk5 expression recently described (Zhang et al., 2020).

Besides a possible involvement of cAMP, 5-HT7 receptors might activate Cdk5 through additional mechanisms. A just-published paper shows that 5-HT7 receptors are physically linked to Cdk5 and stimulate Cdk5 activity in a G protein-independent mode. Of note, using several in vitro and in vivo approaches, the same work shows that abnormally high constitutive activity of 5-HT7 receptors caused Tau hyperphosphorylation, formation of Tau aggregates, neuronal damage, impaired synaptic plasticity and learning deficits that were rescued by knocking down 5-HT7 receptor expression, suggesting that inhibition of 5-HT7 receptor-mediated Cdk5 activity might be used as a therapy for tauopathies (Labus et al., 2021).

Many therapeutic strategies for a potential treatment of Alzheimer's disease and Parkinson's disease aim to reduce excessive Cdk5 activity, focusing on Cdk5 inhibitors (Cheung & Ip, 2012; Gong & Iqbal, 2008). Our present results, together with the work of Zhang et al. (Zhang et al., 2020), indicate that in *Fmr1* KO neurons Cdk5 activity is instead abnormally low, suggesting that activation of Cdk5 might be beneficial in Fragile X Syndrome.

Pharmacological activators of Cdk5 are not available at present. The intracellular membrane-bound kinases p35 and p39 are physiological Cdk5 activators; only few upstream extracellular messengers are currently known to activate p35 and Cdk5, namely BDNF (Cheung et al., 2007), dopamine through D1 receptors (Lebel et al., 2009), and serotonin through 5-HT7 receptors (Speranza et al., 2013, 2015, 2017). We suggest that selective 5-HT7 receptor agonists can

be used to stimulate Cdk5 activity and might become useful pharmacological tools for Fragile X Syndrome. In addition, we suggest that the effects of 5-HT7 receptor agonists might be studied in other conditions associated with reduced Cdk5 expression and function.

ACKNOWLEDGEMENTS

The authors thank Dr. Michael Tranfaglia (Medical Director and Chief Scientific Officer of FRAXA Research Foundation, U.S.A.) for critical reading of the manuscript. The present work was financed by Telethon Foundation (grant GGP13145) and by the University of Catania (grant Chance 2017). We wish to thank Dr. Marco Abbate for veterinary assistance, Mr Giuseppe Valastro and Mr Nicola Pulvirenti for animal care and technical assistance. Images from Motifolio drawing toolkit (www.motifolio.com) were utilized in the graphical abstract preparation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Lucia Ciranna designed the study, analysed data and drafted the paper; Lara Costa and Alessandra Tempio performed experiments and analysed data; Enza Lacivita and Marcello Leopoldo designed 5-HT7R agonist and analysed data.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ejn.15246.

DATA AVAILABILITY

The data that support the findings of this study are openly available in the public repository Figshare at https://doi.org/10.6084/m9.figshare.14431205.v1

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How to cite this article: Costa L, Tempio A, Lacivita E, Leopoldo M, Ciranna L. Serotonin 5-HT7 receptors require cyclin-dependent kinase 5 to rescue hippocampal synaptic plasticity in a mouse model of Fragile X Syndrome. *Eur J Neurosci*. 2021;54:4124–4132. https://doi.org/10.1111/ejn.15246

International Journal of
Molecular Sciences

Communication

Mitochondrial Membranes of Human SH-SY5Y Neuroblastoma Cells Express Serotonin 5-HT$_7$ Receptor

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Received: 20 November 2020; Accepted: 13 December 2020; Published: 17 December 2020

