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Receptor signaling mechanisms in neural progenitor cell differentiation and alterations in a mouse model for Fragile X Syndrome

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Academic Dissertation

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ABSTRACT

Neural stem cells and their arising progenitors create our central nervous system (CNS). The questions of how neural progenitor cells (NPCs) are determined to a certain neuronal fate, in that, how do they mature, migrate, and develop to take on their physiological roles, during the formation of functional networks in the brain, remain fundamentally unanswered. Despite the vast amount of information acquired and accumulated over the last centuries, the interplay between molecular mechanisms that drive brain development have only recently started to unravel. Studies have shown that early brain electrical activity, neurotransmitter-induced responses, and trophic factor signaling, acting through their respective receptors, are implicated as critical regulators of brain development.

By elucidating the mechanisms governing progenitor cell behavior under normal and pathological conditions, such as fragile X syndrome (FXS), the most common cause of intellectual disability and leading genetic cause of autism, will further improve our understanding of brain development, and facilitate the development of CNS cell therapies.

This thesis aims to shed light into the molecular and cellular mechanisms in the developing brain by utilizing *in vitro* neurosphere model to study the differentiation and migration of neural progenitors, by combining gene and protein expression analysis, and immunocytochemical stainings with intracellular calcium imaging and time-lapse video microscopy. In addition, *in vivo* immunohistological staining methodologies, *in situ* hybridization studies and *in utero* electroporation were utilized to study neocortical development in the absence of functional Fragile X mental retardation protein (FMRP), in a mouse model for FXS.

Taken together, results presented in this thesis provide new information on the molecular mechanisms that guide neural progenitor cells and their interactions with radial glia (RG) cells. It sheds key insights into NPC functional responses as they mature and differentiate, identifying key molecular players as well as providing compelling evidence that neural-glia interaction during cortical formation plays an important role in brain development. Additionally, timing and region-specific modulatory role of brain-derived neurotrophic factor (BDNF)-TrkB signaling during neocortical development and abnormalities particularly in glutamatergic neurogenesis in the absence of FMRP was demonstrated.

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LIST OF ORIGINAL PUBLICATIONS:

This thesis is based on the following publications, which are referred to in the text by their corresponding Roman numerals (I-IV):

I Kärkkäinen, V.*#, **Louhivuori, V.*#,** Castrén, M.L., and Åkerman, K.E., 2009. Neurotransmitter responsiveness during early maturation of neural progenitor cells. Differentiation 77: 188-98.

II Louhivuori, L.M., Turunen P.M.*, **Louhivuori, V.***, Yellapragada, V., Nordström, T., Uhlén, P., & Åkerman, K.E., 2018. Regulation of radial glial process growth by glutamate via mGluR5/TRPC3 and neuregulin/ErbB4. Glia, 66: 94–107.

III **Louhivuori, V.,** Vicario, A., Uutela, M., Rantamäki, T., Louhivuori, L., Castrén, E., Tongiorgi, E., Åkerman, K., and Castrén, M.L., 2011. BDNF and TrkB in neuronal differentiation of *Fmr1*-knockout mouse. Neurobiol. Dis. 41: 469-80.

IV Tervonen, T.A.*#, **Louhivuori, V.*#,** Sun, X., Hokkanen, M.E., Kratochwil, C.F., Zebryk, P., Castrén, E., and Castrén, M.L., 2009. Aberrant differentiation of glutamatergic cells in neocortex of mouse model for fragile X syndrome. Neurobiol. Dis. 33: 250-9.

* Equal contribution, # this publication has been used as well as a part of another academic dissertation work

This thesis includes 11 figure legends and 2 tables. In addition to original publications, some unpublished data are presented.

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ABBREVIATIONS:

Ach	acetylcholine
actNotch1	activated domain of Notch1
AMCA	7-amino-4-methylcoumarin-3-acetic acid
AMPA	alpha-amino-3-hydroxy-5-methylsoxazole-4-propionic acid
APP	amyloid precursor protein
ASD	autism spectrum disorder
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BDNF	brain-derived neurotrophic factor
BLBP	brain lipid binding protein
BMP	bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular Ca^{2+}
$[Ca^{2+}]_e$	extracellular Ca^{2+}
CaMKII	calcium-calmodulin-dependent kinase II
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CxP/CP	cortical plate
CREB	cyclic AMP response element binding protein
CYFP1	cytoplasmic FMRP interacting protein 1
Cy3	cyanine3
D(x number)	Differentiation Day x
DAG	diacylglycerol
DAPI	4',6-diaminodino-2-phenylindole
DHPG	3,5-dihydroxyphenylglycine
DMEM/F12	Dulbecco's Modified Eagle Medium
E14	embryonic day 14
eIF4E	eukaryotic initiation factor 4E
EBSS	Earle's balanced salt solution
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ErbB	v-erb-a-erthroblastic leukemia viral oncogene homolog
ER81	ETS variant transcription factor 1
ERK	extracellular signal regulated protein kinase
ESC	embryonic stem cell
FGF-2	fibroblast growth factor 2
FL	fibre layer
FMR1	fragile X mental retardation 1 gene
Fmr1-KO	*Fmr1*-knockout
FXR1	fragile X related 1
FXS	fragile X syndrome
FMRP	fragile X mental retardation protein
FMRP-eGFP	FMRP over expression with eGFP reporter gene
FMRPmt-eGFP	FMRP with I304N mutation in the KH2 domain with eGFP
fura-2AM	fura-2-acetoxymethyl ester
G3	lenti-CMV-eGFP
GABA	α-aminobutyric acid
GE	ganglionic eminence
GFAP	glial fibrillary acidic protein
GLAST	astrocyte-specific glial glutamate transporter
GP	globus pallidus
GPCR	G-protein coupled receptor
Gsk3β	glycogen synthase kinase 3 beta
HBM	hepes buffered media
HBSS	Hank's balanced salts solution
HC	Hippocampus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HVA	high voltage activated
Id	inhibitor of differentiation
ICC	immunocytochemistry
ICH	immunohistochemistry
iGlu	ionotropic glutamate
iGluR	ionotropic glutamate receptor
INM	interkinetic nuclear migration
i.p.	intraperitoneal
IP3	inositol-1,4,5-trisphosphate
IPC	intermediate progenitor/precursor cell
IZ/IMZ	intermediate zone
K^+	potassium
KH	nuclear ribonucleoprotein K Homology
KO	knock-out
ldt/lpt	lateral dorsal/posterior thalamic nucleus
LVA	low voltage activated
LTD	long-term depression
LTP	long-term potentiation
MAP1B	microtubule-associated protein 1B
MAP-2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
mGlu	metabotropic glutamate
mGluR	metabotropic glutamate receptor
mGluR5	metabotropic glutamate receptor 5
MPEP	2-methyl-6-(phenylethynyl) pyridine
mRNA	messenger ribonucleic adic
MZ	marginal zone
NE cells	neuroepithelial cells
NE	noradrenaline
NeuN	neuron specific nuclear protein
NGF	normal goat serum
NGF	neurotrophic growth factor
Ngn	neurogenin
NMDA	N-methyl-D-aspartic acid
NPC	neural progenitor cell
NRG	neuregulin
NSC	neural stem cell
NT	neurotensin
NT-3	neurotrophin-3

NUFIP nuclear FMRP interacting protein
o/n overnight
OPC oligodendrocyte precursor/progenitor cells
P6 postnatal day 6
p75NTR p75 neurotrophin receptor
PAK p21-activated kinase
PBS phosphate buffered saline
PCR polymerase chain reaction
PC progenitor cell
PDGF platelet-derived growth factor
PFA paraformaldehyde
PI phosphoinositide hydrolysis
PI3K phosphatidylinositol-3-kinase
PIP2 phospatidyl-inositol-bis-phosphate
PKC protein kinase C
PLC phospholipase C
PNS peripheral nervous system
PSA-NCAM polysialylated neural cell adhesion molecule
RG radial glia
RGG Arginine-Glycine-Glycine
RISC RNA-induced silencing complex
RT room temperature
RT-PCR real time polymerase chain reaction
S100β calcium-binding protein
SC stem cell
SCF stem cell factor
SD standard deviation
SEM standard error of mean
SGZ subgranular zone
SH2 Src-homology-2
Shh sonic hedgehog
siRNA small interfering RNA
SMAD mothers against decapentaplegic homologue
SNP single-nucleotide polymorphism
SOX2 SRY-related high-mobility group (HMG)-box protein-2
SP substance P
Svet1 subventricular-expressed transcript 1
SVZ subventricular zone
Tbr2 T-box transcription factor 2
Tbr1 T-box transcription factor 1
TGF transforming growth factor
Trk tyrosine receptor kinase
TrkB.T1 truncated 1 isoform of TrkB receptor
TrkB.FL full-length TrkB
TRP transient receptor potential
TRPC3 transient receptor potential channel 3
Tuj1 βetaIII-tubulin
VGCC voltage-gated calcium channel
VZ ventricular zone
7TMRs seven transmembrane receptors

TABLE OF CONTENTS

1. INTRODUCTION

The developing brain consists of a vast number of neural stem/progenitor cells which proliferate, differentiate, migrate, and maturate into functional neuronal cells of the brain. Defects of neural progenitors and disruption of their differentiation affect formation of neural network structures and synaptic plasticity that are important for learning and memory. Disruption of these developmental processes are associated with the pathophysiology of neurodevelopmental disorders such as Fragile X syndrome (FXS).

