Department of Physiology
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Molecular Mechanisms Involved in Altered Differentiation of Neural Progenitors in Fragile X Syndrome

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ACADEMIC DISSERTATION

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To my sister

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Helsinki, February 2018

Swaroop

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1. List of original publications

This thesis is based on the following original publications, which are referred in the text by their roman numerals I-III:

I. **Achuta VS**, Rezov V, Uutela M, Louhivuori V, Louhivuori L, Castrén ML (2014). Tissue plasminogen activator contributes to alterations of neuronal migration and activity-dependent responses in fragile X mice. *J. Neurosci. 34, 1916-1923.*

II. **Achuta VS**, Grym H, Putkonen N, Louhivuori V, Kärkkäinen V, Koistinaho J, Roybon L, Castrén ML (2017). Metabotropic glutamate receptor 5 responses dictate differentiation of neural progenitors to NMDA-responsive cells in fragile X syndrome. *Dev. Neurobiol. 77, 438-453.*

III. **Achuta VS**, Möykkynen T, Peteri UK, Turconi G, Rivera C, Keinänen K, Castrén ML (2018). Functional changes of AMPA responses in human induced pluripotent stem cell-derived neural progenitors in fragile X syndrome. *Sci. Signal. 11, eaan8784.*

Author contributions

I. The candidate maintained mouse neural progenitor cultures; performed calcium imaging and time-lapse live imaging experiments; and participated in data analysis.

II. The candidate generated and maintained human and mouse neural progenitor cultures; performed calcium imaging, immunostainings, western blotting and time-lapse live imaging experiments; and participated in data analysis, prepared tables, and figures and wrote the manuscript together with the supervisor.

III. The candidate generated and maintained human and mouse neural progenitor cultures; performed calcium imaging, immunostainings, quantitative PCR experiments and collected samples for transcriptome analysis; and participated in data analysis, prepared tables, and figures and wrote the manuscript together with the supervisor.

2. Abbreviations

ADAR2	adenosine deaminase acting on RNA 2
aIP	apical intermediate progenitor
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR	AMPA receptor
APP	amyloid precursor protein
Arc	activity-regulated cytoskeleton-associated protein
aRG	apical radial glia
ASD	autism spectrum disorder
BDNF	brain-derived neurotropic factor
bFGF	basic fibroblast growth factor
bIP	basal intermediate progenitor
BLBP	brain lipid binding protein
BrdU	5-bromo-2-deoxyuridine
bRG	basal radial glia
BSA	bovine serum albumin
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNS	central nervous system
CP-AMPAR	calcium-permeable AMPAR
DAG	diacylglycerol
DAPI	4',6-Diamidino-2-Phenylindole
DCX	doublecortin
DG	dentate gyrus
DHPG	*(S)*-3,5-dihydroxyphenylglycine
DMEM/F-12	Dulbecco's modified Eagle's medium nutrient mixture F-12
E14	embryonic day 14
E17	embryonic day 17
EBSS	Earl's balanced salt solution
EGF	epidermal growth factor

ER	endoplasmic reticulum
ESCs	embryonic stem cells
FMR1	*fragile X mental retardation 1*
Fmr1-KO	*Fmr1*-knockout
FMRP	FMR1 protein
FXPOI	fragile X-associated primary ovarian insufficiency
FXS	fragile X syndrome
FXTAS	fragile X-associated tremor/ataxia syndrome
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GLAST	glial glutamate-aspartate transporter
HBSS	Hank's balanced salt solution
hESCs	human embryonic stem cells
hiPSCs	human induced pluripotent stem cells
ID	intellectual disability
INM	interkinetic nuclear migration
iGluR	ionotropic glutamate receptor
IP_3	inositol 1,4,5-trisphosphate
IP_3Rs	inositol triphosphate receptors
iPSCs	induced pluripotent stem cells
IP	intermediate progenitor
KA	kainic acid
$[K^+]_e$	extracellular potassium
LRP	low-density lipoprotein
LTD	long-term depression
MAP1B	microtubule-associated protein 1B
MAP2	microtubule-associated protein 2
mGluR	metabotropic glutamate receptor
miRNAs	MicroRNAs
MPEP	2-methyl-6-(phenylethynyl)-pyridine
MZ	marginal zone

Naspm	naspm trihydrochloride
NBQX	dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide
NECs	neuroepithelial cells
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NPCs	neural progenitor cells
O/N	overnight
oSVZ	outer subventricular zone
PBS	phosphate buffered saline
P7	postnatal day 7
PA1-1	plasminogen activator inhibitor
i.p.	Intraperitoneal
PFA	paraformaldehyde
PhTx	philanthotoxin
PPI	prepulse inhibition
PSCs	pluripotent stem cells
REST	RE1 silencing transcription factor
RG	radial glia
ROCs	receptor-operated channels
RT	room temperature
R_yR_s	ryanodine receptors
SGZ	subgranular zone
SNS	synaptoneurosomes
SOCEs	store-operated calcium entry channels
STEP	striatal-enriched protein tyrosine phosphatase
SVZ	subventricular zone
Tbr2	t-box transcription factor 2
tPA	tissue plasminogen activator
TRPC	transient receptor potential type C
Tuj1	anti-β-III-tubulin

VGCCs	voltage-gated calcium channels
vRG	ventricular radial glia
VZ	ventricular zone
WT	wild-type

3. Abstract

Fragile X syndrome (FXS) is the most common cause of genetically acquired intellectual disability and is strongly associated with autism spectrum disorders. FXS is an X-linked neurodevelopmental disorder, with an incidence of approximately 1 in 5000 males and 1 in 8000 females. It is primarily caused by a trinucleotide repeat expansion in the *Fragile X mental retardation 1* (*FMR1*) gene leading to epigenetic silencing and loss of FMR1 protein (FMRP). Studies using *Fmr1*-knockout (*Fmr1*-KO) mice, modelling FXS, revealed that alterations in glutamatergic signaling play a central role in the aberrances of developmental processes in FXS brain. Tissue plasminogen activator (tPA) is a serine protease that potentiates signaling mediated by glutamate receptors. This thesis explored the effects of tPA and glutamate receptor signaling during early differentiation of FXS neural progenitor cells (NPCs). The differentiation of human and mouse FXS NPCs was characterized using calcium imaging, live cell imaging and immunostaining. Expression of tPA was increased in NPCs and brain of *Fmr1*-KO mice. The increased tPA was involved in altered neuronal migration and activity-dependent changes in FMRP-deficient mouse NPCs. NPCs were functionally characterized based on their responses to activation of type 1 metabotropic and ionotropic glutamate receptors. Increased differentiation of subpopulations of glutamate-responsive cells was observed in FXS NPCs. Treatment with 2-methyl-6-(phenylethynyl)-pyridine (MPEP), rescued abnormal differentiation of glutamate-responsive cells in both human and mouse FXS NPCs. In addition, MPEP treatment corrected morphological defects and migration of *Fmr1*-KO cells. Finally, an increased differentiation was evident for cells expressing calcium-permeable alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors in FXS NPCs and reduced GluA2 expression resulted in increased calcium permeability of AMPA receptors. In summary, this study provides insight into the molecular mechanisms involved in early aberrant differentiation of FXS neuronal cells and will pave the way to develop new therapeutic approaches and biomarkers for FXS.

4. Introduction

The use of neural stem/progenitor cells (NPCs) has created novel possibilities for studies of brain development and disease mechanisms underlying disorders of the central nervous system (CNS). Many signaling pathways are involved in the regulation of stem cell proliferation and differentiation (*1*), making stem cell-based assays valuable for investigation of the developmental mechanisms. Furthermore, the remarkable discovery of reprogramming technologies provides an opportunity for the derivation of induced pluripotent stem cells (iPSCs) with patient-specific properties (*2-5*). The iPSCs can be used to model disorders (*6-8*) to improve understanding of disease pathophysiology and to develop targeted therapies. In this thesis, human iPSC (hiPSC)-derived and mouse brain-derived NPCs were used to model fragile X syndrome (FXS), which is the most common inherited cause of intellectual disability (ID) and a variant autism (*9, 10*). FXS is caused by a triplet CGG repeat expansion mutation in the *Fragile X mental retardation 1* (*FMR1*) gene leading to the absence of FMR1 protein (FMRP) (*11, 12*). FMRP regulates translation of many mRNAs essential for maturation and function of synapses and neuronal networks (*13*). Exaggerated responses to metabotropic glutamate receptor (mGluR) activation and increased local protein synthesis at synapses (*14, 15*) are associated with the excitatory and inhibitory imbalance in FXS brain (*16, 17*). The role of FMRP in early brain development is poorly understood, as most studies on FXS are focused on mature neurons. Indeed, FMRP is highly expressed in NPCs (*18-20*), and the early onset of *FMR1* gene expression in the human fetal brain (*18*) indicates a crucial role for FMRP during early neuronal network formation. Previous studies have shown that the absence of FMRP results in abnormal differentiation and altered fate determination of FXS NPCs (*21-25*). In this context, this thesis contributes to broadening the understanding of glutamate signaling mediated effects on the early differentiation of NPCs lacking FMRP.

5. Review of the literature

5.1 Stem cells

Stem cells are characterized by their self-renewal (proliferation) ability and plasticity (differentiation). Their self-renewal ability preserves their undifferentiated state, even after numerous cycles of cell division. "Plasticity" refers to the capacity of cells to adapt their genetic expression profile and differentiate towards specific cell phenotypes. Stem cells divide asymmetrically to give rise to two cells: one identical to the mother cell that maintains the pool of stem cells; and the second undergoing differentiation, eventually becoming a specialized cell. Stem cell potency can be divided into totipotent, pluripotent, and multipotent. Totipotent stem cells derive from fertilized eggs or morula cells in early-stage embryos, and can differentiate into all cell types of the three germ layers, including placental cells (*26*). Pluripotent stem cells (PSCs) are isolated from the inner cell mass of a blastocyst (*27*) and have the capacity to differentiate into all cell types, except placental cells (*26, 27*). PSCs can also be induced from adult somatic cells by transduction of pluripotency-inducing genes (*2-5*). Multipotent stem cells are isolated from fetus or adults and they can differentiate into limited cell types restricted to same/closely-related tissues of origin (*28*).

5.2 Neural stem/progenitor cells

NPCs are multipotent stem cells derived from developing or adult mammalian CNS (*29*). Interest in NPCs soared when newly generated neurons were first identified in the dentate gyrus (DG) (*30*) and the subventricular zone (SVZ) of the lateral ventricles in the adult brain (*31, 32*). NPCs located in these neurogenic zones continue to produce neurons throughout life (*30-32*). In the prenatal period, neurogenesis exists throughout the brain (*33, 34*). In adulthood, NPCs are located in the subgranular zone (SGZ) of the DG in the hippocampus (*30, 35*) and the SVZ of the lateral ventricles (*31, 32, 36*). Thousands of new cells are generated every day, but the exact number varies with age (*37*). For example,

approximately 17,000 new cells are produced each day in the DG of peri-pubescent rats and approximately 9,000 new cells are produced each day in the DG of young adult rats (*38*). Inhibition of adult neurogenesis in rats and mice has been shown to alter their behavior and affect learning and memory (*39, 40*). Thus, not surprisingly, aberrant neurogenesis is implicated in several neurological disorders, including epilepsy (*41*), Alzheimer's disease (*42*), Parkinson's disease (*43*), and FXS (*44, 45*), highlighting the importance of NPC regulation. NPCs can self-renew and differentiate into committed neural cell types of the CNS, such as neurons, astrocytes, and oligodendrocytes. NPCs can be cultured *in vitro* in an undifferentiated state in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (*36, 46, 47*).

5.3 Cortical neurogenesis

The mammalian neocortex is a well-organized cellular architecture formed by the production and positioning of diverse neuronal populations. It controls nearly all aspects of behavior, including language, perception, and decision-making. The human neocortex contains an immense number of neurons─ approximately 16 billion (*48*). Cortical neurons can be divided into two major groups: glutamatergic excitatory neurons, and γ-aminobutyric acid (GABA)-ergic inhibitory interneurons. There are approximately five times more glutamatergic neurons than GABAergic neurons (*49*), which are supported by glial cells and blood vessels. During embryonic stages, the appropriate production and positioning of excitatory and inhibitory neurons define the proper function of the neocortex.

During early cortical neurogenesis (figure 1), a monolayer of homogenous neuroepithelial cells (NECs) lines the ventricular cavity. NECs constitute the neural tube and are the precursors of all future neurons in the CNS, including neocortical neurons (*50*). NECs undergo symmetric division to produce two

daughter NECs at each division, thereby expanding the thickness and surface area of the cerebral wall forming the VZ. Most NECs are multipotent progenitor cells capable of transforming into apical radial glial (aRG) cells and generating the first group of neurons in the neocortex (*51*). aRG cells are defined by their characteristic bipolar morphology and by the initiation of the expression of astroglial markers, such as glutamate-aspartate transporter (GLAST), brain lipid binding protein (BLBP), and glial fibrillary acidic protein (GFAP) (*52-54*). The bipolar aRG cells stretch between the pial and VZ surface, with a long basal process pointing towards the pial surface and the short apical endfoot reaching the VZ surface (*55, 56*). At the early stages of neurogenesis, aRG cells divide symmetrically to produce two daughter cells at each division (*57, 58*). As neurogenesis progresses, they predominately undergo asymmetric divisions to self-renew and to produce a post-mitotic neuron, a basal progenitor termed as intermediate progenitor (IP) cell (*59, 60*), or an outer subventricular zone (oSVZ) progenitor termed as basal RG (bRG) (*61*).