Abstract: Mitochondria in neurons contribute to energy supply, the regulation of synaptic transmission, Ca^{2+} homeostasis, neuronal excitability, and stress adaptation. In recent years, several studies have highlighted that the neurotransmitter serotonin (5-HT) plays an important role in mitochondrial biogenesis in cortical neurons, and regulates mitochondrial activity and cellular function in cardiomyocytes. 5-HT exerts its diverse actions by binding to cell surface receptors that are classified into seven distinct families (5-HT1 to 5-HT7). Recently, it was shown that 5-HT3 and 5-HT4 receptors are located on the mitochondrial membrane and participate in the regulation of mitochondrial function. Furthermore, it was observed that activation of brain 5-HT7 receptors rescued mitochondrial dysfunction in female mice from two models of Rett syndrome, a rare neurodevelopmental disorder characterized by severe behavioral and physiological symptoms. Our Western blot analyses performed on cell-lysate and purified mitochondria isolated from neuronal cell line SH-SY5Y showed that 5-HT7 receptors are also expressed into mitochondria. Maximal binding capacity (Bmax) obtained by Scatchard analysis on purified mitochondrial membranes was 0.081 pmol/mg of 5-HT7 receptor protein. Lastly, we evaluated the effect of selective 5-HT7 receptor agonist LP-211 and antagonist (inverse agonist) SB-269970 on mitochondrial respiratory chain (MRC) cytochrome c oxidase activity on mitochondria from SH-SY5Y cells. Our findings provide the first evidence that 5-HT7 receptor is also expressed in mitochondria.

Keywords: serotonin; mitochondria; G protein-coupled receptor; 5-HT7 receptor; cytochrome c oxidase

1. Introduction

G-protein-coupled receptors (GPCRs) are the largest family of membrane receptors in eukaryotes. About 800 GPCRs have been identified in humans, of which about half have sensory functions, while

the remaining half include nonsensory GPCRs that mediate signaling by ligands and are the targets for a majority of drugs in clinical usage [1].

The largest majority of studies focused on GPCRs present on the cell surface and their downstream signaling partners. However, a critical new role is emerging for GPCRs to signal from inside the cell. In fact, intracellular GPCRs were localized in the nuclear membrane, endoplasmic reticulum, lysosomes, and mitochondrial membranes [2].

Recent studies unveiled that various GPCRs are associated with mitochondria. Purinergic receptors were among the first GPCRs to be localized to mitochondria, where they contribute to the regulation of mitochondrial Ca^{2+} uptake [3]. Angiotensin receptors AT1 and AT2 were found in the mitochondria of several cell types. The AT2 receptor was localized on the inner mitochondrial membrane, where its activation results in nitric oxide formation and respiration suppression in various cell types including neurons [4]. Serotonin 5-HT3 and 5-HT4 receptors are present on cardiac mitochondria, where they regulate mitochondrial activities and cellular functions [5]. Melatonin MT1 receptors are present on the outer mitochondrial membrane, where melatonin activates Gαi and blocks adenylate cyclase activity, leading to the inhibition of stress-induced cytochrome c release and caspase activation [6]. Altogether, these studies pose the question as to whether many processes previously thought to be mediated by plasma membrane receptors are also mediated by mitochondrial GPCRs [2].

Serotonin 7 receptor (5-HT7R) is a GPCR broadly expressed in the central nervous system, including the hypothalamus, thalamus, hippocampus, prefrontal cortex, striatum, amygdala, and spinal cord. 5-HT7R controls diverse neural functions such as thermoregulation, the sleep–wake cycle, circadian rhythm, nociception, learning, and memory processing. 5-HT7R dysfunction has been related to neuropsychiatric and neurodevelopmental diseases (depression, anxiety, schizophrenia, epilepsy, impulsivity, and autism spectrum disorder) [7].

5-HT7R is a key component of the molecular cascade involved in the organization and reshaping of neuronal cytoarchitecture during prenatal and postnatal development, as well as in the mature brain. The involvement of 5-HT7R in synaptic plasticity was further demonstrated by studies reporting that its activation rescues long-term potentiation or long-term depression deficits in various rodent models of neurodevelopmental diseases [8]. In fact, the activation of 5-HT7R corrects molecular, electrophysiological, and behavioral alterations in mice models of neurodevelopmental disorders, such as Fragile-X syndrome [9], Rett syndrome, and CDKL5 deficiency disorder [7]. In particular, Valenti and coworkers reported that selective 5-HT7R agonist LP-211 [10] had beneficial effects on the neurobehavioral phenotype of two mouse models of Rett syndrome. Interestingly, the effects were associated with the rescue of mitochondrial abnormalities in the brain [11]. The same group also reported that the reactivation of mitochondrial respiratory chain complexes in the brain of a mouse model of CDKL5 deficiency disorder by treatment with LP-211 rescued the defective brain energy status [12]. This finding was consistent with literature data, as mitochondrial dysfunction and altered mitochondrial dynamics were documented in pathologies characterized by impaired neuronal development [13]. The above studies suggested a direct link between mitochondrial functionality and 5-HT7R, but they did not investigate the mechanism through which 5-HT7R elicited the observed effects. Considering the increasing number of studies reporting the presence of GPCRs on mitochondrial membranes, we searched the literature to find if 5-HT7Rs were ever localized on mitochondrial membrane, but we did not find any evidence for any cell type.