Various extracellular cues and intrinsic factors regulate the process of corticogenesis, where the six-layered structure of neocortex is formed (reviewed in, Guillemot et al., 2006; Molyneaux et al., 2007; Ohtaka-Maruyama and Okado, 2015). This is achieved in a highly coordinated temporal and activity-dependent manner, that encompasses a range of activities. These include cell proliferation, differentiation, and migration, the activation of intrinsic signaling transcription factor programs, radial glia (RG)-neuronal interactions as well as the integration of neuronal networks, and apoptosis (reviewed in, Kriegstein and Alvarez-Buylla, 2009; Kawaguchi, 2019). Defects in these processes lead to problems in neuronal cytoarchitecture, circuit formation and are implicated in the development and maintenance of synaptic function and plasticity in FXS, a leading model for autism spectrum disorder (ASD) (for reviews, see, (Richter et al., 2015; Banerjee et al., 2018; Bagni and Zukin, 2019; Zafarullah and Tassone, 2019).
The molecular mechanisms that respond to extracellular guiding cues along with intrinsic programs that control the machinery involved in neuronal differentiation and migrational motility are not yet fully understood. One key meditator of such cellular mechanisms is signaling pathways linked to changes in intracellular calcium (Hagg, 2009; Padamsey et al., 2018). Very strictly controlled free Ca^{2+} is indeed most widely used second messenger in organisms and involved in vast variety of cellular behavior's ranging from development of neural cells to neuronal migration and neocortical formation, as well as in mediating integration to neural circuits, synaptic functions, and programmed cell death.

This thesis addresses the dynamics of receptor activation evoked Ca^{2+}-signaling mechanisms, which influence the fate determination, differentiation and migration of progenitors and mediate RG-neuronal interactions during cortical development. Also, BDNF/TrkB signaling and glutamatergic neurogenesis in the pathophysiology of FXS is addressed in the *Fmr1*-knockout mouse model. "Which functional properties of differentiating progenitors are coupled to fate and migration control?" and, "what signaling pathways are involved in mediating RG-neuronal interactions?" are the critical questions to answer for better understanding of brain development and cell differentiation mechanisms in general, in addition to, developing strategies for brain repair and cell transplantation therapies in the future.
This thesis will firstly introduce the reader to neural stem cells, their progenitors, and then briefly overview cortical neurogenesis, followed by summary of calcium signaling and receptor signaling pathways with relevance to this work. Then, before going through the experimental section and results, discussion, general implications and conclusions, an overview of FXS is presented.

2. REVIEW OF THE LITERATURE

1. Definition of stem and progenitor cells

Stem cells (SCs) are characterized by their ability to *self-renew* and their capability to *differentiate* into a wide variety of specialized cells. The SC pool is replenished primarily by symmetrical divisions, where two new daughter stem cells are produced. SCs destined to differentiate are produced via asymmetrical division, whereby one new daughter SC is born. The *potency* of SCs (i.e., the capacity to differentiate into highly specialized cell types) can be divided into following groups:

[1)] Totipotent SCs have the ability to produce a whole new organism, by differentiating into all embryonic and extraembryonic cell types (for example zygote).

[2)] Pluripotent SCs possess the capacity to produce all cell types of the three germ layers, but they cannot differentiate into extraembryonic cells.

[3)] Multipotent SCs can differentiate only to cell types of a particular cell lineage in their natural conditions. In general, multipotent SCs in the postnatal mammalians participate in the maintenance of tissue homeostasis and are involved in the processes of tissue replacement in the body.

[4)] The descendants of multipotent SCs, named progenitor cells (PCs), they too have the ability to (limited) self-renew, but their capacity to produce differentiated cells is more restricted and typically just differentiated cell of one type of tissue is produced by a certain PC (i.e., neural progenitor cells that are limited in producing all the cell types found in the mammalian brain).

In this thesis, the term **neural stem cell (NSC)** is used to refer to neuroepithelial (NE) cell derived **primary progenitor cells,** known as radial glia (RG) cells during development (or type B cells/SVZ astrocytes during adult neurogenesis). During different developmental stages, these NSCs initiate lineages that lead to the formation of differentiated neurons or glial cells (for review, see Kriegstein and Alvarez-Buylla, 2009). In addition to direct amplification of neuronal cells from NSCs, differentiated cells can be generated indirectly through one or multiple stages of amplification from distinct types of **neural progenitor cells (NPCs)**. These precursor cell pools are a diverse cell group comprising of multipotent, lineage-restricted or fate-restricted daughter cells of NSCs with limited capability to self-renew (for review, see Kriegstein and Alvarez-Buylla, 2009). Given that the attributes of these cell types in the brain overlap in several ways, the precise contours discriminating these cells from each other is ambiguous. As such, this thesis will focus on using the term NPCs to contain all these heterogeneous types of multipotent progenitors, precursors, more lineage-restricted and fate-restricted daughter cell progenies produced with asymmetrical division from NSCs. See **Figure 1** for further information about cell divisions.

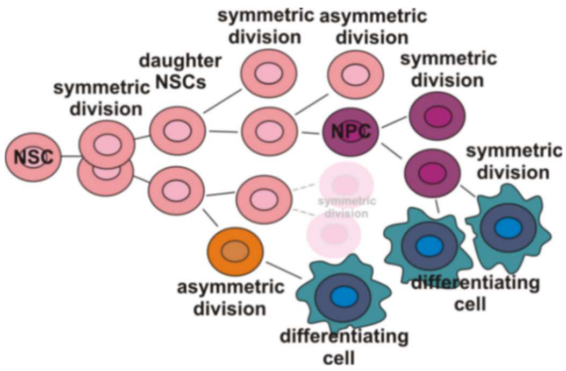

Figure 1. *Generalized view of neural stem cell divisions in the central nervous system (CNS).* With symmetric division two daughter neural stem cells (daughter NSCs) are produced from NSC whereas with asymmetrical division one daughter NSC and another cell type; immediately differentiating cell or neural progenitor cell (NPC) is produced. NPCs can divide few mitotic cycles with symmetric divisions to produce either two other NPCs or two immediately differentiating cells.

2. Introduction to neural stem and progenitor cells

It is well established that the developing and adult mammalian central nervous system (CNS) contains a population of undifferentiated multipotent cells which have a capacity to self-renew throughout the mammalian life span and give rise to different types of neuronal cells; neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992; Gage, 2000; Temple, 2001; Merkle and Alvarez-Buylla, 2006). The unique cytoarchitectural microenvironment surrounding these primary progenitor cells form NSC niches in the brain, where cell characteristics are shaped and defined in synchrony with intrinsic cellular programs whereby self-renewal is maintained, and the differentiation programs are kept on hold (Gage, 2000; Doetsch, 2003) for reviews, see (Pontious et al., 2008; Kriegstein and Alvarez-Buylla, 2009; Obernier and Alvarez-Buylla, 2019).

Classically, in developmental neurobiology, it has been thought that neurons and glial cells derive from different embryonic precursor pools. Nevertheless, now it is well established that a subpopulation of astroglial cells, namely RG cells, function as NSCs. These RG cells give rise to a diversity of neuronal progenitors, serve as scaffolding for neural migration and produce differentiated neurons and glial cells to virtually all brain regions (Malatesta et al., 2003; Anthony et al., 2004; Mori et al., 2005). In the embryonic brain the NSCs are first NE cells (Haubensak et al., 2004), which have been suggested to turn into RG at the onset of neurogenesis (Malatesta et al., 2008; Anthony et al., 2004; Götz and Huttner, 2005). The origin and potency of postnatal NSCs was as well a matter of controversy. However, various studies have convincingly shown that the actual origin of these multipotent cells is astrocyte-like RG cells, hence indicating the same lineage with embryonic originally NE cell derived RG cells (Doetsch et al., 1999; Tramontin et al., 2003; Kriegstein and Gotz, 2003; Doetsch, 2003; Merkle et al., 2004; Merkle and Alvarez-Buylla, 2006).

During development and in the adult brain, the newly born neurons and macroglial cells (astrocytes and oligodendrocytes) are produced either directly from asymmetrically dividing RG cells or indirectly from their daughter cell progenies which include cells entitled IPCs (for review, see Kriegstein and Alvarez-Buylla, 2009; Obernier and Alvarez-Buylla, 2019). IPCs originate from RG NSCs and share a few common characteristics with them. IPCs divide symmetrically at the subventricular zone (SVZ) to enlarge their population, however, they have limited proliferation capacity and they are more lineage-restricted than NSCs and lack the ability for interkinetic nuclear migration (INM), a feature closely linked to self-renewal properties of NE and RG cells (Gage et al., 1995; Weiss et al., 1996; McKay, 1997; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Martinez-Cerdeno et al., 2006). Interestingly, the developmental timing stage and the location of NSCs, a property strongly linked to their NE origin, appear to be a key determinant for the types of progenitors, neurons and glial cells generated at certain time during neocortical development (for review, see Kriegstein and Alvarez-Buylla, 2009).

NSCs and NPCs are produced continuously throughout embryonic development to postnatal and adult life in several areas of the brain in various mammals, including humans, rats and mice (for review, see Qu and Shi, 2009). One of the most active parts of neurogenesis in the adult as well as in the fetus brain is the SVZ of the lateral ventricles (Reynolds and Weiss, 1992; Palmer et al., 1995; Levison and Goldman, 1997; Doetsch et al., 1999). Active neurogenesis has also been shown in various animals in the subgranular zone (SGZ) of the hippocampus (Altman and Das, 1965; Kaplan and Hinds, 1977; Eriksson et al., 1998, Gould et al., 1999; Gage, 2000; Kempermann et al., 2004; Ming and Song, 2011).

See **Figure 2** for an overview of the generation of neuronal cells from progenitors and **Figure 3** for summary of neocortical development.