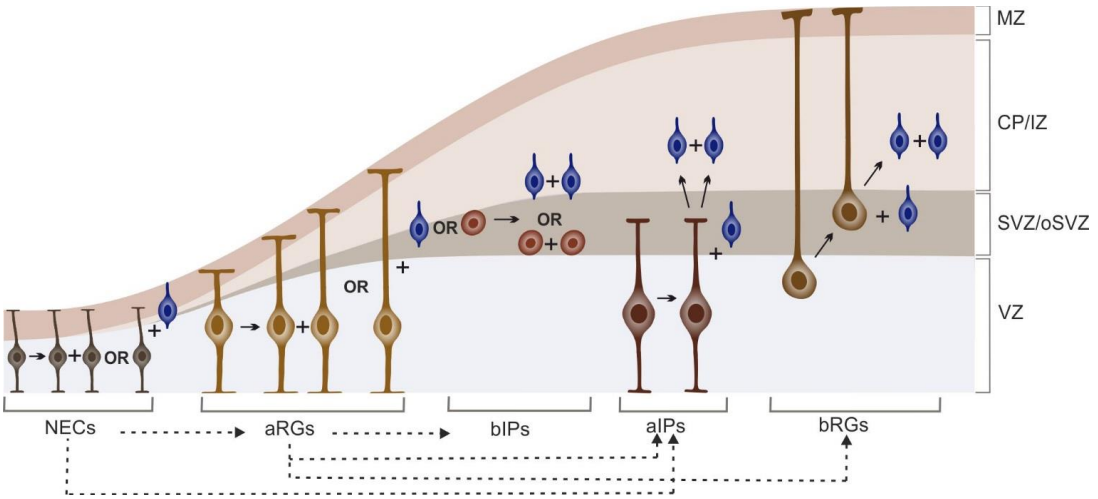

Figure 1: Cortical neurogenesis in human neocortex. During early development, neuroepithelial cells (NECs) generate additional NECs or give rise to the first group of neurons. As neurogenesis progresses, NECs elongate and transit to apical radial glial cells (aRGs). aRGCs expand the progenitor pool or

generate neurons directly or indirectly via intermediate progenitor cells (IPs), IPs further generate neurons. Apical intermediate progenitors (aIPs), progeny of aRGCs that are also known as short neural precursors, divide predominately symmetrically and produce two postmitotic neurons. Basal RG cells (bRGs) likely originate from apical RG cells (aRGs) through oblique division, which generates neurons outside the ventricular zone (VZ). aIPs, apical intermediate progenitors; aRGs, apical radial glial cells; bRGs, basal RG cells; CP, cortical plate; IPs, intermediate progenitor cells; IZ, intermediate zone; MZ, marginal zone; NECs, neuroepithelial cells; oSVZ, outer subventricular zone; SVZ, subventricular zone; VZ, ventricular zone. (Figure prepared by Peteri UK and Achuta VS).

Basal progenitors form the second proliferative zone, the SVZ. In the SVZ, basal IP (bIP) cells undergo symmetric divisions and produce either two postmitotic neurons (*60, 62, 63*) or a pair of bIP cells, which can subsequently generate a pair of neurons (*60, 63*). The bIP cells can be distinguished from aRG cells by their expression of transcription factors such as t-box transcription factor 2 (Tbr2), or cut like homeobox 1 and 2 (*64, 65*). The majority of the VZ progenitors possess a long process extending towards the basal pial surface with a growth cone at their tips. However, a small subpopulation of VZ progenitors has been identified to have either a short basal process or no basal process. These apical IP (aIP) cells were previously termed as short neural precursors (*66*). They are molecularly distinct and display different cycle kinetics compared to RG cells (*66*). aIPs can directly give rise to postmitotic neurons within the VZ (*67*). Recently, distinct self-renewing bRG population was discovered in the oSVZ of developing human and ferret neocortices (*68, 69*). A small proportion of bRG progenitors is also identified in the SVZ of the mouse neocortex (*61, 70*). Unlike aRG cells, bRG cells possess only a basal process pointing towards the pial surface (*68*). In humans, only a small proportion of bRG cells divide into IP cells, whereas the vast majority of bRG cells self-renew (*68*). Therefore, the generation of neurons in the human neocortex is significantly increased by self-renewal of bRG cells (*71*). Production of

secondary NPCs is termed as “transit amplification”, and appears to be a defining feature in humans that might have contributed to the evolutionary expansion of the neocortex (*72*).

The cortical plate develops between the deep subplate and the superficial marginal zone (MZ). It is a six-layered structure formed by the neurons migrating out of the proliferative zones (*73*). The MZ develops into the future layer I and the migrating neurons arriving at the cortical plate form layers II-VI of the cortex in an inside-out manner. Thus, the newborn neurons migrate past the earlier-born neurons. The intermediate zone separates the cortical plate and SVZ. It exists only during corticogenesis and contains the myelinated afferent and efferent axons of the cortex in the mature brain (*74*).

5.4 Modeling neural differentiation

The neurosphere assay has been a valuable tool for studying NPC proliferation and differentiation as well as neural development *in vitro*. Neurospheres are heterogeneous, consisting of multipotent stem cells and restricted progenitor cells with different proliferative, self-renewal, and differentiation capacities (*75*). NPCs isolated from the lateral ventricles of embryonic mouse brain form a non-adherent spherical cluster of cells termed as “neurospheres” in the presence of EGF and bFGF (*36, 46, 47*). Upon removal of growth factors, the neurospheres adhere to the substrate and cells start to migrate out. Cells migrating out of the neurosphere cluster initially display GLAST-positive RG morphology with thick processes followed by maturing neuron-like cells with thin, lengthy processes that show immunoreactivity to makers of immature neurons (*76, 77*). Eventually, the cells migrating further away from the neurosphere cluster will become mature neurons. An identical behavior of differentiating cells has been reported with human fetal-derived neurosphere cultures (*78*). In addition to RG and neuron-like cells, astrocytes and pre-oligodendrocytes are also identified in the culture system but to a lesser extent. Similarly, neurospheres generated from human embryonic stem cells (hESCs) have been shown to differentiate into neurons and

glia (*79*). The hESCs cultured in neural induction and proliferation medium in the presence of bFGF for 42 days showed a temporal increase in expression for neuroectoderm markers (*79*), thus confirming the differentiation to neuroectodermal lineage. During differentiation, the majority of the migrating adherent populations displayed GFAP-positive RG morphology, MAP2-positive neuron-like morphology and very few astrocyte-like morphology (*79*). Also, the majority of the neurons showed immunopositivity to glutamate and few to GABA (*79*). The human FGF-responsive neurospheres are shown to expand slower after an extended period (50-60 days) of culturing (*80*); conversely, neurospheres cultured in a combination of EGF and FGF have longer proliferation capacity (*34*). Similar to any other culture system, neurosphere model has its limitations. Multiple factors like cell plating density, culture medium, the concentration of growth factors (can differentiate towards different lineages), the method of passaging (enzymatic or manual) and high passage number (loss of neurogenic potential) affect the proliferation and differentiation of NPCs (*75*). Notably, the NPCs generated from mouse cortex develop in similarly as they do *in vivo (81)*. Thus, the neurosphere model can be considered as a model containing different progenitor populations to study neural development *in vitro*.

5.5 Basis of calcium signaling

5.5.1 Calcium ion

Calcium ion (Ca^{2+}) is termed as a universal second messenger that regulates many functions in the eukaryotic cell. In the course of evolution, Ca^{2+} ion has been chosen to act as an important universal carrier because of its high affinity to interact with biological molecules. It controls almost all aspects of cell life, starting from fertilization to terminal programmed cell death (*82, 83*). Various buffer and sensor proteins regulate the Ca^{2+} signal, the former by maintaining the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the latter by activating distinct protein targets (*82, 84*). In resting conditions, Ca^{2+} concentration (1-2 mM) outside the cell is 10,000-20,000 times higher than $[Ca^{2+}]_i$ concentration (100 nM). The steep

concentration gradient maintained between the extracellular space and the cell interior is due to highly conserved transporters and the existence of several intracellular compartments (*85, 86*). Using the steep concentration gradient, Ca^{2+} controls a broad range of cellular functions, such as differentiation of specific cell types, muscle contraction, on and off mechanisms in cardiac cells, neuronal migration, and many other physiological functions (*82, 83, 85*). Unlike other secondary messengers, Ca^{2+} signals exhibit unique properties, i.e., programmed cell death and autoregulation at both pre- and post-transcriptional levels (*83, 87*). These diverse functions make Ca^{2+} ions important messengers and understanding the role of Ca^{2+} in cell function, and dysfunction, is vital.

5.5.2 Neuronal Ca^{2+} influx toolkit

Ca^{2+} signal controls numerous neuronal functions, including excitability, exocytosis of neurotransmitters, synaptic plasticity, and gene transcription (*86, 88*). Like many other cell types, neurons use both intracellular and extracellular Ca^{2+} for signal transduction. Ca^{2+} influx is primarily mediated by voltage-gated calcium channels (VGCCs), receptor-operated channels (ROCs), store-operated calcium entry channels (SOCEs), and transient receptor potential type C (TRPC) channels (*82, 85*). VGCCs are activated in response to voltage changes across cell-surface membrane, ROCs are activated upon binding a neurotransmitter to the receptor and SOCEs are activated in response to the release of Ca^{2+} from the endoplasmic reticulum (ER) and store depletion. In addition to Ca^{2+} entry from extracellular space, inositol triphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) mediate Ca^{2+} release from internal stores (*82, 88*). The IP_3-mediated Ca^{2+} release is trigged by neurotransmitters such as glutamate, whereas RyRs can be activated by changes in the cytosolic Ca^{2+} concentration.

5.6 Glutamate

Glutamate is considered to be a principal mediator of signal transmission at excitatory synapses in the CNS. It controls many aspects of mammalian brain function, such as cognition, memory, and learning (*89, 90*). Glutamate acts post-synaptically through ionotropic glutamate receptors (iGluRs), which are subdivided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate receptors, and mGluRs, as shown in table 1. In addition to its role at the synapse, glutamate regulates cell survival, proliferation, differentiation, and migration of cells during early neural development (*91-93*). Glutamate receptor activation causes changes in $[Ca^{2+}]_i$ concentration in the postsynaptic cell, thereby triggering various signaling cascades.

Table 1: Glutamate receptors, their subunit compositions, and signal transmission.

	Types	Subtypes	Subunits	Signal	Transmission
Glutamate receptors	Ionotropic glutamate receptors (ligand-gated)	NMDA	NR1 NR2A-D NR3A-B	Ca^{2+}, Na^{+}	fast excitatory
		AMPA	GluA1-4	Na^{+}, Ca^{2+}	
		Kainate	GluK1-5		
	Metabotropic glutamate receptors (G protein-coupled)	Group I	mGluR1 mGluR5	G_q, PLC; IP_3/DAG	slow excitatory
		Group II	mGluR2 mGluR3	G_i; cAMP	slow inhibitory
		Group III	mGluR4 mGluR6 mGluR7 mGluR8	G_i/G_o; cAMP	

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; NMDA, *N*-methyl-D-aspartate; PLC, phospholipase C.

5.6.1 Glutamate receptors

5.6.1.1 Metabotropic receptors

The mGluRs are seven-transmembrane G protein-coupled glutamate receptors that are found both pre-, post-synaptically on neurons, and glial cells. The glutamate-binding site of mGluRs is present within a large extracellular N-terminal domain (*94*). Upon agonist binding, signal transduction is triggered by a conformational change and altering downstream signaling pathways (*94, 95*). The intracellular C-terminal domain has been shown to mediate interactions with other proteins (*96*). Unlike iGluRs, mGluRs mediate slow excitatory and inhibitory neurotransmissions. The mGluR family can be divided into three groups (Group I-III) based on their amino-acid homology, agonist binding, and activated downstream secondary signaling cascades (*97-99*). Neuronal group I mGluRs (mGluR1 and mGluR5) are generally localized to the periphery of post-synaptic density (*100, 101*). Group II mGluRs (mGluR2 and mGluR3) are located extrasynaptically and involved in inhibition of neurotransmission (*101, 102*). Group III mGluRs (mGluR4, mGluR6, mGluR7, and mGluR8) are predominately located at the presynaptic active zone and function as inhibitory autoreceptors (*103, 104*). When activated, the group I mGluRs couple through Gq/G_{11} and stimulate phospholipase C leading to the subsequent generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (*98, 99, 105*). Both IP_3 and DAG act as second messengers, the former by releasing calcium from intracellular stores that in turn controls various cellular process and the latter by activating protein kinase C that in turn enables many proteins by phosphorylation (*98, 99, 105*). Group II and III mGluRs are coupled to inhibitory G-proteins ($G_{i/o}$) that inhibit adenylyl cyclase/protein kinase pathway leading to a decrease in intracellular adenosine monophosphate and resulting in altered gene expression, inhibition of voltage-gated Ca^{2+} channels, and activation of K^+ channels (*98, 99, 105*).

5.6.1.2 Ionotropic receptors

AMPA receptors (AMPARs) are widely expressed in the CNS and are known to mediate fast excitatory neurotransmission (*106*). In the presence of glutamate, AMPARs mediate entry of Na^+ and Ca^{2+} into the cell. AMPARs are functional tetrameters formed by GluA1-4 subunits (*89, 98, 107*). The combination of subunits confers the properties of AMPARs, such as Ca^{2+} permeability, desensitization kinetics, and channel conductance (*89, 97, 99*). The subunits of AMPARs are encoded by separate genes *Gria1-4*. Further complexity is introduced by alternative splicing to two forms of each subunit, known as "flip" and "flop" forms (*108*). During development, the "flip" variant is highly expressed, whereas both variants are equally expressed in the mature brain. In the CNS, most of the AMPAR complexes contain GluA1 and GluA2 subunits. The presence of the GluA2 subunit and its RNA editing determine Ca^{2+}-ion permeability of the AMPARs (*109-111*). Edited GluA2 subunit forms Ca^{2+}-impermeable channels, whereas unedited GluA2 channels are permeable to Ca^{2+} flow. The GluA2 subunit contains four membrane domains (M1-M4), and the glutamine/arginine (Q/R)-editing site is located on M2 domain. The Q/R-editing of *Gria2* pre-messenger RNA by adenosine deaminase acting on RNA 2 (ADAR2) enzyme renders AMPARs impermeable to Ca^{2+} (*109-111*) . The Q/R-editing is almost 100% in the adult CNS and is necessary for survival (*112*). Higuchi et al. showed that ADAR2 knockout mice displayed altered AMPAR channel properties, including receptor desensitization and increased Ca^{2+} permeability, leading to seizures and premature death of the mice (*112*). AMPARs are assembled in ER and are transported to the plasma membrane. A significant proportion of edited GluA2 remains in the ER before been transported to the synapse (*113*). The exocytosis and endocytosis of AMPARs at synapses is a highly dynamic process. Increased rate of endocytosis results in long-term depression (LTD), whereas enhanced receptor exocytosis leads to synaptic potentiation (*114, 115*).