Thus, we addressed the relationship between 5-HT7R and mitochondrial function by investigating the presence of 5-HT7R on the mitochondrial membrane of the SH-SY5Y cell line. Over the last forty years, the SH-SY5Y cell line has been extensively used as a neuronal model due to experimental limitations caused by the inability of primary neurons to propagate in vitro. Consequently, a wealth of biological research has relied on SH-SY5Y cells as a model to investigate central-nervous-system (CNS) disorders, including neurodevelopmental disorders [14]. It is, therefore, not surprising that SH-SY5Y cells have been used to investigate the effects downstream of the activation of 5-HT7R [15,16].

Consistently, Yuksel and coworkers reported 5-HT7R mRNA expression in SH-SY5Y cells [17], even if no study reported the SH-SY5Y cellular expression of a 5-HT7R protein.

Therefore, we investigated the expression of 5-HT7R in the SH-SY5Y cell line, verifying the presence of the receptor at mitochondrial membranes. Then, we determined the total density (Bmax) of 5-HT7R in SH-SY5Y mitochondrial subfraction via Scatchard analysis. Lastly, we estimated mitochondrial respiratory chain (MRC) cytochrome c oxidase (Complex IV) activity in mitochondria extracted from SH-SY5Y before and after incubation with selective 5-HT7R agonist LP-211 or selective antagonist (inverse agonist) SB-269970 through spectrophotometric assays.

2. Results

2.1. 5-HT7Rs Are Located in Cytosolic and Mitochondrial Fractions of SH-SY5Y Cells

We first investigated the expression of 5-HT7Rs in the SH-SY5Y cell line through immunoblotting analysis of cytosolic and mitochondrial-enriched fractions using a rabbit polyclonal antibody against a sequence identical for all human splice variants of 5-HT7R. As a positive control, we used membranes obtained from HEK 293 cells, stably transfected with cDNA for 5-HT7R that express 5-HT7R. These membranes were the very same used in the radioligand binding assay [18]. Western blot analysis revealed that 5-HT7R was present in both cytosolic and mitochondrial fractions (Figure 1A). Two bands were detected at approximately 40 and 50 KDa in the cytosolic and the mitochondrial-enriched fraction, respectively. This data pattern was observed in at least three independent experiments. Results showed that two forms of 5-HT7R are expressed in SH-SY5Y cells.

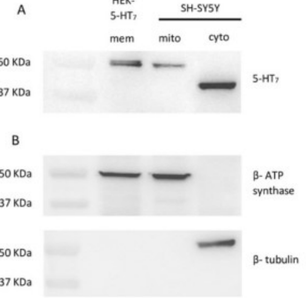

Figure 1. (**A**) Expression of 5-HT7R in cytosolic (cyto) and mitochondrial (mito) enriched fractions obtained from SH-SY5Y cell line. Positive control represented by membranes (mem) obtained from 5-HT7R-stably transfected HEK 293 cells. (**B**) Same fractions of SH-SY5Y analyzed to detect β-ATP synthase (mitochondria marker) and β-tubulin (cytosol marker) expression by sequential reprobing on same blot. Molecular mass markers (KDa) indicated on the left.

The expected range was 43–50 KDa and corresponded with the three known 5-HT7R splice variants. 5-HT7R undergoes alternative splicing at the second intron, located in the carboxyl terminus, giving rise to three splice variants in humans (a,b,d) [19]. In addition, 5-HT7R undergoes different post-translational modifications. This receptor contains two consensus sequences for N-linked glycosylation sites in the extracellular N-terminal region [20] and for attachment of saturated fatty acids (i.e., palmitate) to cysteine residues within the protein via thioesterification (S-palmitoylation) [21]. The 40 KDa cytosolic form suggested the presence of a form of the receptor not subjected to post-translational modifications in SH-SY5Y cells [22].