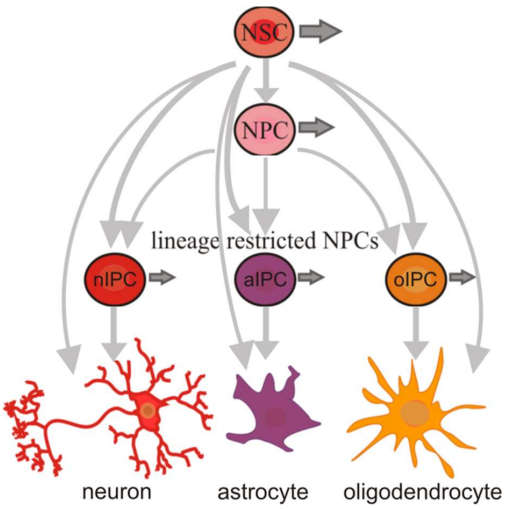

Figure 2. *A simplified illustration of self-renewal (horizontal dark arrows) and generation of neuronal cells; neurons or glial cells (astrocytes or oligodendrocytes) from apical and basal progenitors (grey vertical arrows) which is controlled through complex interplay between intrinsic factors and extracellular cues.*
The neural stem cell (NSC) pool is kept alive with symmetrical and asymmetrical divisions. Multipotent NSCs can generate differentiated neuronal cells directly through asymmetric cell divisions or indirectly through generation of neural progenitor cells (NPCs). Majority of the differentiating cells are produced indirectly from NPCs or lineage- or fate-restricted NPCs, such as neural intermediate progenitor cells (nIPCs) (for reviews see, Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009).

3. Neocortical development and adult neurogenesis

During the time of neocortical development NSCs and NPCs sequentially pass-through phases of expansion: 1) neurogenesis and 2) astrocytogenesis and 3) oligodendrocytogenesis to produce the vast cellular diversity found in the mammalian CNS (reviewed in, Miller and Gauthier, 2007). In the light of current knowledge, these different cell type progenies are produced continuously in a regionally heterogeneous and specified-manner with different developmental peaks that vary between animals in the expansion phase lengths and proliferation capacity (for review, see Kriegstein and Alvarez-Buylla, 2009). The switch from generating subtype-specific progenitors can be modeled as an interplay between region-restricted mechanisms and temporal regulators that determine the neurogenic or gliogenic phases of development, ultimately leading to the formation of neocortical layers and other brain structures in a highly orchestrated manner (for review, see Rowitch and Kriegstein 2010). The last steps of neocortical development, which occur during early postnatal development, are synaptogenesis and formation of neuronal network structures, which are then maintained and shaped throughout life with new synaptic connections and by removing unnecessary ones in an activity-dependent manner (McConnell, 1988; Marin and Rubenstein, 2003; Guillemot et al., 2006).

3.1. Radial glia during brain development and adult neurogenesis

3.1.1. Radial glia origins and dual natured phenotype

At the expansion phase of neocortical formation (till embryonic day (E) 9 in mouse) NSCs (which are referred to NE cells at this developmental stage) divide symmetrically to increase their cell numbers and likely generate the early born layer I neurons (Rakic, 2005). However, as mentioned earlier, these NE cells are thought to turn into RG at the onset of the neurogenic phase (E9-E10 in the mouse) (for reviews, see Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). This conversion from NE cells to RG is thought to happen due the elongation of NE cells, as the brain epithelium thickens and tight junctional complexes that couple NE cells convert to adherens junctions (Aaku-Saraste et al., 1997; Stoykova et al., 1997). Adherens junctional complex components such as cadherins are in fact essential in maintaining RG behavior (Rasin et al, 2007). At the same time with thickening of the brain epithelium and conversion of the junctions, the cells begin to make endothelial cell contacts in an astrocyte-like manner (Takahashi et al., 1990). Indeed,

RG cells share some features with NE cells and some with astroglial phenotypes (for review, see Kriegstein and Götz, 2003; Rowitch and Kriegstein, 2010; Bayraktar et al., 2014; Obernier and Alvarez-Buylla, 2019).

3.1.2. Radial glia self-renewal, cell division and INM

RG cells divide at the apical (ventricular) cortical surface and hence are occasionally termed apical progenitors. RG cells self-renew with symmetrical divisions or generate through asymmetric cell divisions differentiated cells, directly or indirectly through NPCs, as well as undergo apical-basal INM as NE cells (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; 2007). Even though the functional significance of this nuclear migration is not well understood, INM has been linked to cell cycle progression (Tsai et al., 2005; Ueno et al., 2006). During INM, the nuclei undergoing the S phase cell cycle form a nuclei layer away from the ventricle, at the apical side of the VZ, while in M phase the nuclei line up along the surface of the ventricle (basal side). In G1 and G2 phases the nuclei are transitioning between the S and M phases in the middle region (reviewed in, Miyata, 2008). This complex mitotic behavior is one important aspect of NE cell activity retained by RG cells (Huttner and Brand, 1997).

It has been proposed that INM regulates neurogenesis by modulating the exposure of cell nuclei to proliferative or neurogenic signals, particularly to Notch (Del Bene et al, 2008). Notch activation is important in maintaining the proliferative state of RG cells (Dorsky et al., 1997; Gaiano and Fishell, 2002; Silva et al., 2003; Zhang et al., 2008) and RG cell identity and self-renewal, too (Gaiano et al., 2000). Indeed, Notch signaling through target genes, such as *hes1* and *hes5* seems to be particularly important in blocking the cell differentiation and antagonizing proneural genes and therefore maintaining proliferation and self-renewal (Gaiano et al., 2000; Iso et al., 2003; Hatakeyama et al., 2004).

3.1.3. Radial glia contacts, structure and morphological changes

RG contact the ventricle with a single apical primary cilium ending and thus maintain the pseudostratified epithelium in the VZ. They also make contacts through their radial processes to the pial cortical surface, contacting the meninges, basal lamina and blood vessels, therefore having an apical-basal polarity (Boulder Comm, 1970). The mouse homologs for *Drosophila* endocytic protein genes, *Numb* and *Numbl* are critical in maintaining the polarized structure of RG (Rasin et al., 2007). Through their long radial fiber processes, RG cells are thought to guide the migration of young neurons to their destined neocortical layers (Rakic, 1978; Noctor et al., 2004; Kriegstein et al., 2006).

When the progenies generated from RG, NSCs and NPCs move further along to differentiate into the mantel of cortical formation, the brain thickness and thus the radial RG processes elongate further. These morphological changes are also accompanied with expression of astroglial markers, such as brain lipid-binding protein (BLBP), astrocyte-specific glutamate transporter (GLAST) the calcium-binding protein (S100β), glutamine synthase (GS), Sox2 and the adhesion molecule tenascin C (TN-C) (for reviews, see (Campbell and Götz, 2002; Chandrasekaran et al., 2016). In addition, RG cells also start to express a variety of intermediate filament proteins such as nestin and vimentin (Frederiksen and McKay, 1988; Hartfuss et al., 2001; Mori et al., 2005). S100β is considered as a suitable RG marker due its expression throughout the period of neurogenesis and early gliogenesis (Patro et al., 2015). Furthermore, RG cells express also at least in rhesus monkey and human brain astroglial intermediate filament protein called glial fibrillary acidic protein (GFAP) (Choi and Lapham, 1978; Levitt and Rakic, 1980; Imura et al., 2003). Astroglial structural phenotype in RG includes the appearance of glycogen storaging granules as well (Gadisseux and

Evrard, 1985). Importantly, RG cells express transcription factors paired box 6 (Pax6) and Hes (Englund et al., 2005; Cappello et al., 2006).

As the embryonic development ensues, most RG begin to detach from the apical side and convert into multipolar astrocytes (Morest, 1970; Choi and Lapham, 1978; Schmechel and Racic, 1979; Misson et al., 1991; Noctor et al., 2008). This transformation is supported by the vanishing of majority of RG at the time when growing numbers of astrocytes emerge and moreover, shared expression of same markers such as radial glial cell marker 1 and 2; RC1 and RC2 (Mission et al., 1991) for review, see (Rowitch and Kriegstein, 2010; Bayraktar et al., 2014).

3.1.4. Radial glia in postnatal and adult neurogenesis

A subpopulation of RG cells retains apical contacts and the ability to INM even after embryonic development. These cells function as NSCs in the postnatal brain and continue the generation of progenitors. In adults some RG cells convert to ependymal cells and some to SVZ astrocytes (so-called type B cells) which continue to function as NSC in the adult CNS (for reviews, see Götz and Huttner, 2005; Ihrie and Alvarez-Buylla, 2008; Rowitch and Kriegstein, 2010, Gonzalez-Perez and Quinones-Hinojosa, 2012; Bayraktar et al., 2014; Obernier and Alvarez-Buylla, 2019). The type B cells in the adult SVZ are relatively quiescent and produce type C cells which proliferate more actively and function as IPCs (Doetsch et al., 1999b). The type C cells generate immature neuroblasts (type A cells) which migrate in chains through the rostral migratory pathway to the olfactory bulb and differentiate into interneurons (Lois and Alvarez-Buylla, 1993; Belluzzi et al., 2003; Carleton et al., 2003). SVZ type B and C cells are closely associated with blood vessels that extend an extensive extracellular matrix next to them (Mercier et al., 2002; Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008). As well, proliferating SVZ cells are frequently associated with blood vessels (Tavazoie et al., 2008) or the extracellular matrix around them (Kerever et al., 2007), suggesting that factors derived from the vasculature may regulate both cell types in the SVZ.

Similar NSC pattern of adult neurogenesis has been shown in the SGZ of the hippocampus (Seri et al., 2001; Filippov et al., 2003; Fukuda et al., 2003; Garcia et al., 2004; Ming and Song, 2011). Unlike other astrocytes in the SGZ that express only GFAP, these cells express beside GFAP, also nestin (Seri et al., 2004; Steiner et al., 2006). Furthermore, the SGZ is also located near a widespread vascular area (Palmer et al., 2000; Javaherian and Kriegstein, 2009), suggesting that factors derived from blood vessels may indeed greatly influence the behaviour of NSCs.