Kainate receptors are composed of two related sub-families, GluK1-3 formerly known as GuR5-7 and GluK4-5 formerly known as KA1-2 (*97, 107, 116*). Kainate receptors are widely expressed at both the pre- and post-synaptic membrane of the CNS (*89, 117*). They likely form heteromeric assemblies with one or more members of both sub-families. The GluK1-3 subunits can also form homomeric tetramers, whereas GluK4-5 requires GluK1, GluK2, or GluK3 to form functional heteromeric assemblies (*89, 98, 118*). In addition, GluK1-3 subunits undergo alternative splicing and RNA editing thereby increasing the subunit variants and complexity (*89, 98, 118*). Compared to AMPARs, kainate receptors are fast acting, bind with a higher affinity, and recover from desensitization slowly (*119*).

NMDA receptors (NMDARs) are a major type of ionotropic receptors, which are highly permeable to Ca^{2+} and also to Na^{+}. The NMDAR family contains seven subunits NR1, NR2A-D, and NRA-B that are encoded by separate genes (*97, 107, 120*). The NR1 subunit encodes three alternatively spliced exons resulting in eight functional splice variants with different properties and distribution. For functional NMDARs, a compulsory NR1 subunit must be present in two copies in the receptor complex (*120*), along with other subunits. Glutamate binds to the NR2 subunit (*121*). Glycine is a co-transmitter that binds to the NR1 subunit and promotes the function of the receptor (*122*). Binding of both glycine and glutamate in two copies is necessary for the optimal functioning of NMDAR. The activation of NMDAR is voltage-dependent and mediated by AMPA/kainate receptor activation. In normal resting conditions, extracellular Mg^{2+} blocks most NMDARs. The activation of AMPA/kainate receptors results in the Ca^{2+} influx into the cell leading to removal of the internal Mg^{2+} and thereby allowing more Ca^{2+} into the cell. The entry of Ca^{2+} triggers both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms, which regulate AMPARs at the synaptic membrane, synaptic strength, and gene expression (*89, 99, 123*).

5.6.2 Role of glutamate receptors in NPC regulation

Glutamate is proposed to regulate neurogenesis directly through mGluRs or iGluRs expressed by the NPCs (*90, 124*). The AMPA/kainate receptors are the first iGluRs to appear during embryonic development and are expressed throughout the adult CNS (*110, 125*). NMDARs are also highly expressed in both developing and adult brain (*120, 126, 127*). However, they become functional at later stages compared to AMPA/kainate receptors (*126*). There is evidence that they exhibit Ca^{2+} responses and depolarizing currents when the NPCs have differentiated into postmitotic neurons (*126, 127*). NMDAR activation is important for neuronal migration and neurite extension/retraction (*127, 128*). In mouse embryonic (E13-E18) cortical slice cultures, cell migration from proliferative zones (VZ/SVZ) towards CP is shown to be promoted by NMDARs and mGluRs, but not by AMPA/kainate receptors (*127*). Preventing NMDAR function partially blocks the glutamate-mediated migratory effect (*127*), indicating the involvement of NMDARs in migration. In embryonic mouse NPCs cultures, a subpopulation of cells responsive to NMDAR also responded to GABA receptor activation and exhibited enhanced migration in the presence of BDNF (*129*). The majority of cells in mouse NPCs cultures do express mGluRs and AMPA/kainate receptors (*76, 77, 129*). Blocking the AMPA/kainate receptor function by 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) *in vitro* reduced the motility of NPCs (*76*). Also, blocking AMPAR function has been shown to regulate the extension of neurite processes in human fetal NPCs and human neuroblastoma cell line (*130*).

All mGluRs, apart from mGluR6, are expressed in NPCs and/or neuronal cells in both embryonic and adult brain (*105, 131*). Several studies have reported the involvement of mGluR5 in the regulation of cell proliferation, survival, and migration (*76, 131-134*). Blocking mGluR5 function with 2 -methyl-6-(phenylethynyl)-pyridine (MPEP) reduces cell proliferation and increases cell death in rat embryonic (E20) forebrain NPCs (*132, 133*), and a similar adverse effect of MPEP has been shown in the SVZ of

intact adult mice (*132*). Appropriately, the group I mGluR agonist *(S)*-3,5-dihydroxyphenylglycine (DHPG) has been shown to promote cell proliferation in both mouse and human-derived cortical NPCs (*133, 134*). In mouse embryonic NPCs cultures, MPEP treatment has been shown to flatten and distort RG processes and transiently promote the motility pattern of neuron-like cells (*76*). The role of group II and III mGluRs in both embryonic and adult neurogenesis seems to be less clear, however. Most studies conducted on group II and III mGluRs have shown that activation of these receptors has negative effects on NPC proliferation and neuronal differentiation (*135, 136*), whereas, few studies have reported that mGluR3 and mGluR7 promote cortical NPC proliferation (*132, 137, 138*).

5.7 Tissue plasminogen activator

Tissue plasminogen activator (tPA) is a serine protease that catalyzes the conversion of plasminogen into proteinase plasmin. In the vascular system, tPA is primarily responsible for the removal of intravascular fibrin deposits (*139*), as a result, it is used as a thrombolytic agent for stroke and myocardial infarction (*140, 141*). Besides its role in the vascular system, tPA has been implicated in synaptic plasticity, learning and excitotoxic cell death (*142, 143*). In human CNS, tPA is widely expressed in neurons and glial cells of neocortex, hippocampus, cerebellum, hypothalamus, thalamus, medulla, and sympathetic ganglia (*144*). tPA exerts its functions in an either plasmin-dependent or -independent manner. The plasmin-dependent mechanisms include the conversion of the precursor form of brain-derived neurotropic factor (proBDNF) to mature form of BDNF, a crucial step in neuronal maturation and plasticity (*145*). The synaptic functions and excitotoxic effects exerted by tPA are known to be mediated via NMDAR signaling, which occurs both dependent and independent of plasminogen activation (*146-148*). In 2000, Nicole et al. first demonstrated that tPA directly cleaves the NR1 subunit and thereby increases the Ca^{2+} permeability of NMDAR in a plasmin independent manner (*146*). Subsequently, the mutation of Arginine260 within the NR1 subunit was shown as the tPA-mediated cleavage site (*149*). However, the

observations reporting the interplay between tPA and NMDAR remained controversial since the cleavage of NR1 subunit by tPA was shown to be an indirect effect, and in fact mediated by plasmin (*147*). Later, Samson et al. demonstrated the involvement of low-density lipoprotein (LRP) co-receptor in augmenting the NMDA-induced $[Ca^{2+}]_i$ changes caused by tPA (*148*). Supporting the plasmin-mediated mechanisms, few other groups reported the cleavage of N2 subunit of NMDAR by tPA. The cleavage is shown to occur at two sites of the N2 subunit-Lys317 on N2A (*150*) and Arg67 on the N2B subunit (*151*) and thereby enhancing NMDAR activity. Whatever the mechanism, all these studies indicate that tPA increases NMDAR signaling. Thus, tPA plays a vital role in synaptic plasticity, either by proteolytic and nonproteolytic mechanisms involving pro-neurotrophins, NMDAR signaling, or via LRP. Besides, tPA-deficient mice are shown to be resistant to experimentally induced neuronal degeneration (*143*). tPA is also implicated in CNS pathologies, including Alzheimer's disease, infarct formation/injury, stroke, and seizure spreading (*142*).

5.8 Micro RNA

MicroRNAs (miRNAs) are a class of short non-coding RNAs generated from long precursor RNA transcripts through a series of cleavage steps. miRNAs are 18-25 nucleotides long RNAs, which plays a crucial role in regulating gene expression posttranscriptionally. miRNAs bind to a short core sequence in the 3' untranslated region of its target mRNAs or non-coding RNAs (*152*) and results in mRNA degradation or repression of their translation. In 1993, Lee et al. first discovered lin-4 miRNA, which inhibits LIN-14 activity in *Caenorhabditis elegans* (*153*). Since then, over 2000 miRNAs have been identified in humans, and thought to control > 60% of human protein-coding genes (*154*). miRNAs are known to regulate many cellular functions including proliferation, differentiation, and apoptosis (*155*). It is estimated that one-third of the human genes are regulated by miRNAs and alterations in their

expression (overexpression or repression) leads to disease states. miRNAs are implicated in many neuropsychiatric and neurodevelopmental disorders such as Schizophrenia, Tourette's syndrome, ASD, Rett syndrome, FXS and Down syndrome (*156*). Thus, activation or inhibition of miRNAs became a promising tool in the area of therapeutics (*157*).

5.9 Fragile X Syndrome

FXS is a monogenic leading cause of intellectual impairment and is frequently associated with autism spectrum disorder (ASD) (*158*). The prevalence of FXS in the general population is approximately 1 in 5000 males and 1 in 8000 females (*159*). The average age of diagnosis is 35-37 months (boys) and 41.6 months (girls) (*160*). The life expectancy of FXS patients is normal; however, they need support and care throughout their lives. Due to its X-linked heritability, males are more frequently affected than females. Males present with severe symptoms/features, including developmental delay, hyperactivity, cognitive and memory impairments, epilepsy, mild facial dysmorphology such as long face and prominent ears, macroorchidism (*161*) and a unique pattern of neuropsychiatric features. Because of random inactivation of X chromosome, cognitive disability in the phenotype of FXS females is generally milder than males but it associates with emotional problems (*162*). Currently, there is no targeted treatment for FXS, but most procedures are to help to minimize the disease condition. These include proper education, therapy, and medications that improve individual capabilities and skills. The pharmacological treatments prescribed are on individual basis to lessen symptoms of anxiety, aggression and attention deficit hyperactivity disorder (ADHD). Treatment commonly used for FXS patients include stimulants, selective serotonin reuptake inhibitors (SSRIs), and antipsychotics (*163*).

5.9.1 Identification of FXS

FXS is also known as Martin-Bell syndrome, named after Martin and Bell who first reported the sex-linked inheritance of mental retardation in families in 1943 (*164*). More than two decades later, Lubs identified a fragile site in the long arm of X chromosome in a family, diagnosed with ID (*165*). Later, Sutherland found that the ability to visualize the fragile site depended on the specific culturing conditions with reduced folate (*166*). The exact location of the fragile site is found to be Xq27.3 (*167*), and the gene mapped to the constriction site is cloned and termed "*FMR1* gene" (*11*). In most cases, the causative mutation is an expansion of CGG repeats (>200) within the 5'untranslated region of the *FMR1* gene leading to promoter methylation and the absence or deficiency of FMRP (*11*). In normal individuals, the length of the CGG repeats vary between 6-54, whereas the premutation carriers contain 55-200 repeats (*168*). The premutation carriers are at increased risk of developing a condition known as fragile X-associated tremor/ataxia syndrome (FXTAS). FXTAS is more common in males than females. Females are at risk of fragile X-associated primary ovarian insufficiency (FXPOI) (*169, 170*). The FXTAS and FXPOI symptoms are caused by increased transcription of the *FMR1* gene and slightly reduced expression of FMRP (*171*). In both full mutation and premutation individuals, the severity of the phenotype depends on the number of CGG repeats (*172*). The repeat number has the tendency to increase every generation (*173*).

5.9.2 FMRP

FMRP is an RNA binding protein ubiquitously expressed in all mammalian tissues, predominately in brain and testis (*20*). In the brain, FMRP is mainly expressed in the cell body and dendrites of neurons (*20, 174, 175*) and also in axonal compartments (*176, 177*). Initially, the FMRP expression was shown to be restricted to neurons, however, later confirmed in astrocytes, oligodendrocytes and microglia of developing and adult mouse brain (*178*). FMRP is associated with the ribosomes via RNA in the

cytoplasm (*174*). FMRP contains functional nuclear localization and export signals indicating that FMRP shuttles between nucleocytoplasmic space (*179, 180*). In the nucleus, FMRP interacts with its homologous proteins fragile X related proteins 1 and 2, other proteins, and RNA/mRNA to form a ribonucleocomplex, which is likely involved in mRNA export from the nucleus to the cytoplasm (*13*). In the cytoplasm, FMRP along with its nuclear partners interacts with cytoplasmic proteins, such as FMRP-interacting protein 1 and 2 and regulates protein synthesis at synapses (*13*). FMRP selectively binds to as much as 4% of the total mRNAs in the mammalian brain (*181*). It contains four RNA-binding motifs: three KH domains (KH0, KH1, and KH2) and an arginine-glycine-rich box that mediates mRNA transport, stability, and regulation of translation (*181, 182*). FMRP represses the translation of mRNAs that are involved in neuronal and synaptic transmission such as NMDAR subunits, mGluR5, postsynaptic density 93 & 95, Shank 1-3, neuroligins 1-3 (*183*). Some of these mRNAs are identified as candidate genes for autism (*184*). In the absence of FMRP, translational repression of target mRNAs fails, leading to alterations in synaptic plasticity and dendritic spine dynamics, which are thought to underlie the clinical manifestations of FXS (*185*).

5.9.3 Fmr1-KO mouse model

Significant advancements have been made in fragile X research since the generation of the mouse *Fmr1* gene knockout model. *Fmr1*-knockout (*Fmr1*-KO) mice are the most widely used murine model for FXS and were generated by inserting a neomycin cassette in exon 5 of the *Fmr1* gene (*186*). The murine FMRP shows 97% amino acid homology to its human counterpart (*187*). Similar to humans, the life expectancy of *Fmr1*-KO mice is normal. Although the human FXS condition results from expanded CGG repeats, both human and mouse models are functionally identical with no FMRP production. Indeed, the *Fmr1*-KO model recapitulates many phenotypes observed in human FXS patients. They show similar morphological abnormalities in synapse structure, altered synapse function and an abundance of

immature dendritic spines (*188*). In addition, *Fmr1*-KO show learning deficits, hyperactivity and increased risk of seizures (*186, 189*). FMRP deficiency leads to the excessive synthesis of microtubule-associated protein 1B (MAP1B), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and the activity-regulated cytoskeleton-associated protein (Arc) in synaptoneurosomes (SNS) of *Fmr1-KO* mice (*190*), whose function is linked with synaptic plasticity. Subsequently, excessive translation of mRNAs is shown in cortical SNS (*191*), suggesting that absence of FMRP results in exaggerated protein synthesis at synapses.