In order to rule out any cross-contamination from the mitochondrial to the cytosolic fraction and vice versa, the same cell fractions were probed using antibodies as marker proteins for specific cellular compartments: an anti-β-ATP synthase antibody for mitochondria, and an anti-β-tubulin antibody for

cytosol. Results showed no β ATP synthase band in the cytosolic fraction and no β-tubulin band in the mitochondrial fraction (Figure 1), indicating that there was no contamination in the analyzed fractions.

2.2. Saturation-Binding Assay

The presence of 5-HT7R in the SH-SY5Y cell line was investigated with saturation-binding analysis. The assay was performed on both whole SH-SY5Y cell membranes and SH-SY5Y cell mitochondrial fractions. Results demonstrated the presence of 5-HT7R in both preparations, albeit with different expressions. SH-SY5Y cell membrane Bmax was 0.51 pmol/mg of protein (Figure 2A), whereas SH-SY5Y cell mitochondrial fraction Bmax was 0.081 pmol/mg of protein (Figure 2B). Furthermore, experiments gave different Kd values for [^{3}H]SB-269970 in whole SH-SY5Y cells (Kd = 6.55 nM) and SH-SY5Y cells mitochondrial-enriched fraction (Kd = 1.90 nM). For comparative purposes, saturation-binding analysis, performed with membranes obtained from HEK 293 cells stably transfected with cDNA for 5-HT7R, is reported in Figure 2C.

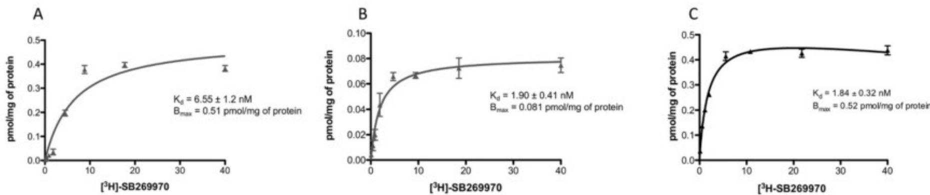

Figure 2. Scatchard analysis with selective 5-HT7R radioligand [^{3}H]SB-269970 on (**A**) whole SH-SY5Y cell membranes, (**B**) mitochondrial-enriched fractions obtained from SH-SY5Y cell line, and (**C**) membranes of 5-HT7R-transfected HEK 293 cells.

Schild regression analysis indicated the presence of a single binding site in the SH-SY5Y cells' mitochondrial-enriched fraction and the presence of an additional binding site in whole SH-SY5Y cell membranes.

2.3. Administration of SB-269970 (but Not LP-211) to Mitochondria Weakly Influences Mitochondrial Respiratory Chain (MRC) Cytochrome c Oxidase Activity

To investigate whether the mitochondrial function is influenced by the activation of 5-HT7Rs located on mitochondria in the SH-SY5Y cell line, mitochondrial respiratory chain (MRC) cytochrome c oxidase activity was measured in mitochondria purified from SH-SY5Y cells after incubation with selective 5-HT7R agonist LP-211 or 5-HT7R antagonist (inverse agonist) SB-269970 (Figure 3). LP-211 or SB-269970 was dissolved in 10% ethanol in H_2O. Mitochondria were incubated for 3 min with LP-211 or SB-269970 (1 μM) before the measurements. Cytochrome c oxidase activity was 258.6 ± 4.28 nmol/min/mg in H_2O and 286.9 ± 29.41 nmol/min/mg in 10% ethanol in H_2O. Subsequently, the effect of selective 5-HT7R agonist LP-211 on cytochrome c oxidase activity was tested. No statistically significant differences between LP-211 treatment and control were observed. Upon treatment with LP-211, cytochrome c oxidase activity was 292.7 ± 39.51 nmol/min/mg.

Lastly, the effect of selective 5-HT7R antagonist (inverse agonist) SB-269970 on cytochrome c oxidase activity was evaluated. The incubation of mitochondria with SB-269970 resulted in a weak increase in cytochrome c oxidase activity compared to control. Upon treatment with SB-269970, cytochrome c oxidase activity was 303.63 ± 30.48 nmol/min/mg (Figure 3).