3.2. NPCs and IPCs during brain development and adult neurogenesis

3.2.1. Characteristics of NPCs

A vast knowledge indicates that various types of RG derived NPCs co-exist during embryonic development. Multipotential progenitors that contribute to both, neuronal and glial lineages and fate-restricted progenitors which may give rise to a diversity of neural cells or just single type of neurons, suggest heterogeneity of RG proliferative behavior (reviewed in, Kriegstein and Alvarez-Buylla, 2009; Gonzalez-Perez and Quinones-Hinojosa, 2012). Although it is very well established that bi- or tri-potent progenitors exist and are present at all developmental stages, their numbers are comparatively low (Walsh and Cepko, 1988, 1992; Parnavelas et al., 1991; Williams et al., 1991; Reid et al., 1995; Williams and Price, 1995; Qian et al., 2000; He et al., 2001; McCarthy et al., 2001; Yung et al., 2002; Shen et al., 2006). Whereas a clonal analysis has revealed that most progenitors are already early committed to neural or glial fates (Luskin et al., 1988, 1993; Price and Thurlow, 1988; Grove et al., 1993; Krushel et al., 1993; Davis and Temple, 1994; Williams and Price, 1995; Mione et al., 1997; Malatesta et al., 2000; Qian et al., 2000; McCarthy et al., 2001; Anthony et al., 2004; Wu et al., 2006; Battiste et al., 2007).

In fact, it has been introduced that this restriction to certain fate is partly present already in NE cells (Qian et al., 2000; McCarthy et al., 2001). Furthermore, the occurrence of early commitments to late cell fates at early stages of cortical development suggests that already-committed progenitors can remain quiescent even for long periods of time. Consequently, the evident progressive lineage switch shown by RG is partly due to the differential expansion of distinct populations of already-committed progenitors in response to intrinsic and extrinsic signals, rather than only to a change in the differentiation pattern of multipotent progenitors (reviewed in, Kriegstein and Alvarez-Buylla, 2009; Gonzalez-Perez and Quinones-Hinojosa, 2012). This observation also prompts the important question of whether it is possible to distinguish progenitors with distinct differentiation capabilities by means of appropriate molecular markers. Indeed, distinct types of NPCs have been distinguished by features of cellular morphology, molecular expression, and mitotic divisions as well daughter cell fates. Technical advances on single-cell transcriptomics techniques which can be used to profile gene expression in individual cells, and uniquely classify neural cell types based on combinatorial gene expression, deliver resources to expand our understanding of the genetic programming underlying cortical development (reviewed in, Poulin et al., 2016).

NPCs differ from RG in the terms of gene expression: they do not express astroglial markers and they show decline in the gene expression pattern of *Pax6* and *Hes* transcription factors (Englund et al., 2005; Cappello et al., 2006). Types of NPCs can be positively identified by the expression of T-box transcription factor (Tbr2) (Englund et al., 2005), cut-like 1 and 2 (*Cux1* and *2*) (Nieto et al., 2004; Zimmer et al., 2004; Conti et al., 2005), *VGlut2* (Schuurmans et al., 2004), *Satb2* (Britanova et al., 2005) and the non-coding RNA subventricular-expressed transcript 1 (*Svet1*) (Tarabykin et al., 2001). As well *Mash1* and *Ngn1* transcription factors have been used to mark NPCs (Landgren and Curtis, 2011). All markers are reviewed in, (Zhang and Jiao, 2015). However, the probable identification of distinct subpopulations of NPC types which would produce only cells with certain highly specific differentiated phenotype or laminar fate is still unachieved, and currently only larger subpopulations of progenitors have been recognized with their appropriate specific markers. Recent advancement on the functional genomics field, the (CRISPR)-associated protein 9 (Cas9) system, that is a powerful tool for editing DNA at specific loci, offers the opportunity to infer cell lineage from gene expression trajectories and this technology can be used to capture a highly detailed picture of cortical cell fate decisions and shall revolutionize developmental studies (Zhang et al., 2018; Ben Jehuda et al., 2018).

3.2.2. IPC cell division and generation of neuronal cells

IPCs which are a type of transient amplifying cells or intermediate progenitors, also known as basal progenitors, divide primarily, in contrast to asymmetrically dividing RG cells, symmetrically at basal, abventricular locations at the SVZ and to some extent in the intermediate zone (IMZ) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; 2007; Wu et al., 2005). However, some IPCs are also present within the VZ, mainly at early time of neurogenesis, prior to the formation of a distinct SVZ (Noctor et al., 2007). Indeed, this mixture of RG cells and IPCs within the VZ may explain previous observations of both RG cells and non-RG cells undergoing mitosis in the embryonic VZ (Levitt et al., 1981; Misson et al, 1988; Gal et al., 2006). IPCs have limited proliferative capacity, from 1-3 mitotic cycles in mouse and they lack the ability for INM (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Importantly, the proportions between apical and basal progenitors and their subpopulations vary during development in different stages and regions and between species and partly depend on the rounds of amplification that IPCs undergo before differentiation. The different populations show heterogeneity in their cell cycle

length, a parameter that is thought to be closely related to their differentiation pattern (Calegari and Huttner, 2003; Calegari, et al., 2005).

Intriguingly, the primate brain which has an expanded abventricular proliferative zone, namely the outer SVZ (Smart et al., 2002), contains a vast number of mitotic cells during development which have been suggested to be IPCs and could contribute to the enormous cortical expansion observed in primate cortex (Kriegstein et al., 2006; Molnar, 2011; Taverna et al., 2014). In fact, it has been shown that many RG-like cells, which have a long basal process but are non-epithelial as they lack contact with the ventricular surface, populate with IPCs the human outer SVZ (Hansen et al., 2010). This establishment of non-ventricular RG-like cells together with their daughter cell IPC progeny may indeed in part explain the increased cortical size and complexity in the human brain (reviewed in, Molnar et al., 2019).

IPC genesis and amplification are regulated with various extrinsic and intrinsic factors such as signals from postmitotic neurons and thalamocortical axons, diffusible morphogens and sequential gene expression programs. In the embryonic brain, similarly to NSCs, IPCs divide near blood vessel branch points suggesting that cerebral vasculature is specifically crucial for proper patterning of neurogenesis and establishes a niche for NSCs and NPCs in the SVZ (Javaherian and Kriegstein, 2009). IPCs can generate neurons (nIPCs) or glial cells, including astrocytes (aIPCs) or oligodendrocytes (oIPCs) whose production differs a bit from others.

Neurogenic IPCs are most prominent during middle and late stages of neurogenesis (E14-E17 in the mouse) when they accumulate at the SVZ although nIPCs are present throughout the entire period of cortical neurogenesis (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Importantly, these nIPCs can be positively identified with Tbr2 transcription factor (Englund et al., 2005). In addition to their own limited self-renewal, nIPC produce neurons to all cortical layers during development (E10.5-P0.5 in the mouse) (Sessa et al., 2008; Kowalczyk et al., 2009). Moreover, an individual neurogenic IPC can contribute neurons to multiple cortical layers (Luskin et al., 1988). A transcription factor expression sequence of *Pax6*, *Ngn2*, *Tbr2*, *NeuroD* and *Tbr1* in developing cells which ultimately mature to glutamatergic pyramidal projection neurons has been found in addition to SVZ in the hippocampus and persist throughout adult neurogenesis and with modifications also in the developing cerebellum (Hevner et al., 2006). Likewise, Tbr2 positive nIPCs has been shown to play a major role in the regulation of cortical SVZ and adult hippocampal neurogenesis (Arnold et al., 2008; Hodge et al., 2008). While the exact functional significance of this cell lineage sequence remains unidentified, its common emergence in embryonic and adult neurogenesis, and in different brain regions, suggests it being a part of a conserved genetic program that specifies general properties of glutamatergic neurons and fascinatingly that a similar transcriptional program controls neurogenesis in the embryonic SVZ as in the adult SGZ (Hevner et al., 2006; Hodge et al., 2008) for review, see (Pontious et al., 2008; Hevner, 2019).

3.3. Astrocytogenesis and oligodendrocytogenesis

Astrocytes and oligodendrocytes are originally NE cell-derived, generated from RG with major gliogenic peak following neurogenesis and subsequently oligodendrocytogenesis at early postnatal stage and represent the principal types of macroglial cells found throughout the mature CNS (reviewed in Farhy-Tselnicker and Allen, 2018). Whereas the other group of glia; microglia are hematopoietic in origin and will not be introduced here in detail since they are out of scope of this thesis. Astrocytes, a major type of glial cell, are important regulators of synapse formation and function during development (reviewed in Farhy-Tselnicker and Allen, 2018). Ultimately, mature astrocytes provide structural support for neuronal structures in the brain, regulate water balance

and ion distribution, and maintain the blood–brain barrier. Additionally, they play important part in cell-cell signaling by regulating Ca^{2+} flux, releasing d-serine, generating neuropeptides and modulating synaptic transmission. Astrocytes represent a diverse population of cells with complex and functionally diverse dynamics and functions in specific neural circuits (reviewed in, Khakh and Sofroniew, 2015). Astrocytes are generated directly from RG and indirectly through aIPCs. Like previously mentioned, majority of RG cells also directly convert to astrocytes during late embryonic development (for reviews, see Ihrie and Alvarez-Buylla, 2008; Kriegstein and Alvarez-Buylla, 2009).