5.9.4 FMRP and glutamatergic signaling

Extensive preclinical evidence on animal models of FXS has demonstrated that aberrant signaling via group 1 mGluRs has important consequences on the mechanisms involved in dysregulated synaptic plasticity in the absence of FMRP. Group 1 mGluRs include mGluR5 that is highly expressed in cortex, striatum, and CA1 and CA3 regions of the hippocampus and mGluR1 mainly expressed in the cerebellum, thalamus, and CA3 region of the hippocampus (*192*). Weiler et al. first demonstrated the connection between FMRP and mGluR pathways; they observed that activation of group 1 mGluRs by DHPG increased protein synthesis, including FMRP, in SNS (*175*). Later, Huber et al. showed that the absence of FMRP leads to increased mGluR-dependent protein synthesis and altered synaptic plasticity, including enhanced LTD in the postnatal and adult brain of *Fmr1*-KO mice (*14*). They also showed that NMDA-dependent LTD is normal in *Fmr1*-KO mice suggesting that the phenotype is linked exclusively to mGluR-dependent LTD (*14, 193, 194*). This led to the mGluR theory of FXS (*15*), which postulates that the absence of FMRP results in overactive glutamatergic signaling via mGluR5. The activation of group I mGluRs stimulates protein synthesis responsible for the internalization of synaptic AMPARs and NMDARs (*195, 196*). Thus, in the normal brain, activation of mGluRs increases FMRP synthesis, which then negatively regulates the protein translation involved in AMPAR internalization during LTD (*13*).

In *Fmr1*-KO mice, the absence of FMRP increases translation of LTD proteins leading to aberrant glutamate receptors trafficking and consequently causing changes in synaptic plasticity (*13, 197-199*). Unlike normal mGluR-LTD, the *Fmr1*-KO mGluR-LTD does not require new protein synthesis because of basally elevated proteins levels in the absence of FMRP (*200, 201*).

Proteins regulating AMPAR endocytosis such as MAP1B, Arc, striatal-enriched protein tyrosine phosphatase (STEP) and amyloid precursor protein (APP) are upregulated in the brain of *Fmr1*-KO mice (*190, 198, 202*). AMPARs surface expression is decreased during hippocampal mGluR-LTD in *Fmr1*-KO mice (*162, 200, 203*) in agreement with the increased expression of AMPAR endocytosis proteins. Furthermore, in *Fmr1*-KO mice, GluA1 is decreased in the cortical synapses (*204*), synapse membrane (*205, 206*) and hippocampal neurons (*207*). The GluA1 and GluA2/3 levels are reduced in the SNS fractions of one-week-old *Fmr1*-KO mice, and at later developmental stage only GluA1 is reduced in SNS, while GluA2/3 and GluNR2B levels are reduced in cortical homogenates (*208*). Both protein and mRNA expression levels of GluA1 are decreased in the cerebral cortex, hippocampus and amygdala synaptosome extracts of *Fmr1*-KO mice (*204, 209*). In addition to the changes in AMPAR subunits expression, abnormal NMDAR expression patterns are observed in FXS. The GluNR1, NR2A, and NR2B levels are significantly reduced in homogenates and SNS fractions extracted from the prefrontal cortex (*210*) and in DG (*211*) of *Fmr1*-KO mice. Moreover, a significantly lower AMPA/NMDA ratio is observed just before the closure of the normal critical period in *Fmr1*-KO mice (*212*). In another *Fmr1*-KO2 mouse model, Pilpel et al. also reported lower AMPA/NMDA ratio at postnatal day 14, which is related to the upregulation of NMDAR component paralleled with downregulation of AMPAR component (*213*), suggesting that the AMPAR/NMDAR function is dysregulated in *Fmr1*-KO mice.

Genetic reduction of 50% mGluR5 signaling in *Fmr1*-KO mice corrects dendritic spine density, ocular dominance plasticity, inhibitory avoidance extinction, audiogenic seizures, body weight loss, and excessive protein synthesis (*214*). Complete *Grm5* knockout mice show impaired brain function (*215*); therefore it is vital to study heterozygous mice ($Fmr1^{-/y}$; $Grm5^{+/-}$) with 50% reduction in mGluR5 expression (*214*). Following the identification of a potent and selective, noncompetitive antagonist MPEP (*216, 217*), several groups reported the beneficial effects of antagonizing mGluR5 in FXS. In *Fmr1*-KO mice, MPEP treatment resulted in suppression of audiogenic seizure phenotype (*218, 219*), reduction in repetitive-like behavior (*219, 220*), rescued prepulse inhibition (PPI) (*221*), and normalized the increased density of immature spines and excessive protein synthesis (*214, 221*). MPEP administration in 2-week-old *Fmr1*-KO mice rescued the immature morphology of pyramidal neurons in the somatosensory cortex (*222*). Other mGluR5 antagonists, such as fenobam or mavoglurant, are also shown to have positive effects on FXS phenotype (*223-225*). These findings support the mGluR theory by showing that either by genetically reducing or by pharmacologically antagonizing mGluR5 signaling, the main phenotype of FXS can be completely rescued.

5.9.5 FMRP and neural progenitors

FMRP is widely expressed during embryonic development in both mouse and human (*18-20*), particularly in the regions where proliferating NPCs and newly born neurons are found. In normal mice, FMRP expression is highly detected at the end of the first postnatal week, and decline gradually thereafter (*226, 227*), indicating that FMRP plays a crucial role in early development. Altered NPCs proliferation and differentiation are reported in FXS. However, the biology of NPCs in FXS is not well characterized as very few groups have focused on studying early neuronal development. The studies of mouse NPC proliferation showed variations, which may depend on different methodologies, brain regions and developmental stages used. Neural colony-forming cell assay (*228*) has shown that the proliferation of

primary progenitors did not differ in neurospheres derived from the embryonic brains of *Fmr1*-KO and WT mice (*21, 229*). Similarly, no alterations have been reported in NPC proliferation in DG of *Fmr1*-KO mice (*25*), and in human FXS fetal cortex derived NPCs (*230*). However, the proliferation of secondary progenitors has been shown to be increased both *in vitro* and *in vivo* (*21, 22*). Increased neuronal differentiation and decreased astrocyte differentiation is observed in NPCs derived from mouse embryonic brain, human fetus, and in DG of *Fmr1*-KO mice (*21, 25*). In contrast, increased astrocyte differentiation and decreased neuronal differentiation is observed both *in vitro* and *in vivo* of DG of 8-10 week-old mice and NPCs derived from human embryonic stem cells (ESCs) (*23, 24, 231*). Callan et al. showed excessive proliferation of NPCs and accumulation of an increased number of neurons in *dFmr1* mutant brain (*232*). Furthermore, lack of FMRP is shown to suppress transition of RG cells into IP cells via an actin-dependent mechanism (*233*), in line with the reduced production of RG (*21, 25, 44*). Another line of research shows that neurons generated from NPCs of DG of adult *Fmr1*-KO mice display impaired NMDAR-dependent synaptic plasticity (*211*). However, neurons in the CA1 region of the hippocampus showed no alterations in NMDAR-dependent synaptic plasticity (*211*). NMDAR-dependent activity is inversely correlated with NPC proliferation in the DG, indicating that impaired NMDAR-dependent activity might also lead to NPC dysregulation (*234*). Altogether, these results highlight the importance of FMRP in NPC maintenance and fate determination in both the developing and adult brain.

Recently, many research groups have generated NPCs from FXS hiPSCs to study the mechanisms involved in altered neuronal function, to identify the compounds that can reactivate a silent *FMR1* locus, and to restore *FMR1* gene expression. Gene expression and DNA methylation profiling studies demonstrated that NPCs generated from FXS hiPSCs exhibit alterations in expression of genes that are responsible for neurodevelopment, migration, and axon guidance (*235*). Few other groups succeeded in deleting the elongated CGG repeat in FXS hiPSC and undifferentiated cells using CRISPR/Cas9 genome

editing method (*236-238*). The deletion of CGG repeats resulted in promoter demethylation and restoration of *FMR1* gene expression in FXS hiPSCs and sustained throughout the neural rosette formation and in mature neurons (*236-238*). Bar-Nur et al. showed that DNA methyltransferase inhibitor 5-Azacytidine reactivated *FMR1* gene expression in FXS hiPSC derived neurons (*239*). Accordingly, high-throughput screening of FXS hiPSC derived NPCs revealed 5-Aza-2-deoxycytidine and 5-Azacytidine as positive hits (*238*). All these findings emphasize the importance of studying early developmental time stages, where FMRP is highly expressed, to understand the contribution of NPCs in altered neuronal and circuit excitability observed in FXS. The hiPSC studies reported in FXS are summarized in table 2.

Table 2: FXS hiPSC studies

Models	Alterations and treatment/modification effects	References
hiPSCs & hESCs	FXS-hESCs express *FMR1* gene and become silent upon differentiation. Unlike FXS-hESCs, hiPSCs carry a completely silent *FMR1* locus.	(*6*)
hiPSCs	The CCG repeat length is responsible for methylation status, *FMR1* gene, and FMRP expression. FXS-hiPSCs generate fewer neurons with short neuritis and more glial cells.	(*240*)
hiPSCs	Treatment with DNMT inhibitor (5-Aza-C) cause demethylation of *FMR1* promoter leading to reactivation of *FMR1* gene in FXS hiPSCs and neurons.	(*239*)
hiPSCs & peripheral blood	Genome-wide analysis reveals that hypermethylation is unique to the *FMR1* locus and no other genomic regions show aberrant methylation.	(*241*)
hiPSCs	Forebrain FXS neurons exhibit defects in neurite length, neurite outgrowth rate, and the number of neurites per sphere.	(*242*)
hiPSCs	UFM fibroblasts express *FMR1* gene, while hiPSCs and NPCs show complete methylation leading to no detectable levels of *FMR1* mRNA.	(*243*)
hiPSCs	FXS neurons show an increase in *REST* expression that result in suppression of genes essential for neural differentiation and axon guidance. Transfection of hsa-mir-382 into mature FXS neurons reduces *REST* expression thereby upregulating expression of axonal guidance genes.	(*244*)

hiPSCs	Using TR-FRET assay, 6 compounds (Out of 5000) that induce moderate *FMR1* gene expression are identified in FXS-hiPSCs derived NPCs.	(*245*)
hiPSCs & hESCs	Using Cas9 nuclease, abnormal CGG repeats are deleted leading to promoter demethylation and restoration of *FMR1* expression. Both demethylation and *FMR1* expression sustained throughout the neural rosette formation and in mature FXS neurons.	(*236*)
hiPSCs	Out of 50000 compounds screened, 2099 compounds are found (identity not disclosed) to induce a weak FMRP signal in FXS NPCs.	(*246*)
hiPSCs & Somatic hybrid cell lines	Excising expanded CGG repeats results in demethylation and reactivation of *FMR1* and FMRP expression in hiPSCs and somatic hybrid cells (containing human fragile X chromosome).	(*237*)
hiPSCs	High-throughput RNA-seq analysis reveals 1559 differentially expressing genes during differentiation of FXS-hiPSCs into neurons. Most importantly, up-regulation of transcription factors involved in neuronal differentiation and down-regulation of genes encoding potassium channels, glutamatergic synapse.	(*247*)
hiPSCs	Both hiPSCs and neurons derived from UFM individuals express *FMR1* gene. Only the iPS clones with >400 CGG repeats show full methylation, and *FMR1* gene remains silent. Neurons show accumulation of ubiquitin-positive inclusion bodies suggesting that the UFM individuals might develop FXTAS.	(*248*)

hiPSCs & hESCs	hESCs/hiPSCs are used to establish *FMR1*-Nluc reporter cell lines and further differentiated into NPCs. High-throughput screening of NPCs reveals DNMT inhibitors (5-Aza-dC and 5-Aza-C) as positive hits that can reactivate *FMR1* gene expression.	(*238*)
hiPSCs and mouse embryonic brain-derived stem cells	FXS NPCs derived from hiPSCs and mouse brain shows an increase in differentiation of cells responsive to glutamate receptors activation. MPEP (mGluR5 antagonist) treatment corrects the enhanced differentiation of a distinct subpopulation of FXS NPCs.	(*249*)
hiPSCs	Gene expression and DNA methylation profiling demonstrate dysregulation of genes responsible for neurodevelopment, migration, neurite extension, cell motility, and axon guidance in FXS neurons. Also, the aberrant methylation patterns are shown to affect the gene expression profile during differentiation of FXS neurons.	(*235*)

5-Aza-C, 5-Azacytidine; 5-Aza-dC, 5-Aza-2-deoxycytidine; DNMT, DNA methyltransferase; *FMR1*, *fragile X mental retardation 1*; FMRP, FMR1 Protein; FXS, fragile X syndrome; FXTAS, fragile X-associated tremor/ataxia syndrome; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; MPEP, methyl-6-(phenylethynyl)-pyridine; NPCs, neural progenitor cells; REST, RE1 silencing transcription factor; TR-FRET, time-resolved fluorescence resonance energy transfer; UFM, unmethylated full mutation.

6. Aims of the study

The overarching aim of my doctoral thesis was to identify novel targets for pharmacological intervention in FXS. The study mainly focused on investigating the involvement of glutamate signaling in early neuronal differentiation and migration of NPCs lacking FMRP. The findings obtained with NPCs derived from FXS hiPSC lines were compared with those derived from *Fmr1*-KO embryonic mouse brain.

The specific aims of the study were as follows:

I. To understand the role of tPA in the differentiation, migration and activity-dependent responses of FMRP-deficient NPCs

II. To functionally characterize differentiation of glutamate-responsive cell populations and to identify mGluR5-mediated abnormalities during differentiation of human and mouse FMRP-deficient NPCs

III. To investigate the mechanisms involved in enhanced differentiation of cells with Ca^{2+}-permeable AMPARs in human and mouse FMRP-deficient NPCs

7. Materials and Methods

7.1 Mouse model (*I-III)*

7.1.1 Fmr1-knockout mice

Fmr1-KO (B6.129P2-*Fmr1*$^{tml/Cgr}$/J, Jackson Laboratory) and wild-type (WT) mice were maintained in C57BL/6J genetic background (*I-III*). *Fmr1*-KO mice were generated (*186*) and genotyped by tail-PCR as described previously (*21*). Female *Fmr1*$^{-/+}$ mice were crossed with male WT mice and embryos with *Fmr1*-KO, and WT genotypes were used in the study. All animals were group-housed by the guidelines of the National Institute of Health at the Animal Centre, University of Helsinki. The experiments were accomplished in accordance with the European Economic Community Council Directive and protocols were approved by the Experimental Animal Ethical Committee of the National Laboratory Animal Center, Finland.