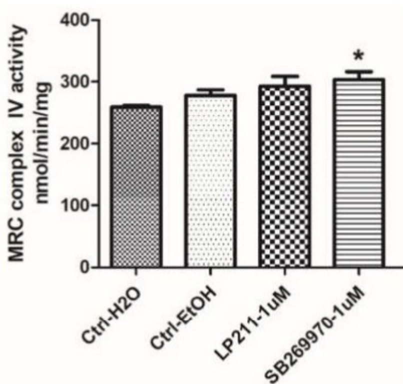

Figure 3. SB-269970 showed a weak stimulating effect on cytochrome c oxidase activity, which was spectrophotometrically measured in mitochondrial fractions from SH-SY5Y cells administered with LP-211 and SB-269970 3 min before measurements. Values represent mean rates (nmol/min/mg) ± SEM obtained from at least four independent experiments. * $p < 0.05$, nonparametric Wilcoxon test between mitochondria administered with SB-269970 and nontreated mitochondria in two controls. Ctrl-EtOH, 10% EtOH in H_2O.

3. Discussion

5-HT7Rs are expressed in discrete areas of the CNS at the neuronal and astrocyte levels. These receptors are postsynaptically located, and are positively coupled with a Gs or G12 protein [7]. Several studies highlighted the role of 5-HT7R in neuronal plasticity as a key component of the signaling cascade that regulates several processes in various stages of brain development [8]. Studies conducted with selective 5-HT7R agonist LP-211 showed that 5-HT7R activation can correct molecular, electrophysiological, and behavioral defects in various mouse models of neurodevelopmental diseases [7]. Two of the studies showed that 5-HT7R activation is able to reactivate mitochondrial dysfunction in mouse models of Rett syndrome and CDKL5 deficiency [11,12]. The mechanism by which LP-211 had a positive effect on mitochondrial function was not investigated. Serotonin has a role in the biogenesis of mitochondria. In fact, 5-HT2A receptors are responsible for such an effect [23]. There are also studies that provide evidence of the presence of GPCR on mitochondrial membranes, where stimulation of these receptors has an influence on mitochondrial function [2]. This posed the question of whether the observed effect could be mediated by 5-HT7R expressed into mitochondria. From a search of the literature, the presence of 5-HT7R on the mitochondrial membrane has never been investigated. Thus, to address this fascinating issue, we focused on the SH-SY-5Y cell line, which was used to study the cellular effect of 5-HT7R stimulation [15,16]. We first investigated the expression of 5-HT7Rs in the SH-SY5Y cell line through immunoblotting analysis of the cytosolic and the mitochondrial-enriched fractions using a rabbit polyclonal antibody against a sequence identical for all human splice variants of 5-HT7R. Western blot analysis revealed that 5-HT7R was present in both cytosolic and mitochondrial fractions (Figure 1A). Two bands with molecular masses of approximately 40 and 50 KDa were detected, the former present in the cytosolic fraction and the latter in the mitochondrial fraction. The 45–50 KDa range was consistent with the expected molecular mass of 5-HT7R, which has two sites for N-linked glycosylation in the amino terminal region and several sites for phosphorylation. Thus, two protein forms of 5-HT7R could be expressed in SH-SY5Y cells reflecting different levels of glycosylation and/or phosphorylation [22].

Quantification of the 5-HT7R protein was performed by Scatchard analysis in both membranes from whole SH-SY5Y cells and mitochondrial-enriched membranes of SH-SY-5Y cells. We selected radioligand [^{3}H]SB-269970 because it shows greater 5-HT7R selectivity compared to that of [^{3}H]5-CT and [^{3}H]LSD, which are used in routine radioligand binding assays with 5-HT7R-transfected cell

lines [24]. 5-HT7R was detected in both preparations at different concentrations. Bmax values were 0.51 pmol/mg of protein (membranes from whole SH-SY-5Y cells) and 0.081 pmol/mg of protein (mitochondrial-enriched membranes of SH-SY-5Y cells). Scatchard analysis agreed with Western blot analysis regarding the expression of 5-HT7R in the mitochondrial membranes of SH-SY5Y cells. The K_d value of $[^3H]$SB-269970 in the mitochondrial-enriched fraction was 1.9 nM, close to the literature value (K_d = 1.7 nM in guinea pig cortex membranes) [25]. In the membranes of whole SY-SH-5Y cells, the K_d value of $[^3H]$SB-269970 was 6.55 nM, different from the literature data. This difference prompted us to investigate whether the radioligand was interacting with one or more binding sites. Hill plot analysis indicated the presence of a single binding site in the mitochondrial-enriched fraction of SH-SY5Y cells (h = 1.5) and the presence of more than one binding site in membranes of whole SH-SY5Y cells (h = 3.4). Considering that $[^3H]$SB-269970 has measurable affinity for 5-HT5a receptor (HT5a Ki = 63.1 nM; 5-HT7 Ki = 1.3 nM) [24], the presence of 5-HT5a receptor protein in membranes of whole SH-SY5Y cells cannot be ruled out.