The glial cells are a diverse group of different types of cells which can be distinguished based on their morphological characteristics and expression of several different markers. To mention few key ones, astrocytes express astrocyte markers called aldehyde dehydrogenase 1 family member L1 (ALDh1L1), RC1, RC2 and GFAP (for reviews, see Rowitch and Kriegstein, 2010; Chandrasekaran et al., 2016). Mature astrocytes can be roughly divided into two groups: fibrous "star-like" astrocytes that populate white matter and protoplasmic astrocytes that are found in the grey matter and possess more irregular processes and typically fewer glial filaments than the fibrous ones. These cells contact neuronal cells and ensheath synapses with their extended thin processes, some of which also contact blood vessels. Interestingly, it has been suggested that protoplasmic and fibrous astrocytes might arise also from independent progenitor pools (Rowitch and Kriegstein, 2010). However, this has not yet been fully elucidated, but provides one possible control mechanism in the generation of diverse glial cell types along with spatial and temporal regulation. Indeed, astrocytes display an astounding diversity in terms of positional identity, partly inherited from their RG ancestors (Bayraktar et al., 2014; John Lin et al., 2017). Glial cells also maintain some of the original patterning information from their RG ancestors making them an attractive target for neural reprogramming (Falk and Götz, 2017; Grade and Götz, 2017, Barker et al., 2018, Wang and Zhang, 2018, Mattugini et al., 2019). Interestingly, similar regional specification for production of astrocytes (Hochstim et al., 2008) and oligodendrocytes (Kessaris et al., 2006) has been shown as earlier for neurons (Desai and McConnell, 2000). However, recent study of the development of cortical astrocytes discovered that local environment likely determines clonal expansion of astrocytes and their final morphotype, suggesting that non-specified astrocyte progenitors produce plastic, intermixed clones, whose daughter cells may adopt different morphotypes through interactions with their environment (Clavreul et al., 2019).

RG produce oIPCs also named oligodendrocyte precursor cells (OPCs) to generate oligodendrocytes (Fogarty et al., 2005; Casper and McCarthy, 2006). OPCs can originate from numerous locations during development (Kessaris et al., 2006; Menn et al., 2006). The OPCs preserve the proliferative capability during development and in the adult and they may remain quiescent at their locations and proliferate symmetrically in response to local signals (Noble, 2000; Aguirre et al., 2007; Barres, 2008; Rivers et al., 2008). Therefore, differing from other IPCs that actively proliferates in the VZ or SVZ. In adult SVZ, oligodendrocytes are produced by two independent pathways. At first, the type B cells in the SVZ which produce transit-amplifying cells (known as type C cells) produce OPCs in addition to neurons (for reviews see, Götz and Huttner, 2005; Ihrie and Alvarez-Buylla, 2008; Rowitch and Kriegstein, 2010). These OPCs then generate oligodendrocytes and OPCs which travel to populate the white and grey matter while remaining the ability to produce new oligodendrocytes in their location. Interestingly, it has been shown that OPCs form synapses with neurons, suggesting even greater degree of complexity than previously anticipated in the interactions between neurons and oligodendroglia in the mammalian brain (Lin and Bergles, 2004). Oligodendrocytes also support neuronal functions, by providing the electrical insulation around neuronal axons by myelin sheath formation.

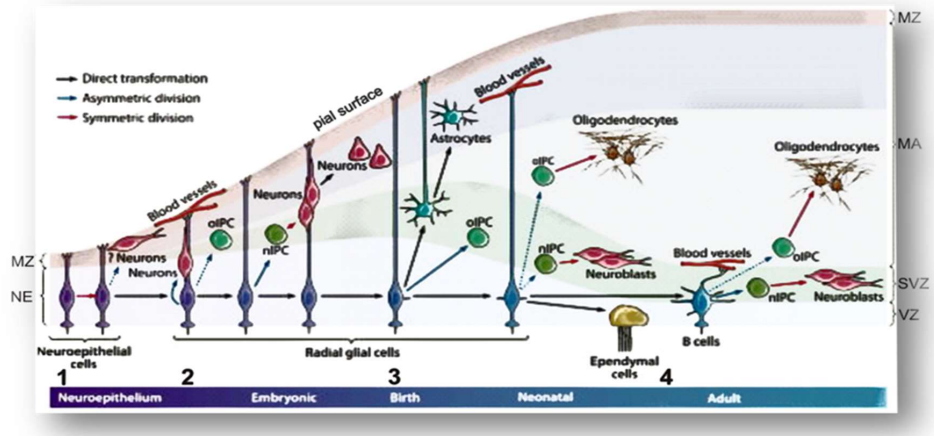

Figure 3. *An overview into the generation of neuronal cells from neural stem and progenitor cells in the mouse neocortex throughout development and in postnatal and adult neurogenesis* 1) At the expansion phase of neocortical formation, neural stem cells (NSCs) are neuroepithelial (NE) cells that produce the first neurons into the marginal zone (MZ) with asymmetric divisions and through symmetric divisions increase their own cell numbers at the apical locations of the ventricular zone (VZ). 2) On the neurogenic phase of the cortical development, some NE cells convert to radial glial cells (RG) due the cell elongation as the brain epithelium thickens along with conversion of cell junctions and establishment of endothelial cell contacts near the pial surface. These RG NSCs produce most neuronal cells, either directly through asymmetric divisions or indirectly by producing types of progenitors, which divide symmetrically at basal locations, in the subventricular zone (SVZ). RG cells also take part in guiding the migration of neurons to their intended cortical layers through their long radial fiber processes. 3) During late neurogenic phase, most of the RG cells convert through direct transformation into astrocytes. However, a population of RG cells retains the RG identity (apical contact and ability to interkinetic nuclear migration) and thus continues to function as NSCs in the postnatal brain. 4) At postnatal phase, some RG cells convert to ependymal cells and some to type B cells, also known as SVZ astrocytes, which continue to function as NSC in the adult SVZ. B cells produce IPCs through asymmetric divisions. Neural IPCs generate through symmetric divisions immature neuroblasts which migrate through the rostral migratory pathway to the olfactory bulb and differentiate into interneurons. MA, mantle. Modified illustration reproduced with permission from Kriegstein and Alvarez-Buylla, 2009.

3.4. Corticogenesis, the development of cortical structure

The adult neocortex consists of six layers, into which two main types of neural cells; the pyramidal neurons and cortical interneurons are organized during development (reviewed in, Molnar et al., 2006; Molnar, 2011). Pyramidal excitatory projection neurons synthesize neurotransmitter called glutamate and extend their processes from the area in which their cell body is located to distant parts of the CNS. Whereas, inhibitory interneurons, a diverse group of cells that display a range of morphologies and molecular identities, synthesize GABA and neuropeptide(s), and their processes mainly remain within the boundaries of their area of the cortex.

Cortical development involves the formation of layers and several discrete areas that uniquely process different kinds of information and these areas are characterized by their specific networks, inputs and outputs (for review, see Sur and Leamey, 2001). Endless interplay between intrinsic and extrinsic factors at all stages of development affects this process. The specification of cortical layers and later on in the development; areas and internal networks, requires multiple cues that involve regional and/or graded expression of molecules along with spatial and temporal signals which are in part regulated by thalamic afferents and patterned electrical activity (for review, see Sur and Leamey, 2001). Nevertheless, the process of neocortical development and layer and area

specification is not yet fully understood but it is crucial for understanding the brain function and to the development of treatments for neurological diseases. Various transcription factors have been found to be expressed in specific subsets of cells or layer specifically and can be therefore used as their markers (for reviews see, Guillemont, 2007; Molyneaux et al., 2007). One particularly interesting is the transcription factor ER81, which is specifically expressed in cortical layer V neurons (de Launoit et al., 1997; Yoneshima et al., 2006).

3.4.1. Formation of six-layered structure

The six layers of the neocortex are generated during development from heterogeneous population of RG NSCs and NPCs present in the VZ and SVZ. The time of neurogenesis in the proliferative zones regulates the laminar location (Angevine and Sidman, 1961; Rakic, 1974; Luskin and Shatz, 1985). Numerous birthdating studies, using nucleotide analogues such as tritiated thymidine and BrdU, which incorporate themselves into newly synthesized DNA, show that the six-layered structure is basically formed in inside out manner, where cells which exit the cell cycle early form the deeper cortical layers, whereas cells which are born later, form progressively the more superficial layers. The first cohort of cells to leave the VZ forms a structure called the preplate (PP) which will eventually form the superficial layer I of the cortex. The second wave of migrating cells splits the PP into two layers, called the marginal zone (MZ; layer I) and the subplate (SP), and the subsequent waves of migrating cells then form cortical plate (CP) structures and ultimately layers II-VI. The innermost layer VI is formed first and afterward upper layers V, IV, and last II/III, meaning that each wave of cells must migrate past the ones before to reach their correct cortical position (Rakic, 1972). The intermediate zone (IMZ), which forms between the SP and SVZ, begins to fill with the processes which extend from cells that have already settled in the developing cortex. The white matter tracts in IMZ connect the cortical cells from different regions to each other and as well to subcortical structures.

See **Figure 4** for a generalized overview of the cortical layer formation.

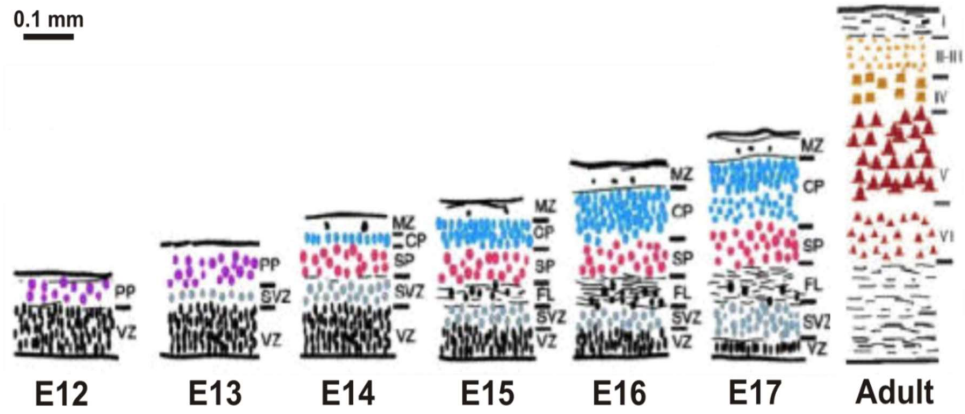

Figure 4. *Picture representation of cortical layer formation in mouse.* At embryonic day (E) 12, preplate (PP) structure which eventually forms marginal zone (MZ) or layer I of the neocortex is already formed by waves of migrating cells which have exited the mitotic cell cycle in the ventricular zone (VZ). Subsequent day of cortical development, a subventricular zone (SVZ) which is another distinctive proliferative region above VZ is clearly distinguished. Next waves of migrating cells split the PP into two layers: MZ and subplate (SP). As well fibre layer (FL) or structure known intermediate zone (IMZ) is formed when the cells which have already settled to their correct cortical position in the developing cortical plate (CP) extend their processes between SP and SVZ. Cortical layers II-VI are formed in an inside out fashion, where cells must migrate past earlier migrated ones before reaching their correct cortical position. Therefore, meaning that the deepest layer VI is formed first, then V, IV, III and upper layer, II last. Illustration reproduced with permission from Molnar et al., 2006.