For studies of early cortical neurogenesis (*II*), heterozygous female *Fmr1*$^{-/+}$ mice carrying embryos received intraperitoneal (i.p.) injections of 5-Bromo-2-deoxyuridine (BrdU; 50 mg/kg) at embryonic day 14 (E 14). Two heterozygous pregnant female *Fmr1*$^{-/+}$ mice received i.p. injections of MPEP (10 mg/kg) along with BrdU injections.

7.1.2 Brain tissue

Pregnant mice were sacrificed by cervical dislocation and pups by decapitation after being anesthetized with CO_2. Cell cultures were prepared from the mouse brain tissues at E14 (*I-III*). The embryonic day 17 (E17), postnatal day 7 (P7), and adult brains were collected for paraffin sectioning (*I,II*). Freshly collected brains were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA) at +4^{0}C overnight (O/N) followed by dehydration with ethanol series: 70% for 2 h, 96% for 2 h, and 100% at +4^{0}C O/N. Brains were washed with xylene for 2 h and immersed in paraffin at +60^{0}C O/N. On the

following day, paraffin molded brains were sectioned into 20 µm coronial sections onto cover glasses using a MICROM HM 355 microtome (Thermo Fisher Scientific).

7.1.3 Generation and culturing of neural progenitors

Cortical NPCs were used to examine intracellular calcium ($[Ca^{2+}]_i$) responses, cell motility, whole-cell currents, protein expression, and mRNA expression levels (*I-III*). NPCs were propagated from the wall of lateral ventricles of WT and *Fmr1*-KO mice as previously described (*250*). The dissected brain tissue (E14) was dissociated in (mg/ml) 0.2 kynurenic acid, 0.7 hyaluronidase, and 1.33 trypsin, in Hank's balanced salt solution (HBSS) containing 2 mM glucose at $+37^0$C for 30 min. After titration, the cells were centrifuged at 200 X g for 5 min, and the pellet was resuspended in HBSS containing 0.9 M sucrose and centrifuged at 750 X g for 10 min. Thereafter, cells were resuspended in Earl's balanced salt solution (EBSS) and centrifuged through a gradient of 12 ml EBSS containing 4% bovine serum albumin (BSA) at 200 X g for 7 min. The cell pellet was resuspended in Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM/F-12) containing B27 supplement, L-glutamine (2 mM), HEPES (15 mM), penicillin (100 U/ml), and streptomycin (100 U/ml) and cultured in the presence of bFGF (10 ng/ml) and EGF (20 ng/ml). Cells were grown as free-floating aggregates known as neurospheres (*46*) in a 5% CO_2-humidified incubator at $+37^0$C. The culture medium was refreshed (50% of the medium) twice and growth factors were added thrice per week. The neurospheres were passaged by manual trituration approximately once a week, and all the experiments were performed on neurospheres between passages 3 and 10.

7.2 Human model *(II,III)*

7.2.1 FXS induced pluripotent stem cells

Seven hiPSC lines were used in the study: HEL100.1, HEL100.2, HEL70.3, and HEL69.5 (full mutation; FXS), and HEL46.11, HEL23.3, and HEL11.4 (control) (*II,III*). With informed consent, human samples were collected from boys diagnosed with FXS (age range: 6-13 years) and from healthy individuals (age range: neonatal and 83). hiPSC lines were reprogrammed at the Biomedicum Stem cell center (University of Helsinki) using CytoTune-iPS Sendai Reprogramming Kit. The hiPSCs were grown as monolayer cultures on Matrigel-coated plates in Essential 8 medium and maintained in a 5% CO_2-humidified incubator at $+37^0$C. The culture medium was replaced every alternate day, and the cell colonies were passaged approximately once every 4-5 days with 0.5 mM EDTA in PBS. All iPSC lines used for generation of NPCs were between passages 15 and 40.

7.2.2 Generation and culturing of neural progenitors

Neural differentiation was induced as previously described (*79*). NPCs were generated from both control and FXS hiPSC lines and further used to examine $[Ca^{2+}]_i$ responses, protein expression, mRNA expression levels, and transcriptional profile (*II,III*). hiPSC colonies were grown on low adherent plates in neuronal differentiation medium containing DMEM/F-12: Neurobasal (1:1), Glutamax (2 mM), B27 and N2 supplement in the presence of bFGF (20 ng/ml). Under these conditions, the hiPSCs form free-floating aggregates (neurospheres) in 2-4 days. After the first week, the culture medium was replaced completely, and growth factors were added every 2-3 days. Neurospheres were grown for 6-8 weeks and manually passaged approximately once a week, and all the experiments were performed on neurospheres between passages 6 and 8.

7.3 Neurosphere differentiation (*I-III*)

For experiments, human (*II,III*) and mouse (*I-III*) neurospheres (average size 200-250 µm) were plated on poly-D-lysine/laminin and poly-DL-ornithine coated cover glasses in the absence of growth factors, respectively. Neurospheres were differentiated in the presence or absence of tPA blocker, MPEP, Philanthotoxin (PhTx), or Naspm trihydrochloride (Naspm) for the indicated periods of time. Growth factors were always added to the cells a day before experimentation and cell cultures were tested regularly to ensure that they were free from mycoplasma contamination.

7.4 Calcium Imaging (*I-III*)

Ca^{2+} imaging was performed (*21*) to analyze functional responses in human and mouse NPCs (*I-III*). Cells were stimulated with different glutamate receptor agonists/antagonists and changes in the $[Ca^{2+}]_i$ levels were recorded. For experiments, cells were loaded with 4 µM fura-2 acetoxymethylester (fura-2/AM; Ca^{2+} indicator) at $+37^0$C for 20 min in HEPES-buffered medium (in mM): 137 NaCl, 5 KCl, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 10 glucose, 2 $CaCl_2$, and 0.5 $MgCl_2$. Thereafter, coverslips were attached to a temperated perfusion chamber and cells were perfused continuously with an external solution at 2 ml/min at $+37^0$C. The fluorescence intensities were recorded after continuous excitation of the measured cells at 340 nm and 380 nm using a 430 nm dichroic mirror and 510 nm barrier filter with an integrating charge-coupled device camera (Olympus). An image (340/380 nm) was acquired every second. One neurosphere per coverslip (4-10 coverslips/group) was analyzed and $[Ca^{2+}]_i$ rise in the soma of 40-100 cells derived from each neurosphere was measured. Cells were stimulated with agonists, and after recording the response, cells were washed with HBM to recover the basal $[Ca^{2+}]_i$ levels. The data were analyzed using InCyt 4.5 software (Intracellular Imaging Inc.) or Cell R software (Olympus) and further processed with Origin 6.0 software (OriginLabCorp.). All agonists and antagonists are listed in table 3.

Table 3: Agonists/Antagonists used in the study

Compound	Agonist/Antagonist	Manufacturer	Final concentration (μM)	Publication
DHPG	Group I mGluR agonist	Tocris	10	II,III
Kainic acid	Kainate/AMPAR agonist	Tocris	50	II,III
NMDA	NMDAR agonist	Tocris	50	II,III
Glycine	NMDAR potentiator	Tocris	10	II,III
AMPA	AMPAR agonist	Tocris	50	III
Naspm	AMPAR antagonist	Tocris	10	III
PhTX	Kainate/AMPAR antagonist	Tocris	10	III

AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; DHPG, *(S)*-3,5 dihydroxyphenylglycine; Naspm, Naspm trihydrochloride; NMDA, N-methyl-D-aspartate; PhTx, philanthotoxin.

7.5 Time-lapse live imaging (*I,II*)

The Cell-IQ live imaging system (Chip-Man Technologies) was used to monitor cellular movement during early differentiation of mouse NPCs (*I,II*). The Cell-IQ system is a cell-culturing instrument combined with phase contrast microscopy, automation, and environmental control. The system contains an integrated incubator, precision movement stages (x, y-axes: ± 1μm; z-axis: ±0.4 μm), two gas flow controllers, and an automated optics module fully controlled through machine vision-based firmware and analysis software. Cell movement was monitored continuously during first 24 h period in two 6-well

plates attached to an integrated plate holder in the incubator. Image analysis was performed using ImageJ or onboard analyzer software.

7.6 Immunostaining (*I-III*)

7.6.1 Immunohistochemistry

Paraffin sections (*I,II*) were deparaffinized in xylene and rehydrated in ethanol series and water. Sections were subjected to antigen retrieval by boiling in 10 mM citrate buffer for 15 min and blocked with 0.5% Triton X-100 and 20% normal goat serum (NGS) in PBS at room temperature (RT) for 1 h. Thereafter, sections were incubated with primary antibodies at $+4^0$C O/N followed by incubation with secondary antibodies at RT for 1 h. The cell nuclei were counterstained with 4',6-Diamidino-2-Phenylindole (DAPI). The fluorescent images were obtained with AxioPLAN 2 imaging microscope, and LSM Pascal laser scanning confocal microscope (both from Zeiss) and processed using ImageJ software. All primary and secondary antibodies are listed in table 4 and 5.

7.6.2 Immunocytochemistry

Differentiated human and mouse NPCs (*I-III*) were fixed with 4% PFA at RT for 10 min. Cells were permeabilized and blocked using 10% NGS and 1% BSA in PBS containing 0.1% Triton X-100 at RT for 45 min. Thereafter, cells were incubated with primary antibodies at $+4^0$C O/N followed by incubation with secondary antibodies at RT for 1 h. Finally, cells were washed with PBS and counterstained with Vectashield mounting medium containing DAPI. The fluorescent images were obtained with AxioImager (Zeiss) and processed using ImageJ software (ImageJ developers). All primary and secondary antibodies are listed in table 4 and 5.

Table 4: Primary antibodies used in the study

Antibody	Host	Manufacturer	Dilution	Publication	Method
Anti-PLAT	Rabbit	Proteintech	1:50	I	IHC
Anti-PAI-1	Rabbit	Abcam	1:500	I	IHC
Anti-GFAP	Chicken	Abcam	1:1000	I	IHC
Anti-TrkB	Rabbit	Santa Cruz Biotechnology	1:150	I	IHC
Anti-tPA	Mouse	American Diagnostica	10mg/ml	I	ICC
Anti-GFAP	Rabbit	Millipore	1:1000	I,II	ICC
Anti-BLBP	Rabbit	Millipore	1:750	I,II	ICC
Anti-DCX	Guinea pig	Millipore	1:100	I,II	ICC
Anti-MAP2	Mouse	Millipore	1:500	I,II,III	ICC
Anti-Tbr2	Rabbit	Chemicon	1:2000	II	IHC
Anti-BrdU	Mouse	Amersham	*	II	IHC
Anti-mGluR5	Rabbit	Millipore	1:50	II	ICC
Anti-FMRP	Rabbit	Abcam	1:500	II	WB
Anti-TRA1-60	Mouse	Thermo Fisher	1:500	II,III	ICC
Anti-OCT4	Rabbit	Cell signalling	1:500	II,III	ICC
Anti-SSEA3	Rat	Millipore	1:100	II,III	ICC
Anti-GluA2	Mouse	Millipore	1:100	III	ICC

ICC, Immunocytochemistry; IHC, Immunohistochemistry; WB, Western Blotting.

*Dilution used according to manufacturer's instructions.

Table 5: Secondary antibodies used in the study

Antibody	Species reactivity	Manufacturer	Dilution	Publication	Method
Alexa Fluor 568	Rabbit	Invitrogen	1:500-1000	I	ICC
Alexa Fluor 488	Chicken	Invitrogen	1:500-1000	I	IHC,ICC
Alexa Fluor 568	Guinea pig	Invitrogen	1:5000	I,II	ICC
Alexa Fluor 488	Rabbit	Invitrogen	1:2000	I,II,III	IHC,ICC
Alexa Fluor 488	Mouse	Life technologies	1:500	I,II,III	ICC
HRP conjugate	Rabbit	GE Healthcare	1:10000	II	WB
Alexa Fluor 488	Rat	Life technologies	1:500	II,III	ICC
Alexa Fluor 568	Mouse	Invitrogen	1:5000	II,III	ICC

ICC, Immunocytochemistry; IHC, Immunohistochemistry; WB, Western Blotting.

7.7 Quantitative PCR (*II,III*)

Gene expression levels were quantified in human and mouse NPCs, and in mouse prefrontal cortex, using qPCR (*II,III*). Total RNA was extracted with the Norgen's RNA purification kit followed by cDNA synthesis (1 µg of total RNA) with iScriptTM or Transcriptor First Strand cDNA synthesis kit. qPCR was performed with SYBR Green I kit using Light Cycler 480 II Real-Time PCR system (Roche). For miRNA assays, cDNA was prepared from 100 ng of total RNA with TaqMan microRNA RT kit followed by qPCR with TaqMan universal Mmix II. All samples were run in triplicates and data were analyzed

using second derivative max method determining the threshold cycle (Ct) (*251*). *GAPDH* and *miR-191* were used as internal controls. PCR Primers are listed in table 6.

Table 6: PCR primers used in the study

Gene	Forward primer	Reverse primer
FMR1	5'-GAAAACAACTGGCAGCCTGATAG-3'	5'-CATTTGCTCTGGAATACACCTC AAC-3'
GRIA1	5'-CCCTGAGAGGTCCCGTAAAC-3'	5'-ACTTCCGGAGTCCTTGCTTC-3'
GAPDH	5'-TGTTCCAATATGATTCCACCC-3'	5'-CTTCTCCATGGTGCGTGAAGA-3'
mGluR5	5'-AGCGCCTTCACAACCTCTAC-3'	5'-CCTGTCGCTGTGCATCCT-3'
Gria1	5'-GGGGTCCGCCCTGAGAAATCCA-3'	5'-TGGAGTCACCTCCCCCGCTG-3'
Gria2	5'-CGGGGGAGGTGATTCCAAGGAAAAG-3'	5'-CCAAACCAAGGCCCCCGACA-3'
Gapdh	5'-AACGACCCCTTCATTGAC-3'	5'-TCCACGACATACTCAGCAC-3'

microRNA	Primer	miRBase Accession number
miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU	MI0000289
miR-181a-3p	ACCAUCGACCGUUGAUUGUACC	MI0000289
miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	MI0000465

7.8 Western Blotting (*II*)

The FMRP expression in hiPSCs was measured using Western blotting. Cells were lysed in ice-cold RIPA buffer and supplemented with protease and phosphatase cocktail. An equal amount (90 µg) of protein was separated by polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane,

and blocked with 5% nonfat dry milk at RT for 1 h. Thereafter, the membrane was incubated with primary antibody (Table 4) at $+4^0$C O/N followed by incubation with horse peroxidase-conjugated secondary antibody (Table 5) at RT for 1 h. The immunoreactive FMRP was detected using G: BOX Chemi XX6 imaging system (Syngene).