To our knowledge, this is the first demonstration that 5-HT7R is expressed in the mitochondrial membrane of SH-SY5Y cells.

Once we had detected the presence of 5-HT7Rs on mitochondrial membranes, we tested if 5-HT7R agonist LP-211 or antagonist (inverse agonist) SB-269970 had an effect on the activity of cytochrome c oxidase, which is a critical regulator of oxidative phosphorylation and used as a marker of neural functional activity [26,27]. A recent study showed that stimulation of mitochondrial cannabinoid receptor 1 in a mouse's hippocampus, coupled with an intramitochondrial Gαi protein, inhibits a soluble adenylyl cyclase, thereby reducing intramitochondrial cAMP levels. This caused a decrease in oxidative phosphorylation system functions and ATP production. These events led to a decrease in brain mitochondrial function required for the acute effects of cannabinoids on synaptic depression and consequent amnesia [28]. Our test showed that 5-HT7R antagonist (inverse agonist) SB-269970 weakly increased cytochrome c oxidase activity, as estimated on mitochondria isolated and purified [29] from the investigated cells. Results indicated that the increase in cAMP caused by LP-211 had no significant effect on cytochrome c oxidase activity. On the other hand, the weak increase in cytochrome c oxidase activity elicited by 5-HT7R inverse agonist SB-269970 might be linked to a reduction in the intramitochondrial levels of cAMP. This might be compatible with findings showing variations of intramitochondrial cAMP levels may upregulate or downregulate cytochrome c oxidase activity [30].

4. Materials and Methods

4.1. Drugs

SB-269970 ((2R)-1-([3-Hydroxyphenyl]sulfonyl)-2-(2-[4-methyl-1-piperidinyl]ethyl)pyrrolidine hydrochloride—CAS no. 261901-57-9) was purchased by Tocris Bioscience, Bristol, UK. LP-211 (*N*-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide—CAS no. 1052147-86-0) was provided by Enza Lacivita and Marcello Leopoldo.

4.2. Cell Culture

SH-SY5Y neuroblastoma cells (cat. CRL-2266, ATCC, LGC Standards, Sesto San Giovanni, Italy) were cultured in a 1:1 mixture of Eagle's Minimum Essential Medium (cat. 15-010-CVR, Corning, SIAL, Roma, Italy) and Ham's F12 Medium (cat. 10-080-CVR, Corning). This medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (cat. 35-079-CV, Corning), 1% (v/v) glutamine (cat. ECB3000D, Euro Clone, Pero, Italy) and 1% (v/v) penicillin–streptomycin (cat.30-002-CI, Corning). Cells were cultured in T75 flasks at 37 °C with 5% CO_2 at saturated humidity and kept below 25 passage to avoid senescence.

4.3. Mitochondrial-Enriched Fraction

Cells grown in T75 flasks were detached and centrifuged at 125 *g* for 5 min, the supernatant was discarded, and cells were resuspended in Ringer NaCl buffer (135 mM NaCl, 20 mM HEPES, 0.8 mM $MgSO_4$, 3 mM KCl, 1.8 mM $CaCl_2$, 11 mM D-glucose, pH = 7.5) [31]. Afterward, cells were centrifuged at 125 *g* for 5 min, suspended in 2 mL of A buffer (sucrose 320 mM, Tris-HCl 5 mM, EGTA 2 mM, pH = 7.4), and homogenized with a glass–Teflon grinder kept in ice. The homogenate was centrifuged at 4 °C for 6 min at 2000 *g* to remove nuclei and tissue particles, while the supernatant was collected and centrifuged at 4 °C for 15 min at 12,000 *g* to pellet mitochondria. Lastly, the pellet was washed with a buffer in order to reduce the cytosolic contamination.