3.4.2. The dependence of layer specification on progenitor zone heterogeneity

The dynamics of cell cycle show regional variation and contribute to the neocortical layer specification (Dehay et al., 1993; Polleux et al., 1997; Kohwi and Doe, 2013). Interestingly, there seems to be a progressive restriction of cell fate already in the precursor population: early progenitors can give rise to neurons which can ultimately reside any cortical layer, whereas later born progenitors can give rise only to neurons of more superficial layers (Luskin et al., 1988; Price and Thurlow, 1988; Desai and McConnell, 2000). The laminar fate of a neuron is thought to be sealed in the late S or early G2 phase of the final round of mitosis and just before this, during the S phase of the final mitotic division the cell fate is thought to be plastic and therefore could be affected by various environmental cues (McConnell and Kaznowski, 1991). The nature of these extrinsic factors varies depending on the area and stage of development. Also, the commitment to certain neuronal phenotype can be influenced with those extrinsic signals which are present during the final cell cycle (Eagleson et al., 1997). Additionally, the intrinsic gradients of gene expression are thought to regulate the initial arealization of the neocortex (Walther and Gruss, 1991, Stoykova and Gruss, 1994; Gulisano et al., 1996; Bishop et al., 2000).

Indeed, the initial broad specification of the cortex is regulated by intrinsic molecular determinants that are present already in the proliferative zones (Rubenstein et al., 1999; Long et al., 2009). It has been shown that different types of neurons are derived from RG in different subregions of the VZ and timing affects this process. This segregation of progenitor zones has been observed in the developing forebrain, where discrete areas were associated with distinct transcription factor expression (Campbell, 2003; Guillemot, 2005; Flames et al., 2007; Long et al., 2009). Therefore, depending on the set of transcription factors expressed, RG cells are highly heterogeneous in their progenitor function. *Pax6, Emx1, Gsh1, Gsh2, Er81, Sp8, Nkx2.1, Dlx1, Dlx2, and Olig2* have been implicated in the generation of different subsets of forebrain neurons (for reviews see, Guillemot, 2007; Molyneaux et al., 2007). However, these likely represent just a small subset of a larger group of transcription factors (Long et al., 2009) that, together with proneural genes such as *Ngn2* and *Mash1* (Guillemot, 2007) mostly determine the specific cell subtype generated from RG NSCs and NPCs. In fact, the RG NSCs and NPCs in different locations undergo a stereotypic sequence by which specific types of neurons are produced at different developmental stages (Desai and McConnell, 2000) and this phenomenon appears to be cell-autonomous (Shen et al., 2006). Intriguingly, similar regional specification pattern for production of oligodendrocytes (Kessaris et al., 2006) and astrocytes (Hochstim et al., 2008) has been shown and furthermore, astrocytes take cortical positions that mirror the inside-out laminar birthdate pattern of cortical neurons with later-born glia positioning to more superficial cortical layers (Ichikawa et al. 1983).

As combined, to produce the enormous diversity of neuronal types within the forebrain the NE cells, RG NSCs and NPCs are obliged to interpret the changing environmental cues and in response unfold unique programs of transcription factor expression. Consequently, these findings indicate that location and timing dependent heterogeneity in RG NSC function is involved in specifying the functional subdivisions of the mature cortex and, those events that occur in the VZ can contribute to regional differences in the cortical cytoarchitecture and thus, a proto map of the cortex may already exist in the VZ as suggested by Rakic already in the 80's (Rakic, 1988).

4. Cell fate determination of NSCs and NPCs

In developmental biology one major aim is to comprehend exactly how the fate of particular cell type is determined and how this cell type develops into the final, specific, highly differentiated cell. Cell proliferation, specialization, interaction and movement are the four key processes taking part in cell fate determination at the cellular and tissue level and ultimately in creating the whole living

organism. The determination of a cell to a particular fate can be divided into two major states: [1] specified (committed) state and [2] determined state. In the specified or committed state, the cell type is not yet determined and any bias the cell has toward a certain fate can be reversed or transformed to another fate. In contrast to committed state, in a determined state the fate of the cell cannot be reversed or transformed. In general, this means in natural conditions that a cell determined to differentiate into a neural cell cannot be transformed to for example a fibroblast cell of the skin. The determination is followed by a cell differentiation, meaning the actual changes in biochemistry, structure, appearance and function that result in a specific cell type.

Even though quite a long time it was generally thought that primarily the microenvironment in which the cells settle and mature determines their neuronal phenotype, knowledge indicates that neuronal characteristics, including their unique morphology, are determined mainly by intrinsic cellular programs that are already established to a major degree as the new cells are generated (for review, see Kriegstein and Alvarez-Byulla, 2009). This revelation leads to a progression where continuous interplay with changing environmental cues and morphogen gradients direct the unfolding of unique intrinsic programs of transcription factor sets that in turn guide the alterations in the maintenance and fate determination which eventually lead to terminal proliferation, apoptosis or survival, migration and differentiation of NE cells, NSCs and NPCs derived cells (reviewed in, Kriegstein 2006; Guillemot et al., 2007; Molyneaux et al., 2007; Kawaguchi, 2019). Essentially, spatiotemporally controlled dynamic expression of a diverse transcription factor sets plays a central role in the process of cell fate determination and takes part in a correct cortical structure formation (for reviews, see Guillemont, 2007; Molyneaux et al., 2007). Additionally, various other intrinsic mechanisms, like epigenetic regulation by DNA methylation and histone modifications and non-coding RNAs (reviewed in, Sanosaka et al., 2009; Ma et al., 2010) along with extrinsic ques have been implicated in the fate determination of NSCs and NPCs. In fact, a huge variety of different neurotransmitter, cytokine and growth factor receptors are expressed and functional in progenitors and maturing neuronal cells (reviewed in, Mattson, 2008; Hagg, 2009; Nakamichi et al., 2009; Young et al., 2011).

To control the correct structure formation and cell numbers and to prevent depletion of the NSC pool or on the other hand tumor formation several events need to take place. The self-renewal, proliferation, survival and conversely apoptosis, as well maturation and migration of NSCs and NPCs must be strictly controlled with the surrounding microenvironment and intrinsic sequential expression of diverse transcription factor sets (reviewed in, Kriegstein 2006; Guillemot et al., 2007; Molyneaux et al., 2007; Kawaguchi, 2019).

5. Modes and control of neural migration in the developing telencephalon

The migrating neurons find their place in the six neocortical layers in an inside out fashion and mature during this process to exhibit various neural phenotypes. Three major types of migration have been implicated in corticogenesis: [1] Cajal-Retzius (CR) cell migration, [2] radial migration of mainly excitatory neural precursor cells of the cortical VZ and [3] tangential migration of interneurons from the ganglionic eminence (GE) of the ventral telencephalon (reviewed in McConnell, 1988; Marin and Rubenstein, 2003; Guillemot et al., 2006; Huang, 2009).

The locomotion of neural cells involves three basic processes and consists of two discrete steps which are coupled through centrosome mediated manner: [1] growth of the leading process and followed by [2] nuclear and cell body translocation. At first, the cell extends a leading process which explores the environment for guidance cues that provide information about the direction of migration. This process involves the reorganization of the cytoskeleton and can be prevented by

blocking the polymerization of actin filaments. Then the nucleus of the cell moves into the leading process in a microtubule-dependent manner (this process is called nucleokinesis). Finally, the cell retracts its trailing process, and the sequence of these events starts again (reviewed in, Tsai and Gleeson, 2005). The cell motility includes these similar steps in both; radial as in tangential migration (Kriegstein and Noctor, 2004; Marin et al., 2006).

5.1. Radial migration

During corticogenesis pallial neocortical neurons migrate radially in a ventral to dorsal direction from the VZ towards the pial surface (Rakic, 1971; Marin and Rubenstein, 2003). There are at least two modes of radial migration that cortical neurons use to transport their cell bodies: [1)] somal translocation and [2)] glia-guided locomotion (Borrell et al., 2006; Rakic, 2007). Cells undergoing somal translocation have a long, branched leading process which contacts with the pial surface and a short and transient trailing process. These cells move continuously during their migration and the pial process progressively thickens and shortens as the cell proceeds on its voyage to correct laminar position. The other type of radial migration is glia-guided locomotion which occurs along radially aligned fibers of RG cells which have their cell bodies in the VZ, and their fibers span the thickness of the neural tube, thus providing a scaffold for migrating neurons (Miyata et al., 2001; Noctor et al., 2008). After exiting the cell cycle in the VZ, the cells recognize their migratory substrate, the glial fibre and adhere to it via various cell surface receptors, and afterward also the microenvironmental cues are used to instruct the direction of migration. Cells migrating by this mechanism have a short leading process which does not encounter the pial surface, and they move slowly, with short migratory bursts interrupted with pauses. Once they approach the final stages of their journey into the correct laminar position, the cells detach themselves from the radial fibers and adopt the somal translocation migratory mode (Marin and Rubenstein, 2003). During radial migration some migrating cells make also retrograde moves toward the ventricle before heading for the CP, as well tangential moves are frequently seen and some cells acquire a transient multipolar phenotype while migrating (Tabata and Nakajima, 2003; Noctor et al., 2004).