7.9 Patch clamp recordings (*III*)

Patch clamp recordings were performed in mouse NPCs to measure whole-cell currents using Axopatch 1B patch clamp amplifier (Molecular devices). Coverslips with cells were placed onto the microscope and perfused continuously with external recording solution (in mM): 150 NaCl, 2.5 KCl, 10 glucose, 10 HEPES, 2.5 $CaCl_2$, and 1 $MgCl_2$. All agonists/antagonists were diluted in the recording solution and applied to the cells through the piezo-driven applicator (Siskiyou Corporation). The recording micropipettes (3-4 MΩ) were filled with internal solution (in mM): 100 *N*-methyl-D-glucamine, 100 CH_3SO_3H, 40 CsF, 10 HEPES, 10 $MgCl_2$, and 5 EGTA. For *I-V* curve recordings, spermine was added to the internal solution. Data were low-pass filtered at 1 kHz and acquired at 5 kHz, and analyzed using Clampfit 10.2 software (Molecular devices) and Prism 4.02 software (GraphPad). Glutamate receptor agonists/antagonists used in the study included: 1 mM L-glutamate; 100 μM Cyclothiazide; 10 μM NBQX; 10 μM Naspm.

7.10 Transcriptional profiling (*III*)

Gene expression profiling was performed with human NPCs using Affymetrix Human Clariom D array (Thermo Fisher Scientific). Biotinylated cRNA was prepared from 100 ng of total RNA using the GeneChip WT plus reagent kit. Following fragmentation, 5.2 μg of cRNA was hybridized to Affymetrix Human Clariom D arrays and scanned using a Hewlett-Packard GeneChip Scanner 3000. The CEL files

were processed and normalized using Signal Space Transformation – Robust Multichip analysis. Affymetrix Expression Console software was used to analyze the processed gene data files.

7.11 Statistical analysis (*I-III*)

The statistical comparisons were carried out using two-sided Student's unpaired *t*-test (*I-III*), Fisher's exact test (*II,III*), One-way ANOVA followed by *post hoc* Tukey/Bonferroni test (*I,III*), and Mann-Whitney test (*III*). Levene's and Shapiro-Wilk test was used to check the homogeneity of variances and normality of the data, respectively. All statistical tests were performed using IBM SPSS analysis software (IBM Analytics). *P* value < 0.05 was considered statistically significant.

7.12 Experimental setup

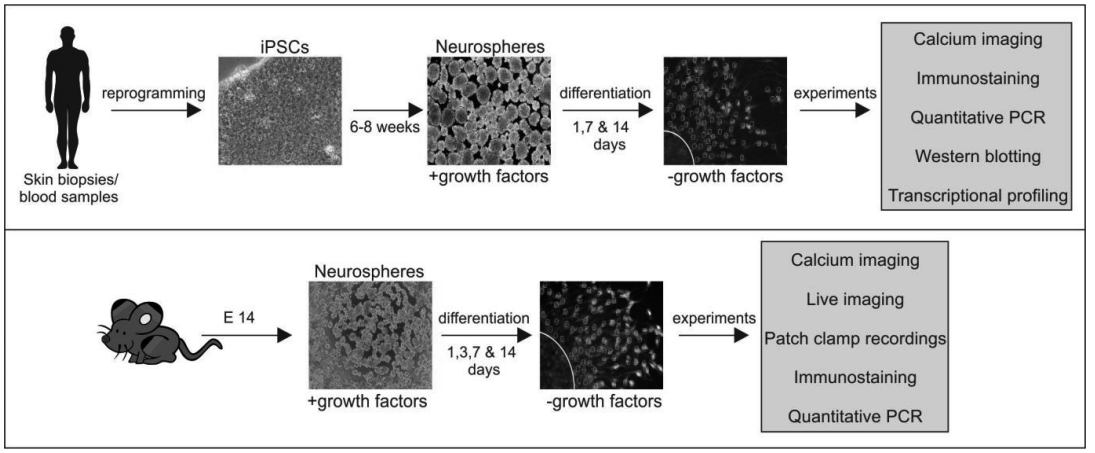

Figure 2: The experimental setup used to study human and mouse cellular models. iPSCs, induced pluripotent stem cells.

8. Results

8.1 Tissue plasminogen activator contributes to alterations of neuronal migration and activity-dependent responses in fragile X mice (I)

8.1.1 tPA expression in NPCs and in brain of Fmr1-KO mice

We compared the tPA expression in embryonic cortical NPCs, in developing and mature brain of *Fmr1*-KO mice to that in WT controls. A significant increase was seen in the number of tPA-immunoreactive cells in FMRP-deficient neurospheres at day 7 and 14 of differentiation (**Article I**: Figure 1A,B). The tPA-positive cells mainly co-expressed GFAP in both WT and *Fmr1*-KO cells (**Article I**: Figure 1B,D), however, tPA expression was not seen in MAP2-positive cells (**Article I**: Figure 1C). Furthermore, an abnormal expression pattern was observed for tPA in the developing brain of *Fmr1*-KO mice (**Article I**: Figure 4A). The proportion of tPA-immunoreactive cells was increased in cortical layers I-III (1.8 fold) and decreased in layers IV-VI (0.7 fold) at P7 (**Article I**: Figure 4C,D). The number of tPA-positive cells was increased (3.2 fold) paralleled with reduction of PA1-1 expressing cells (0.7 fold) in the DG of *Fmr1*-KO mice (**Article I**: Figure 4E,F). Also, the tPA expression was significantly increased in somatosensory cortex, visual cortex, and DG of the adult brain of *Fmr1*-KO mice (**Article I**: Figure 5A-D). Likewise in FMRP-deficient neurospheres, tPA-immunoreactive cells colocalized with GFAP in both cortex and hippocampus of *Fmr1*-KO mice (**Article I**: Figure 5C,D).

8.1.2 Effects of tPA on migration and intracellular calcium responses to depolarization in mouse NPCs lacking FMRP

In the neurosphere model, cells migrating out of the cluster initially display RG morphology followed by maturing neuron-like cells (*76, 77*). The morphology of the cells correlated with immunocytochemical stainings showing that the majority of RG cells are immunopositive to BLBP, and neuron-like cells are immunopositive to DCX (**Article I**: Figure 2B). Using time-lapse live imaging, neuron-like cells lacking

FMRP were observed to migrate longer distances from the edge of the neurosphere after 24 h of differentiation (**Article I**: Figure 2A). Treatment of FMRP-deficient cells with tPA-neutralizing antibody normalized the distance migrated by neuron-like cells to WT levels (**Article I**: Figure 2A). Similarly, treatment with tPA blocker normalized the distance migrated by DCX-immunoreactive FMRP-deficient NPCs (**Article I**: Figure 2B,C). Besides, blocking tPA activity rescued the distance traveled by RG and the velocity of the interkinetic nuclear migration (INM) of FMRP-deficient RG (**Article I**: Figure 2D-F). Furthermore, the involvement of tPA on $[Ca^{2+}]_i$ responses to depolarization during early NPC differentiation is reported. To understand the possible correlation between distance migrated and functional responses the whole migration was divided into three zones (21 μm/each zone, from the edge of the neurosphere) (**Article I**: Figure 3A). Depolarization with 17 and 75 mM extracellular potassium ($[K^+]_e$) caused changes in $[Ca^{2+}]_i$ levels in most cells migrating out of the neurosphere in both FMRP-deficient and WT neurospheres. The amplitude of response to depolarization with 17 and 75 mM $[K^+]_e$ was enhanced in the outer migration zones of FMRP-deficient cells (**Article I**: Figure 3C,D). Treatment with tPA-neutralizing antibody lowered the Ca^{2+} responses only in FMRP-deficient NPCs (**Article I**: Figure 3E). In summary, the results implicate a vital contribution of glial cells to altered neuronal differentiation in FXS (*252*) and indicate the involvement of tPA-mediated mechanisms in the aberrant migration and functional changes of FMRP-deficient RG.

8.2 Metabotropic glutamate receptor 5 responses dictate differentiation of neural progenitors to NMDA-responsive cells in Fragile X syndrome (II)

8.2.1 Functional characterization of glutamate-responsive NPCs and MPEP effects on mouse FMRP-deficient cells

To improve understanding of altered mGluR signaling in FXS (*14, 15*), mGluR5-mediated changes were studied during early differentiation of NPCs derived from *Fmr1*-KO mice. Consistent with the spatial distribution of cell populations observed with immunostainings, the $[Ca^{2+}]_i$ responses to DHPG

dominated in the cells closer to the neurosphere. After longer differentiation, $[Ca^{2+}]_i$ responses to DHPG decreased, and responses to activation of iGluRs (kainate, AMPA, NMDA) increased towards the outer migration zone.

The $[Ca^{2+}]_i$ responses to activation of glutamate receptor agonists were functionally characterized in *Fmr1*-KO neurospheres and responses were compared with those in WT controls. In the absence of extracellular Ca^{2+}, DHPG caused only a transient rise in $[Ca^{2+}]_i$, whereas a sustained Ca^{2+} response was observed in the presence of extracellular Ca^{2+} (**Article II**: Figure 1B). At day 7 of differentiation, the proportion of DHPG-responsive cells remained unaltered before and after treatment with MPEP (a specific mGluR5 antagonist) between *Fmr1*-KO and WT neurospheres (**Article II**: Figure 1C). To understand the distribution of DHPG-responsive cells, the migration area was divided into three zones (**Article II**: Figure 1D) (as described in study I). The proportion of DHPG-responsive cells was increased in zone 1 and reduced in zone 3 of FMRP-deficient cells (**Article II**: Figure 1E). MPEP treatment reduced the cell population in zone 2 and normalized in zone 3 (**Article II**: Figure 1D), without changes in the total DHPG-responsive cell population. Based on $[Ca^{2+}]_i$ responses to mGluR and iGluRs activation, the differentiating NPCs were distinguished into three subpopulations (figure 3). In the type 1 subpopulation, the majority of cells showed a robust response to activation of mGluR and minor/no response to iGluRs (**Article II**: Figure 1F). The type 2 cell population showed a substantial response to activation of both mGluR and iGluRs (**Article II**: Figure 1H), and type 3 displayed a prominent response to the activation of iGluRs and no response to mGluR. The proportion of type 1 (**Article II**: Figure 1G) and type 3 subpopulations of cells did not alter between WT and FMRP-deficient NPCs, even after MPEP treatment. However, a significant increase was seen in the type 2 cell population in FMRP-deficient cells, and MPEP treatment normalized the abnormal differentiation of the subpopulation (**Article II**: Figure 1I). In addition, the type 2 cell population was accumulated in zone 2 and displayed unipolar morphology

(**Article II**: Figure 1J,K). Treatment with MPEP normalized the accumulation and reduced the number of cells with unipolar morphology in FMRP-deficient cells (**Article II**: Figure 1J,K). In agreement with the enhanced differentiation of FMRP-deficient glutamate-responsive cells, a significant increase was observed in the magnitude of DHPG responses in all three zones of migration area of FMRP-deficient NPCs (**Article II**: Figure 2A).

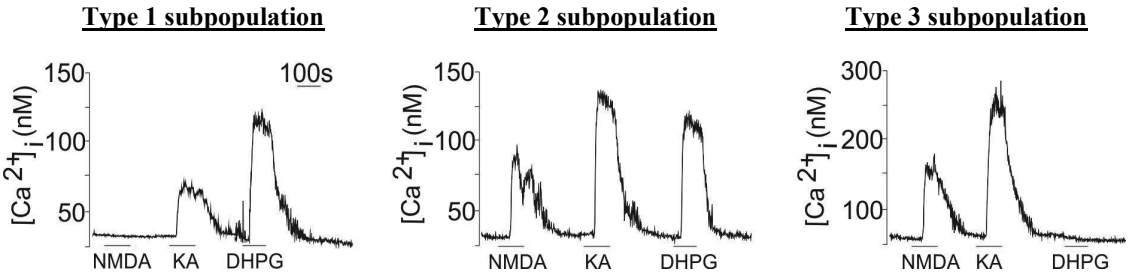

Figure 3: Neural progenitor subpopulations based on their responses to activation of glutamate receptors. DHPG, *(S)*-3,5-dihydroxyphenylglycine; KA, kainic acid; NMDA, *N*-methyl-D-aspartate.

8.2.2 Effects of MPEP on motility and morphology of differentiating NPCs, and on cortical neurogenesis of Fmr1-KO mice

We next examined the effects of MPEP on motility pattern and morphology of NPCs during early differentiation. Using time-lapse live imaging, the freely moving neuron-like cells were monitored in the outer border of migration area during the first 24 h of differentiation. The motility pattern was analyzed by the dividing the cell movement into periods of stalling (movement < 30μm/h) and surges (movement > 30μm/h) in WT and *Fmr1*-KO neurospheres without and with MPEP treatment (**Article II**: Figure 4A-D). The motility pattern (surges/stalling), mean velocity, and the distance traveled by neuron-like cells were increased in FMRP-deficient neurospheres (**Article II**: Figure 4E-G). MPEP treatment normalized the altered pattern and migration in only *Fmr1*-KO NPCs (**Article II**: Figure 4E-G). Furthermore,

treatment with MPEP increased the neurite length of MAP2-positive cells and rescued the number of multipolar GFAP-positive cells in FMRP-deficient neurospheres (**Article II**: Figure 5A-D). Administration of MPEP to E14 *Fmr1*-KO mice triggered a significant reduction in the number of Tbr2- but not BrdU-positive cells at E17 (**Article II**: Figure 6A-C). Tbr2 is expressed in VZ/SVZ of the neocortex during glutamatergic cortical neurogenesis (*253*). These results suggest that MPEP possibly corrects the aberrant neurogenesis in *Fmr1*-KO mice by reducing the increased differentiation of the glutamatergic cell population.