4.4. Western Blot Analysis

The mitochondrial-enriched fraction, as described above, was obtained, and treated with RIPA buffer (cat. R0278, Sigma Aldrich, SIAL, Roma, Italy) and protease inhibitor cocktail (cat. P8340, Sigma Aldrich). The mitochondrial lysate was centrifuged at 4 °C for 15 min at 12,000 *g*, and protein concentration in the supernatant was dosed with DC Protein Assay (cat. 500111, Bio-Rad, Bio-Rad Laboratories, Segrate, Italy). Denatured proteins were separated through SDS-PAGE using Mini Protean TGX Stain-Free gels at 10% polyacrylamide (cat. 456-8034, Bio-Rad) and transferred in a 0.2 um PVDF membrane (cat. 1704156, Bio-Rad) using Trans Turbo Blot Transfer System. Membranes were blocked with 5% nonfat milk in TBS-Tween 20 0.1% for 1 h at room temperature and incubated overnight with an anti-5-HT7 (cat. IMG-368, dilution 1:125, Imgenex, Bio-TECHNE, Milano, Italy), anti-β-tubulin (cat. T8328, Sigma Aldrich, dilution 1:5000) and anti-β-ATP synthase (cat. MABS1304, EMD Millipore, dilution 1:1000) antibodies. Membranes were rinsed three times in TBS-Tween 20 0.1% and incubated with either antimouse (cat. G-21040, dilution 1:2000, ThermoFisher Scientific, Life Technologies Italia, Monza, Italy) or antirabbit (cat. AP307P, dilution 1:2000, EMD Millipore, Sigma Aldrich) antibody. Blots were revealed using Clarity Western ECL Substrate (cat. 170-5060, Bio-Rad) through UVITEC Cambridge Chemiluminescence Imaging System.

4.5. Cytochrome c Oxidase Activity Measurements

To estimate cytochrome c oxidase (Complex IV) activity, we performed spectrophotometric assays with or without mitochondrial treatment with selective 5-HT7R agonist LP-211 (1 μM) or 5-HT7R antagonist SB-269970 (1 μM), using a standard method [29] with some modifications. LP-211 or SB-269970 was dissolved in 10% ethanol in H_2O because of low solubility in pure H_2O. To evaluate the effect of ethanol in the medium, the activity of cytochrome c oxidase was measured by incubating mitochondria for 3 min using H_2O or 10% ethanol in H_2O. No significant difference in cytochrome c oxidase activity was observed between the two tests. Mitochondria, obtained as described above, were subjected to three cycles of freeze and thaw in hypotonic potassium phosphate buffer (20 mM, pH = 7.4) to maximize the enzymatic rates. Then, 50 μg of mitochondria was incubated for 3 min with LP-211 (1 μM) or SB-269970 (1 μM) in 10% ethanol in H_2O or the medium alone, as the control condition in a solution composed by 250 μL of potassium phosphate buffer (0.1 M, pH = 7.5), 5 μL of n-dodecyl-β-D-maltoside (150 mM), and H_2O to reach the volume of 950 μL in cuvette. The reaction began by adding 50 μL of reduced cytochrome c (1 mM). The decrease in absorbance at λ = 550 nm due to the oxidation of cytochrome c was monitored. Cytochrome c oxidase specific activity was checked by adding 20 μL of KCN 60 mM.

4.6. SH-SY5Y Membrane Preparation for Saturation-Binding Assay

The membrane preparation was carried out as described by Colabufo et al. with minor modifications [32]. Briefly, SH-SY5Y cells were cultured to 80% confluence; then, the medium was removed, and cells were rinsed in PBS. After detaching, cells were suspended in ice-cold 10 mM Tris-HCl (pH 7.4), containing 0.32 M sucrose and homogenized in a Potter-Elvehjem homogenizer

(Teflon pestle). The homogenate was centrifuged at 31,000 *g* for 15 min at 4 °C, and the supernatant was discarded. The final pellet was resuspended in ice-cold 10 mM Tris-HCl (pH 7.4) and stored at −80 °C until use.