5.2. Tangential migration

The GABAergic interneurons are at first specified in the medial or caudal ganglionic eminence (MGE; CGE) of the ventral telencephalon also called subpallium, and afterwards subsequently invade the cerebral cortex through a long-distance migration by using two well-defined tangential migratory routes located in [1)] MZ and [2)] the SVZ/IZ and then integrate into the CP via radial migration (Marin and Rubenstein 2001; Polleux, 2002; Batista-Brito and Fishell 2009). Their migration from the MGE and CGE to the MZ and IZ take place probably travelling along the tangentially arranged axonal bundles of the developing corticofugal fiber system, whereas cells destined for the CP might continue along the axons of the pioneer cells of the MZ that span the thickness of the developing cortex (Parnavelas, 2000). Like radially migrating cells, interneurons also adopt an inside-out pattern, with early waves of migrating cells populating the deepest layers of the developing neocortex. Once these cells reach the cortex, they actively migrate toward the VZ, upon their arrival; they pause for a while, before migrating back towards the pial surface and settlement to their final positions. It is still unclear why interneurons undergo this ventricle-directed migration, but it has been suggested that this is done to acquire positional information to instruct their arrangement to correct cortical layer. Tangentially migrating cells can acquire a variety of morphological appearances with branched, short or long elongated processes (Marin, 2006).

5.3. Regulation of migration

Radial migration and neuron-RG interactions during cortical development are controlled by a variety of factors, however the molecular mechanisms are still poorly understood. Particularly, a glycoprotein called Reelin which is secreted by Cajal–Retzius cells positioned strategically in the MZ is crucial for proper radial neuron migration and cortical layer formation. Reelin signaling is controlling cell-cell interactions and cell positioning in the developing cortex through Notch and various cytoplasmic proteins that may link Reelin signaling to cytoskeletal changes (for review, see Gaiano, 2008). As well calcium-signaling through activation of various neurotransmitter, cytokine and growth factor receptors plays a part in orchestrating neuronal migration in a cortical layer-specific manner (for reviews, see, Komuro and Kumada, 2005; Young et al., 2011). One of the most common classes of cortical malformations due defects in cell migration is classical lissencephaly and a vast majority of these cases can be attributed to mutations in one of two genes: *LIS1* and *Doublecortin* (*DCX*) (Hattori et al., 1994; des Portes et al., 1998; Gleeson et al., 1998).

Tangential migration shares some similarities with radial migration, both include cyclic steps of leading process extension and nuclear translocation, thus some regulatory conservation seems likely. Indeed both; *Lis1* and *Dcx* genes are required as well for tangential interneuron migration (McManus et al., 2004; Kappeler et al., 2006; Friocourt et al., 2007). In addition, the homeobox genes *Dlx1* and *Dlx2* are well known to be involved in the migration of interneurons, but also play a part in the maturation of interneuron progenitors (Anderson et al., 1997; Cobos et al., 2007). The role of *Dlx1/2* in regulating interneuron migration depends on its ability to restrain neurite outgrowth and these effects are mediated by *Dlx1/2* dependent repression of several genes involved in regulating cytoskeletal dynamics, such as PAK3 and MAP2. Neuregulin-1 (NRG1), a molecule with various other functions in neural development, plays a major role in guiding the dorsal migration of maturing interneurons by acting as a chemoattractant and its membrane bound NRG1 isoform provides a permissive passageway along the SVZ from lateral GE (Flames et al., 2004), whereas Sema3A and 3F prevent inappropriate interneuron invasion into the developing striatum (Marin et al., 2001).

Additionally, a vast number of cortical motogenic factors that promote interneuron motility have been implicated in the regulation of interneuron migration. These include: BDNF and NT-4 (Polleux et al., 2002), glial-derived neurotrophic factor (GDNF) (Pozas and Ibanez, 2005), hepatocyte growth factor (HGF) (Powell et al., 2003) and Cxcl12. Interestingly, the chemokine Cxcl12 is highly expressed in the meninges and along the SVZ/IZ boundary (two main streams of tangential interneuron migration in the CP), whereas its receptor, Cxcr4 is expressed in interneurons. Studies have shown that Cxcl12 signaling regulates not only tangential interneuron migration but changes in Cxcl12 responsiveness are responsible for controlling the timing of interneuron invasion to CP and a delayed entry through Cxcr4 receptor expression is essential for their proper integration into the cortical circuitry (Stumm et al., 2003; Tiveron et al., 2006; Lopez-Bendito et al., 2008). Intriguingly, very recently it was shown that glutamatergic Tbr2 expressing nIPCs regulate the cerebral cortex expansion by controlling the amplification of pallial glutamatergic neurons and with attraction of the subpallial GABAergic interneurons into the cortical SVZ migratory route through a non-cell-autonomous mechanism (Sessa et al., 2010). In fact, the Tbr2 interneuron attractive activity was moderated by Cxcl12 signaling which forced expression in the Tbr2 mutant mice could to some extent rescue defective SVZ cell migration (Sessa et al., 2010). These findings thus suggest that nIPCs can control simultaneously the increase of glutamatergic and GABAergic neuronal pools and intrinsically balance their relative accumulation to the developing neocortex by the means of Cxcl12 signaling.

6. NSCs and NPCs in neurosphere model *in vitro*

NSCs and NPCs were initially isolated from the CNS of embryonic mammalian (rat, mouse) (Temple, 1989; Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993). Subsequently, NSCs and NPCs were propagated from adult animals (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993). In addition to embryonic NSC extraction from the ventricular wall of lateral ventricles, adult NSCs and NPCs have been isolated and cultured from other mammalian brain regions, including caudal portions of the SVZ, cortex, striatum, olfactory bulbs, hypothalamus, septum, corpus callosum, spinal cord, optic nerve, and retina (Palmer et al., 1995; Weiss et al., 1996; Shihabuddin et al., 1997; Palmer et al., 1999; Pagano et al., 2000; Tropepe et al., 2000; Lie et al., 2002). Neural precursors or NSCs and NPCs isolated from the subventricular wall of lateral ventricles can be grown as free-floating cell cultures and kept on mainly proliferative stage with the presence of instructing mitogens; epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) (Reynolds and Weiss, 1992). These single cells form upon proliferation cell aggregates also known as neurospheres; a heterogeneous mixture of undifferentiated original NSCs, NPCs, and dying cells and likely a small number of neuronal cells at more mature stages of differentiation (reviewed in, Jensen and Parmar, 2006). When differentiated *in vitro*, with the withdrawal of the mitogens, the cells start to migrate out from the neurospheres and differentiate into neurons and glia whilst some cycles of cell divisions are seen during this process. This resembles the *in vivo* process of generating neurons in a precise temporal and laminar order (Caldwell et al., 2001; Shen et al., 2006). Single cells isolated from E10 mouse cortex and grown in cell culture are multipotent and sequentially generate neuronal and then glial-restricted progenitors, resembling the *in vivo* developmental order (Qian et al., 2000). Similarly, embryonic SCs can be turned into NSCs that are capable to generate first neurons and then astroglial cells and moreover, sequentially over time produce subtypes of neurons whose identities match the layer-specific temporal pattern seen *in vivo* (Gaspard et al., 2008).

7. NSCs in disease and therapy

NCSs or NPCs can be exploited to study a variety of disease models which carry an identified pathological defect. Especially for neurodevelopmental disease research, it may be of great use to investigate the effects of certain pathological abnormality at different developmental stages and therefore might lead to revelation of the underlying mechanisms causing these defects (Martinat et al., 2004; Singec et al., 2007). Additionally, NSCs have been considered for use in cell replacement therapies in various neurodegenerative diseases. Alterations of specifically in hippocampal adult neurogenesis which have been associated to hippocampus-dependent learning, memory and emotion (Shors et al., 2002; Paizanis et al., 2007; Zhao et al., 2008), have been implicated in several neurological disorders. Thus, restoring defected NSC functions and potentially enhancing hippocampus-related learning and memory might be important targets for gene therapy. Alterations in NSCs functions or neurogenesis have been associated for example with FXS (reviewed in, Callan and Zarnescu, 2011; Li and Zhao, 2014), neurodegenerative diseases such as Huntington's, Parkinson's and Alzheimer's (Winner et al., 2011), epilepsy (Parent et al., 2007) and seizures (Kokaia, 2011). In addition, a link has been found between aberrant NSC functions and depression (Dranovsky and Hen, 2006), neuroinflammation (Monje et al., 2003) and with brain tumor formation (Palm and Schwamborn, 2010). Furthermore, unanticipated and potentially valuable NSC characteristics for use in cell replacement therapies is their migratory capacity and the fact that they appear to be attracted to areas of brain pathology such as ischemic and neoplastic lesions (reviewed in, Lederer and Santama, 2008). As a conclusion, NSC based cell therapies may prove valuable in treating various CNS disorders.

8. Principles of calcium signaling

Calcium is essential for living organisms, particularly in cell physiology, where the movements of the calcium ions (Ca^{2+}) into and out of the cytoplasm functions as signals for many cellular processes. Ca^{2+} plays an equally central role in practically every cell type, from growth to movement and apoptosis, and hence is vital for both: non-excitable and excitable cells, such as neurons. As such, it is tightly regulated in all cell types, by membrane and cytoplasmic proteins that control the influx and efflux of Ca^{2+} as well as buffering cytoplasmic $[Ca^{2+}]_i$. Ca^{2+} influx pathways can be activated by several plasma membrane receptor families, including both ionotropic and metabotropic G-protein coupled receptors, that directly or indirectly open Ca^{2+} permeable ion channels, voltage-gated calcium channels (VGCCs), store operated calcium channels, or tyrosine kinase receptor family members (for reviews see, Berridge and Lipp, 2000; Berridge et al., 2003; Clapham, 2007). Furthermore, Ca^{2+} discharged from internal stores, i.e the endoplasmic reticulum (ER), can also modulate the cytoplasmic $[Ca^{2+}]_{i..}$ Such pathways utilize the production of inositol metabolites that induce the release of $[Ca^{2+}]$ through IP_3 receptors (for review, see Padamsey et al., 2019). A classic example is G-protein coupled receptors linked to Gαq signaling (Gαq – PLC – IP_3).