8.2.3 Functional characterization of human iPSC-derived NPCs, and MPEP effects on FXS glutamate-responsive cell populations

Following the identification of mGluR5-mediated alterations in *Fmr1*-KO neurospheres, the same was examined in hiPSC-derived NPCs. Three controls and four FXS hiPSC lines were used in the study. All cell lines expressed pluripotency markers and FXS cell lines did not express *FMR1* and FMRP (**Article II:** Figure S1**)**. Most cells migrating out of the differentiating human neurosphere showed immunopositivity to MAP2 at day 1 of differentiation (**Article II**: Figure 3A) indicating that the cells were committed to the neuronal fate. In line with the enhanced differentiation, an increase in a DHPG-responsive cell population was observed, coincident with an increased magnitude of responses to DHPG in FXS NPCs at day 7 of differentiation (**Article II**: Figure 3C,I). Similarly, the type 2 and type 3 cell population were more abundant in FXS NPCs (**Article II**: Figure 3D,E). Furthermore, both $[Ca^{2+}]_i$ responses and the proportion of cells responding to activation of iGluRs were increased in FXS NPCs (**Article II**: Figure 3F,G,J,K).

Unlike in mouse neurospheres, MPEP treatment further increased the DHPG-responsive and type 2 cell population in human FXS cells (**Article II**: Figure 3C,D). However, the type 3 cell population was

normalized after MPEP treatment (**Article II**: Figure 3E), which was almost negligible in mouse NPCs. MPEP treatment also normalized the FXS-specific increase in NMDA-responsive cells (**Article II**: Figure 3F), and reduced the amplitude of responses and cells responding to KA in FXS NPCs (**Article II**: Figure 3G,J). Altogether, these results strikingly demonstrate an FXS-specific increase in a subpopulation of NPCs and MPEP treatment showing cell-type specific effects on maturation of glutamate-responsive cells.

8.3 Functional changes of AMPA responses in human induced pluripotent stem cell-derived neural progenitors in fragile X syndrome (III)

8.3.1 Mechanisms involved in altered differentiation of CP-AMPAR expressing human FXS NPCs

This study was set forth to investigate the molecular mechanisms involved in aberrant differentiation of glutamate-responsive cells in FMRP-deficient NPCs derived from hiPSCs. After differentiating neurospheres for 1 and 7 days, all cells in the migration area were sequentially challenged with NMDA, KA, and DHPG (**Article III**: Figure 1B). The proportion of glutamate-responsive cells was increased in FXS neurospheres at both day 1 and 7 of differentiation compared to controls (**Article III**: Figure 1C,D), in line with previous studies showing altered kinetics of FXS NPCs (*45*). Importantly, the $[Ca^{2+}]_i$ responses and percentage of cells responding to KA were more abundant in FXS neurospheres (**Article III**: Figure 1F,G). KA is a potent AMPAR agonist that does not cause receptor desensitization (**Article III**: Figure 1E), whereas it exhibits transient effects on kainate receptors (*254, 255*). An identical abnormal increased response was seen when stimulated with specific AMPAR agonist in FXS NPCs (**Article III**: Figure 1H). A more detailed analysis revealed that the enhanced responses to KA were mainly detectable in the cell population that was responsive to either NMDA or DHPG (**Article III**: Figure 7B), suggesting a coordinate regulation of glutamate receptors in aberrant functional maturation of glutamate-mediated responses in FXS.

Next, the effects of Ca^{2+} permeable AMPAR (CP-AMPAR) blockers were tested on the increased Ca^{2+} permeability observed in FXS NPCs. PhTx, a blocker of CP-AMPARs, fully inhibited the sustained Ca^{2+} response to KA in 69 ± 5% FXS cells and 49 ± 4% control cells when applied through bath solution or treated prior (**Article III**: Figure 2A,B). Likewise, Naspm, a blocker of CP-AMPARs lacking GluA2 subunit completely blocked the AMPA responses in 53.3 ± 3% FXS cells and 38.4 ± 7% control cells (**Article III**: Figure 2D,E). Since GluA2 subunit has been shown to regulate Ca^{2+} permeability (*256, 257*), these results indicate that reduced GluA2 expression led to increased Ca^{2+} permeability in NPCs lacking FMRP (figure 4). Cells sensitive to CP-AMPAR blockers predominantly co-expressed NMDA receptors in FXS cells (**Article III**: Figure 2C,F), which correlated with enhanced differentiation of glutamate-responsive cells in FXS. The reduction of GluA2 protein expression in FXS neurospheres was confirmed using immunocytochemical analysis (**Article III**: Figure 5A). Furthermore, the normal developmental regulation of *GRIA1* transcript levels shown in WT NPCs (*77, 111*) was absent in FXS neurospheres (**Article III**: Figure 5C), suggesting aberrant maturation of AMPARs during early differentiation of FXS NPCs.

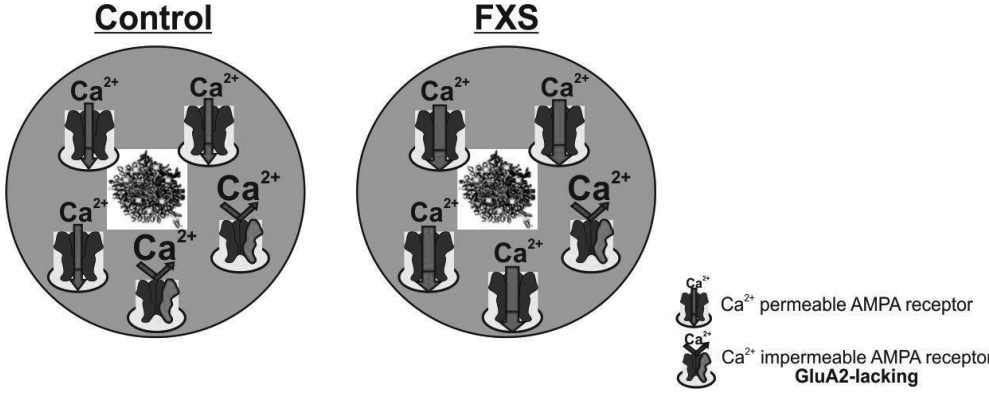

Figure 4: Reduced GluA2-lacking AMPA receptors caused increased calcium permeability of human FXS neural progenitors.

To shed further light on the underlying mechanisms responsible for reduced GluA2 expression, transcriptome analysis was undertaken, showing that *MIR-181A1* host gene expression was increased ($P = 0.018$) in FXS NPCs. The non-coding *MIR-181A1* host gene generates *miR-181a* and *miR-181b* microRNAs, which regulate GluA2 expression (*258, 259*). Particularly, *miR-181a* has been shown to have a direct interaction with GluA2 subunit and inhibits its activity posttranscriptionally (*258, 260*). Using qPCR, the mRNA expression of *miR-181a* was validated and the *miR-181a* transcript expression was found to be significantly increased in FXS NPCs (**Article III**: Figure 6B,C). Taken together, the results indicate that increase in *miR-181a* expression could be implicated with reduced GluA2 activity leading to enhanced Ca^{2+} permeability in FXS.

8.3.2 Mechanisms involved in altered differentiation of CP-AMPAR expressing mouse Fmr1-KO NPCs

Similar to human FXS NPCs, the glutamate-responsive cell populations were increased in mouse FMRP-deficient cells, at both day 1 and 7 of differentiation (**Article III**: Figure 3A-D). AMPAR blockers completely abolished the KA/AMPA responses in a larger proportion of *Fmr1*-KO cells at day 7 of differentiation (**Article III**: Figure 3E-F). In addition, the effects of NBQX and Naspm on glutamate-evoked currents were assessed using whole-cell patch clamp recordings. Confirming the presence of AMPARs in differentiating neurospheres, the glutamate-evoked currents were entirely blocked by NBQX in both WT and *Fmr1*-KO cells (**Article III**: Figure 4A,B). Following the application of Naspm, the glutamate-evoked currents showed substantial inhibition in *Fmr1*-KO neurospheres (**Article III**: Figure 4A,B). Consistent with GluA2-lacking AMPARs exhibiting strong inward rectification (*261, 262*), a significant reduction was observed in inward rectification index (I_{+40}/I_{-60}) in FMRP-deficient cells (**Article III**: Figure 4C-E). These results support the findings demonstrating the presence of more GluA2-lacking AMPARs in *Fmr1*-KO neurospheres than in controls. Comparable to human FXS NPCs, the GluA2 protein expression was reduced, and an abnormal developmental pattern of *Gria1* transcript levels

was observed in FMRP-deficient neurospheres at day 7 of differentiation (**Article III**: Figure 5B,D). In addition, *Gria2* and *Gria1* mRNA expression levels were decreased in the adult prefrontal cortex of *Fmr1*-KO mice (**Article III**: Figure 5E). In summary, the alterations in the functional properties of AMPARs, depending on GluA2 subunit expression, demonstrated a novel Ca^{2+}-dependent mechanism in FXS NPCs, which might contribute to the hyperexcitability in the FXS brain (*263*).

9. Discussion

The present work was centered upon the investigation of glutamate receptor-mediated alterations during differentiation of FXS NPCs, as well as the study of the effects of specific mGluR5 and AMPAR antagonists on the early aberrances. In addition, the involvement of tPA-mediated processes on neuronal migration and activity-dependent changes of *Fmr1*-KO NPCs were investigated.

9.1 Altered differentiation of FMRP-deficient neural progenitors

The loss of FMRP results in increased protein synthesis and altered synaptic plasticity, including exaggerated mGluR-mediated LTD (*9, 14, 15, 214, 264*). Excessive activity of group I mGluR signaling is thought to underlie the main phenotype of *Fmr1*-KO mice. The high expression of mGluR5 in the active neurogenic zones of pre- and postnatal brains (*131*) highlights its importance during neurogenesis. The $[Ca^{2+}]_i$ responses to DHPG were enhanced in both human and mouse FXS NPCs during early differentiation, in line with previous studies showing inappropriate group I mGluR signaling in *Fmr1*-KO mice (*14*). The $[Ca^{2+}]_i$ responses to activation of iGluRs were also augmented in human FXS NPCs, suggesting the excessive activity of glutamate signaling already at early stages of NPCs differentiation. However, the amplitude of responses to activation of iGluRs are not similarly increased in mouse *Fmr1*-KO NPCs, which may reflect progenitor type-dependent differences between human and mouse. Both plasmin-dependent and -independent mechanisms of tPA have been proposed to potentiate NMDAR signaling (*146, 147*). For instance, treatment with exogenous tPA increases NMDA-induced $[Ca^{2+}]_i$ response in mouse cortical neurons (*148*). The enhanced NMDAR activity of responses can be associated with increased tPA expression in *Fmr1*-KO NPCs. Moreover, dysregulated NMDAR signaling is implicated in altered mGluR-mediated LTD in *Fmr1*-KO mice (*193*).

Previous studies have shown abnormalities in the differentiation of NPCs propagated from human FXS fetus and ESCs, embryonic brain, and adult DG of the hippocampus of *Fmr1*-KO mice (*21, 23-25, 44, 231*). Accordingly, enhanced differentiation of cells responsive to activation of glutamate receptors was evident in human and mouse FXS NPCs. Notably, the type 2 cell population, which exhibited a prominent response to activation of both mGluR and iGluRs was increased in human and mouse FXS NPCs. In mouse neurospheres, the cell population was accumulated in zone 2 of the migration area and displayed unipolar morphology similar to that of bRG (*59, 60*). The bRG cells primarily reside in the oSVZ, which is present in the developing primate brain but not generally in rodent brain. Even though, rodents lack oSVZ, a small subpopulation of unipolar bRG cells reside in SVZ of mouse brain (*61, 70*). The aRG cell divisions can generate bRG cells in a cleavage angle-dependent manner. The vertical aRG cell divisions give rise to either a self-renewal aRG or an IP daughter cell (*265, 266*). In contrast, the division of aRG cells with a horizontal cleavage angle generates a self-renewed aRG that inherits apical end foot and bRG cell that inherits basal fiber (*265, 266*). Intriguingly, mouse aRG cells predominately divide with vertical cleavage angle, whereas human aRG cells divide both vertically and horizontally in equal proportions (*265-269*). These data suggest that the evolutionary shift from vertical cleavage angle to both vertical and horizontal cleavage angles of aRG cells may have contributed to the increased production of bRG and expansion of oSVZ in humans. In human FXS NPCs, DHPG-responsive RG and type 3 subpopulations that showed a substantial response to only iGluRs were increased, but remain unaltered in mouse *Fmr1*-KO cells. Although the 2D cell culture model has proven to be a valuable model for cell-based studies, it has its limitations. Therefore, further studies on hiPSC-derived organoid cultures are necessary to examine the altered differentiation and migration of NPCs in more physiologically relevant 3D *in vitro* model.

Increased numbers of Tbr2-positive IPs in the VZ/SVZ of the embryonic brain of *Fmr1*-KO mice (*22*) suggests that loss of FMRP has a great impact on differentiation of NPCs to glutamatergic lineages. Saffary et al. showed that the absence of FMRP increases the transition of RG cells to IPs thereby causing depletion of RG in the neocortex of *Fmr1*-KO mice (*233*). The increased number of Tbr2-positive cells and the enhanced differentiation of mGluR/iGluR expressing cells are in line with the increased NeuroD1 expression reported in NPCs derived from rat cortex (*270*) and hESCs (*45*). NeuroD1 is an essential regulator of glutamatergic neuronal differentiation (*253*). During early neuronal development, the activity-dependent changes in Ca^{2+} signaling alter neurotransmitter specification (*271*). The work presented in this thesis demonstrates that the $[Ca^{2+}]_i$ responses to high $[K^+]_e$ were significantly enhanced in *Fmr1*-KO NPCs. These findings implicate that activity-dependent mechanisms contribute to the altered glutamatergic differentiation of FXS NPCs. In line with the enhanced neuronal differentiation, recent studies demonstrated an increase in activity of transcription factors involved in neuronal differentiation (*247*) and alterations in expression of neurodevelopmental genes (*235, 244*). In another study, Telias et al. found that ESCs lacking FMRP display aberrances in the expression of genes critical for normal neurogenesis (*231*). Taken together, the data suggests that glutamatergic differentiation is accelerated in FXS human and mouse NPCs at early developmental stages.