4.7. Saturation-Binding Assay

Saturation experiments were carried out as previously reported with minor modification [18]. 5-HT7Rs were radiolabeled using [^{3}H]-SB269970 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) at concentrations in the range of 0.1–20 nM. Samples containing 100 µg of SH-SY5Y cells membranes or 70 µg of SH-SY5Y cells mitochondrial-enriched fraction, radioligand, and 10 µM SB-269970 (Tocris Bioscience, Bristol, UK) to determine nonspecific binding were incubated in a final volume of 0.5 mL (50 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 0.1% ascorbic acid, 10 µM pargyline hydrochloride) for 20 min at 37 °C. The suspension was filtered through a Whatman GF/C glass microfiber filter (presoaked in 0.3% polyethylenimine for at least 20 min prior to use). Filters were washed 3 times with 1 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4). Scatchard parameters (K_d and B_{max}) and Hill slope (n_H) were determined by nonlinear curve fitting, using Prism version 5.0 GraphPad software.

4.8. Statistical Analysis

Scatchard analysis data were analyzed by applying one-way repeated-measures analysis of variance (ANOVA test), and unpaired t test followed as a post hoc test. Results were reported as mean ± SEM (standard error of the mean) of at least two to three independent experiments, performed in triplicate. Statistical significance was accepted at $p < 0.05$. Similarly, cytochrome c oxidase activity data represent mean rates (nmol/min/mg) ± SEM obtained from at least four independent experiments. *, $p < 0.05$, nonparametric Wilcoxon test between mitochondria administered with LP-211 and SB-269970, and nontreated mitochondria.

5. Conclusions

The data presented here are the first evidence that 5-HT7R is expressed in mitochondria on the human neuroblastoma SH-SY5Y cell line. These results are of great relevance in future studies to investigate the expression and functional role of 5-HT7R on the mitochondria of primary neuronal cultures. These aspects are particularly fascinating considering the role of 5-HT7R in neural circuit development and structural plasticity [33], and the role of mitochondria in synaptic transmission [34] in physiological conditions and pathologies characterized by mitochondrial dysfunction, such as Alzheimer's disease, Parkinson's disease, and Fragile X syndrome.

Author Contributions: M.L., E.L., and C.L.P. conceived the study and designed the experiments. M.L., E.L., and C.L.P. supervised the study. A.T., L.L., and L.T. prepared the mitochondrial membrane; A.T. and M.F. performed Western blotting experiments; M.N. performed saturation binding experiments; A.T., D.M., and G.P. performed experiments on MRC cytochrome c oxidase activity. L.C. revised the statistical analysis. E.L., M.L., and C.L.P. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was partially support by a grant from the Alzheimer's Association (AARG-NTF-18-565227) to E.L.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

5-HT	5-hydroxytryptamine
cAMP	cyclic Adenosine MonoPhosphate
CNS	Central Nervous System
GPCR	G-protein-coupled receptor
MCR	Mitochondrial respiratory chain

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ACKNOWLEDGEMENTS

I would like to acknowledge my supervisor Prof. Lucia Ciranna for giving me the opportunity to take part to this Ph.D program and for passing down her love for research. Her knowledge, guidance and advice have encouraged me in all the time of my academic research.
I am extremely grateful to Dr. Lara Costa for her support during all these years and for offering encouragement with a perfect blend of insight and humor.

Special thanks to Prof. Marcello Leopoldo as well as Prof. Enza Lacivita for their guidance during my first PhD year and for providing valuable guidance and feedback. I am deeply indebted to Dr. Maria Favia, who taught me to trust myself.

I would like to express my deepest gratitude to Dr. Barbara Bardoni for all the support during my abroad period and wise suggestions for my future carrier in this field. I am looking forward to working with you again. I warmly thank Marielle Jarjat, for each constructive scientific advice and comments. She always encouraged me and she made me feel in the laboratory as in a family.

I could not have undertaken this journey without my colleagues Mariarita Spampinato, Giuseppe Carota, Alfio Distefano, Lia Emma and Virginia Fuochi who have been friends and siblings. Words cannot express my gratitude to Serena Abatematteo and Margherita Mastromarino: thank you for being there during this journey. My thanks also go to my colleagues Asma Boulksibat and Sébastien Delhaye, I promise that I will correctly pronounce “Globules”.

Finally, I must express my very profound gratitude to my parents and to my sister Elena. Their trust in me has kept my spirits and motivation high during these years. This accomplishment would not have been possible without them. Thank you.