AMPARs are essential for neuronal development, cognitive function and synaptic plasticity (*272-274*). The modulation of AMPAR function and trafficking is crucial for many forms of synaptic plasticity and the absence of FMRP appears to have a significant impact on AMPAR-induced synaptic strength and excitability (*198, 275*). The subunit composition of AMPAR is tightly regulated during development and in a cell type-dependent manner (*276, 277*). For instance, changes in subunit composition are involved in the developmental switch of CP-AMPARs in pyramidal neurons (*278*), and synapse-specific expression of CP-AMPARs exists in the neocortex (*279*). Functional GluA2 subunit changes have not

previously been reported in FXS, although the GluA1 and GluA2 expression is reduced in the somatosensory cortex of *Fmr1*-KO mice (*208*). The protein and mRNA expression levels of GluA1 are decreased in the cerebral cortex, hippocampus, and amygdala synaptosome extracts of *Fmr1*-KO mice (*204, 209*). Similarly, a reduction in *Gria1* and *Gria2* mRNA expression was seen in the prefrontal cortex of *Fmr1*-KO mice. GluA2 protein expression was reduced, and the normal developmental increase in *Gria1* expression during early differentiation (*77*) was absent in both human and mouse FXS NPCs, suggesting that the AMPAR subunit composition is dysregulated in FXS. Using CP-AMPAR antagonist in this study resulted in a reduced proportion of GluA2-lacking AMPARs contributing to the increased Ca^{2+} permeability in human and mouse FXS NPCs. The strong inward rectification in FMRP-deficient neurospheres confirmed the delay in the switch of CP-AMPARs to CI-AMPARs in FXS. In FXS hiPSC-derived neurons, dysregulated RE1 silencing transcription factor (REST) has been shown to suppress genes essential for proper neural differentiation and axon guidance (*244*). REST is required for repression of many synaptic genes in NPCs, out of which *Gria2* is one of the primary targets (*280*). The increase in REST correlated with reduced GluA2 expression in FXS NPCs observed in the study. Furthermore, the absence of FMRP dysregulated the expression of several miRNAs at early NPCs differentiation, in line with the previous studies demonstrating altered miRNA expression in FXS (*281*). The increased miRNA expression levels of *miR-181a* in FXS NPCs suggested that *miR-181a* contributes to the post-transcriptional downregulation of GluA2 protein activity leading to increased Ca^{2+} permeability of AMPARs in FXS. In summary, this novel body of work provided a mechanistic insight into the Ca^{2+} dependent mechanisms responsible for enhanced differentiation of FXS NPCs and identified a role for dysregulated miRNAs in FXS.

9.2 Aberrant neural migration and neurite outgrowth in FXS

The migration of neurons to their target regions followed by extensions of neurites is essential for the normal formation and function of synaptic connections in the CNS. Receptor-mediated or spontaneous $[Ca^{2+}]_i$ signaling have been shown to associate with neuronal cell differentiation, migration, neurite outgrowth, and growth cone dynamics (*282-286*). The migration of DCX-immunoreactive cells was shown to be enhanced and motility pattern was altered in *Fmr1*-KO NPCs. In addition, neurite outgrowth in MAP2-positive cells was reduced in FXS NPCs. The FXS hiPSC-derived neuronal cells have been shown previously to display reduced neurite outgrowth and fewer number of neurites per sphere (*240, 242*). Treatment with Naspm reduced neurite outgrowth in both control and FXS NPCs, in line with the previous studies showing the involvement of CP-AMPARs in the regulation of neurite outgrowth (*111*). Even though the increased CP-AMPAR expressing cells could contribute to alterations of neurite outgrowth, other dysregulated mechanisms are likely to be involved too. For instance, GluA1 is a regulator of neuronal maturation, and its absence decreases dendritic growth (*206, 287*). Thus, the abnormal developmental expression pattern of *Gria1* suggests that GluA1 could contribute to the reduced neurite outgrowth in FXS NPCs.

During neurogenesis, nuclei of RG cells display periods of movements between the apical and basal surfaces termed as INM (*265, 266*). It has been speculated that the mechanisms involved in INM of vRG cells in VG are similar to the mitotic somal translocation displayed by oRG cells in oSVZ. The speed of the salutatory movements of nuclei correlate positively with the amplitude and frequency of Ca^{2+} transients changes (*288, 289*). The velocity of INM in FMRP-deficient NPCs was significantly increased during early NPCs differentiation in this study, consistent with the increased Ca^{2+} activity in FXS NPCs. The accelerated nuclear movement might increase the rate at which the progenitor cells migrate to

VZ/SVZ. Altogether, these findings support the role of Ca^{2+}-dependent mechanisms on the neurite extension, migration, and nuclear movement in FXS NPCs.

9.3 tPA-mediated effects on *Fmr1*-KO neural progenitors

The expression of tPA is found in many neurons and glial cell types, including astrocytes, and microglia (*143, 290, 291*). Substantial experimental evidence demonstrates that tPA mediates neuronal migration (*292*), neurite outgrowth (*293*), and synaptic plasticity (*294*) in the CNS. Blocking tPA activity in this study normalized the enhanced migration of DCX-immunoreactive *Fmr1*-KO cells and significantly reduced the increased velocity of INM in *Fmr1*-KO cells. This data in conjunction with the published data, suggests that blocking tPA activity retards the migration of neuronal cell populations. In addition, treating *Fmr1*-KO NPCs with tPA-neutralizing antibody reduced the enhanced $[Ca^{2+}]_i$ responses to depolarization. The aberrances in RG differentiation and migration of FMRP-deficient NPCs are in line with previous studies that demonstrated RG defects in FXS (*21, 44, 233*). Furthermore, tPA colocalization with GFAP-positive cells both in NPCs and in the adult brain of *Fmr1*-KO mice implicates its possible role in RG aberrances observed/reported in FXS. It is evident that tPA is a key player for both physiological and pathological conditions in the CNS (*142*). Therefore, further studies on proteolytic and nonproteolytic functions of tPA are necessary to understand the role of tPA in the pathophysiology of FXS and its potential link to a new therapeutic target in FXS.

9.4 mGluR5-mediated effects on FMRP-deficient neural progenitors

The genetic reduction and pharmacological block of mGluR5 corrects the main phenotypes of *Fmr1*-KO mice (*9, 180, 205, 214, 218, 221*) confirming the crucial role of mGluR signaling in the pathophysiology of FXS. The study of Dölen et al. supported the use of mGluR antagonists for treatment of FXS by demonstrating that 50% reduction in mGluR5 protein levels corrected typical FXS phenotypic features

in mice (*214*). In *Fmr1*-KO mice, MPEP rescues the audiogenic seizure phenotype (*218, 219*), reduces repetitive-like behavior (*219, 220*), and normalizes the increased density of immature spines and excessive protein synthesis (*214, 221*). In this study, MPEP treatment normalized the abnormal differentiation of type 2 NPC population and the accumulation of these cells in zone 2 of *Fmr1*-KO neurospheres. Treatment with MPEP rescued the altered motility of neuron-like cells and morphological defects in FMRP-deficient neurospheres. Furthermore, MPEP administration at E14 normalized the total number of Tbr2-positive cells in the SVZ of *Fmr1*-KO mice, suggesting that by correcting the neuronal motility and glutamatergic differentiation, MPEP might have restored the balance of NPC differentiation in the brain of *Fmr1*-KO mice. Although similar alterations in the differentiation of glutamate-responsive cell populations were observed, MPEP exhibited differential effects on human and mouse NPCs. In contrast to mouse FXS NPCs, MPEP treatment further increased the differentiation of the type 2 cell population and reduced the type 3 subpopulation of cells in human FXS NPCs. In addition, treatment with MPEP corrected altered differentiation of a cell population that was responsive only to NMDAR activation, in line with the previous studies showing the involvement of mGluR-mediated mechanisms in dysregulated NMDA signaling (*193*). The alterations observed between human and mouse NPCs populations may reflect differences in the developmental processes. The period of neurogenesis is months in human and weeks in mouse, and the differentiation time point selected for investigations may represent different developmental stages in human and mouse NPCs cultures. The difference may also support the notion that the higher-order brain functions are associated with the neuronal subpopulations present in the brain of primates but not in the rat neocortex (*266, 295*). Despite the fact that neurospheres containing heterogonous NPC populations were used to study the altered differentiation in FXS, the origin of NPCs and the cell culturing techniques differed between human and mouse. However, the hiPSC findings presented in this thesis are in agreement with the enhanced glutamatergic signaling

reported in murine models. Therefore, this thesis could be considered as a human-cell based study to understand the pathophysiology in FXS patients.

Taken together, this study puts a step forward in understanding the mechanisms involved in altered neural differentiation in FXS and emphasize the differences between human, and mouse NPCs differentiation (figure 5). The fundamental problem in pre-clinical studies is to model human disease in animals. The main cause of failed clinical trials with mGluR5 antagonists in FXS patients (*296*) might be likely because of the timing of the therapy where therapeutic interventions are targeted at too late stages when the FXS phenotypes are well established. Considering the fact that any given response in animal models does not occur in the same way in humans, the FXS-specific changes identified with hiPSC-derived NPCs will provide insights into common interventions that might alleviate the neuropathological conditions in other ASD.

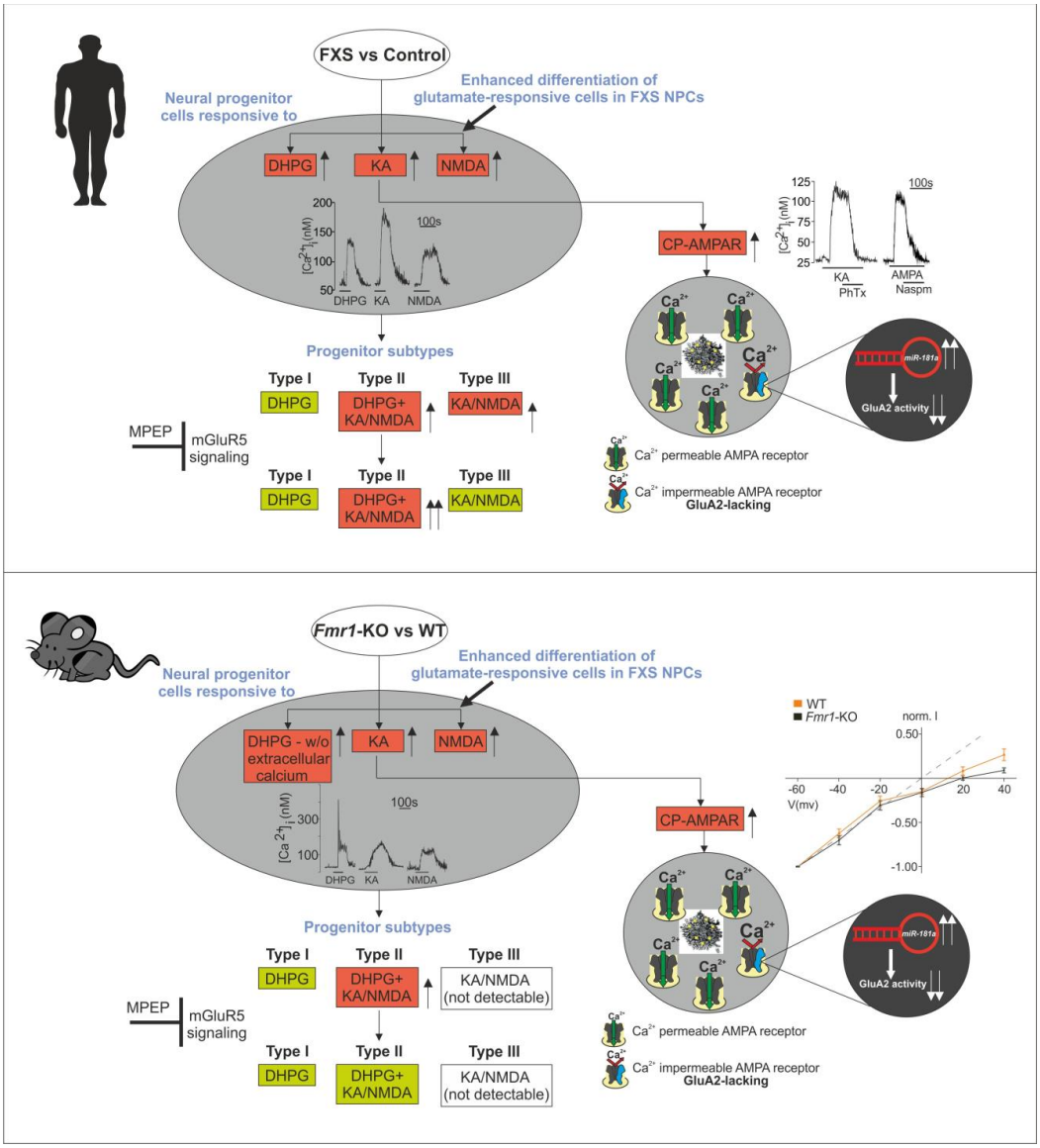

Figure 5: A schematic representation summarizing the similarities and dissimilarities observed between human and mouse NPCs during differentiation of glutamate-responsive cells. Colors in boxes represent no change/normalized (green) and augmented (red). CP-AMPAR, calcium-permeable AMPA receptor; DHPG, *(S)*-3,5-dihydroxyphenylglycine; KA, kainic acid; NMDA, *N*-methyl-D-aspartate.

10. Concluding Remarks

Although extensive research has been carried out using FXS animal models, it remains unclear how FMRP regulates neurogenesis and neuronal development in humans. This thesis provides new insights into the understanding of FXS NPCs differentiation in the human context. Both human and mouse NPCs showed enhanced neuronal differentiation to glutamate-responsive cell populations. However, MPEP treatment exhibited differential effects on distinct human and mouse NPCs populations. The differences observed between human and mouse cellular models could reflect differential responses of species-specific NPCs populations or different developmental stages of NPCs. The investigation of mechanisms resulting in the enhanced differentiation of CP-AMPARs led to the clue that the increased activity of *miR-181a* might have post-transcriptionally inhibited GluA2 expression in FXS NPCs. In addition, these studies demonstrate that tPA could have a role in early RG defects observed in *Fmr1*-KO NPCs.

The initial pharmacological therapies targeting mechanisms underlying FXS focused on antagonizing mGluR5. The results were not very encouraging (*296*), indicating that there is a need for identification of new therapeutic targets to treat FXS. The effectiveness of therapies in humans can be improved by further understanding the molecular deregulations in FXS using human-based models and also by combining two or more drugs targeting different pathways disturbed in FXS. The findings presented here highlight the importance of studies investigating early developmental alterations and provide clues for developing new targets. Furthermore, the differences observed between human and mouse NPCs populations strongly support the use of human cells to identify molecular mechanisms involved in the pathophysiology of FXS and other related neurodevelopmental disorders.

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