The induced local and global alterations in $[Ca^{2+}]_i$ has an essential role in the differentiation process of NPCs (Ciccolini et al., 2003) and in determining the neurotransmitter phenotype of the developing NPCs (Spitzer et al., 2004; Rosenberg and Spitzer, 2011). For example, several studies have revealed that neurotransmitters play a vital role in neuronal differentiation and cell migration in the developing telencephalon by activating G-protein-coupled receptors (GPCRs) or ionotropic receptors (iGluRs) to cause local or global changes in $[Ca^{2+}]_i$ (Emerit et al., 1992; Nguyen et al., 2003; Hagg, 2009; Young et al., 2011). Additionally, several neurotransmitters and their activated receptor activation pathways have been associated in the control of neurogenesis (reviewed in, Hagg, 2009; Young et al., 2011). These Ca^{2+} transients and intracellular fluctuations induced by activated ion channels and metabotropic receptors are also implicated to play a major role in neurogenesis and controlling neuronal migration in a cortical layer-specific manner (for reviews, see, Komuro and Kumada, 2005; Young et al., 2011).

9. Neurotransmitters and their receptors

Neurotransmitters are chemical messengers produced by the nervous system in order to transmit a nerve impulse from one neuronal cell to another. Neurotransmitters include small molecules with functional amine groups, such as acetylcholine (Ach), certain amino acids, amino acid derivatives, peptides and others like adenosine triphosphate (ATP). For example, amino acid tyrosine is converted into the catecholamine neurotransmitter; dopamine and norepinephrine (NE) or into epinephrine through series of chemical reactions. Other examples derived from amino acid derivatives include for example: γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain, which is made from glutamate using the enzyme L-glutamic acid decarboxylase and pyridoxal phosphate (an active form of B6 vitamin). Another one is serotonin which is made from tryptophan and is important in the regulation of mood, appetite, sleep, as well as muscle contraction. Peptide neurotransmitters include among other endorphins, encephalins and substance P (SP).

See **Figure 5** for a generalized summary of neuronal synaptic transmission.

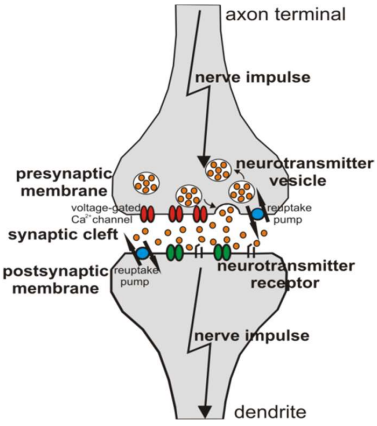

Figure 5. *Basic illustration of synaptic transmission between neurons*. Neurotransmitters are released to synaptic cleft from neurotransmitter-containing vesicles in the presynaptic axon terminal due to action potentials opening voltage-gated Ca^{2+}-channels. The released neurotransmitter causes receptor activation at the postsynaptic membrane of the contacted dendrite.

9.1. Ligand gated ion channels

Some neurotransmitters are referred to as fast-acting because their cellular effects occur only few milliseconds after the substance binds to its target receptor. These neurotransmitters use direct control of ion channels by inducing a conformational change in the receptor (for review, see Unwin, 1993). These receptors are often called ligand-gated ion channels (i.e., ionotropic receptors) since the channel opens only when the ligand is bound correctly. Direct chemical gating of ion channels can produce rapid, transient changes in neuronal activity, which may then lead to long-term changes in neural cell function and ultimately to structural changes of dendritic synaptic structures (for example changes in N-methyl-D-aspartic acid (NMDA) receptor function).

9.2. G-protein coupled receptors

Slower-acting neurotransmitters operate by binding to proteins commonly called GPCRs (for review, see Fredriksson et al., 2003). The conformational change produced upon ligand binding, causes the G-protein to become activated. When activated, the G-protein subunits dissociate and diffuse along the intracellular membrane surface to open or close an ion channel or to activate or inhibit an enzyme that will, in turn, produce a second messenger. Activation of second messenger systems allows a vast flexibility and diversity of responses, ranging from opening or closing of ion channels, through activation of enzyme systems to alteration in gene expression. Second messengers include Ca^{2+}, inositol-1,4,5-phosphate (IP_3) and diacylglycerol (DAG) as well as cyclic nucleotides, such as cAMP and cGMP (see figure 6). They activate enzymes known as protein kinases. Protein kinases in turn act to phosphorylate a variety of proteins within a cell. Protein phosphorylation is a common mechanism used in a cell to activate or inhibit the function of various proteins. Interestingly, GPCRs have been shown to be able to transactivate a family of tyrosine kinase receptors, known as v-erb-a-erthroblastic leukemia viral oncogene homolog (ErbB) family members (Ohtsu et al., 2006) which have been implicated in modulating NPC migration (Anton et al., 1997) via their endogenous ligands the NRGs.

9.3. ErbB receptors and their ligands

The family of ErbB receptors belong to the receptor tyrosine kinase superfamily and are composed of four subtypes: ErbB1-4. ErbB receptors become functional as dimers, and ErbB subtypes can form homodimeric or heterodimeric complexes (Yarden et al., 2001). The first step is ligand binding, which causes the transphosphorylation of the dimers followed by the recruitment of

effector proteins which contain phosphotyrosine binding sites for Src homology-2 (SH2) domains which in turn activate downstream signaling pathways (Mei et al., 2014). Ligands for ErbB1, also known as the epidermal growth factor (EGF) receptor, make up the EFG ligand family, which includes EGF, transforming growth factor alpha, amphiregulin, betacellulin, epiregulin, heparin-binding EFG-like growth factor, and epigen. Whereas NRG1-4 compose a family of ligands for ErBb2, ErbB3 and ErBb4. Different NRGs show varied affinities for ErbB3 and ErbB4, but none for ErbB2. As such, ErbB2 is indirectly activated by NRGs by interacting with other ErbB receptors. Interestingly, ErbB2 is the preferred dimerization partner for all ErbBs (Yarden et al., 2001). NRGs play a part in neuronal migration and RG integrity (Anton et al., 1997; Rio et al., 1997; Lopez-Bendito et al., 2006). NRG and ErbB4 receptors are expressed in RGCs of SVZ during embryonic development and NRG knock-down disturbs RG growth (Anton et al., 2004, Li et al., 2011), and further, selective knock-down of ErbB4 in RG disturbs neuronal migration and appropriate cell placement (Schmid et al., 2003).

9.4. Glutamate and glutamate receptors

Glutamate is the major excitatory neurotransmitter in the brain; glutamatergic transmission is mediated through two distinct classes of receptors; G-protein-coupled metabotropic glutamate receptors (mGluRs) and ligand-gated ionotropic glutamate receptors (iGluRs) (reviewed in, Conn and Pin, 1997; Niswender and Conn, 2010; Traynelis et al., 2010). Although glutamate will bind onto all different types of glutamate receptors (GluRs), each receptor is characterized by its sensitivity to specific glutamate analogues and by the features of their glutamate-elicited current. Developmental expression patterns of GluR subunits have been described (for review, see, Lujan et al., 2005). It has been proposed that different kind of GluRs might be important in the regulation of survival, proliferation and differentiation of developing CNS neurons. Indeed, glutamate seems to play a vital role in NPC fate determination. Glutamate enhances survival and proliferation of SVZ derived NPCs (Brazel et al., 2005). Furthermore, both iGluRs and group I mGluRs have been shown to support neurogenesis and neuronal survival for reviews, see Mattson, 2008; Nakamichi et al., 2009).

See **Figure 6** for a general illustration cell transmembrane receptors for glutamate neurotransmitter.

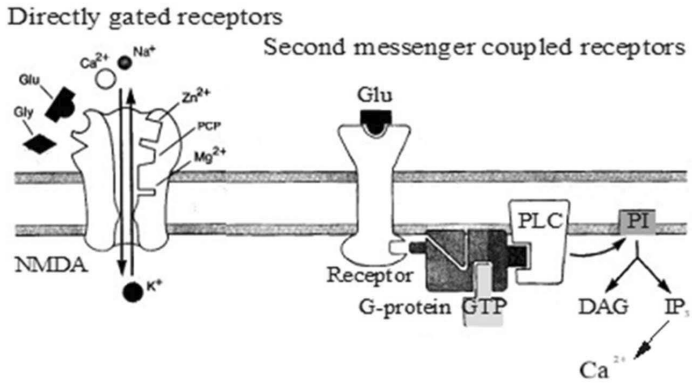

Figure 6. *Simplified picture representation of two different kinds of cell membrane receptors for neurotransmitter glutamate.* On the left side, is an example of directly ligand gated ionotropic NMDA receptor, showing different binding sites, ligands and ions which are involved in the receptor activation. On the right side, is an example of G-protein coupled receptor (GPCR) and its activated signaling pathway. For review, see Conn and Pin, 1997. Illustration adapted and modified from Berne & Levy, Principles of Physiology, Ch. 4.

9.4.1. Ionotropic glutamate receptors

Glutamate binding onto an ionotropic receptor directly influences ion channel activity and fast synaptic transmission between neurons can be achieved. Each ionotropic glutamate receptor (iGluR) is formed from the co-assembly of individual subunits. Several different combinations of subunits exist, resulting in many different types of channels with different characteristics (Moyner et al., 1992). Three iGluR types have been identified based on their structural properties and pharmacology; AMPA, NMDA and kainate receptors. However, iGluRs can be more generally classified to non-NMDA and NMDA receptors. NMDA receptors bind glutamate and the glutamate analogue N-Methyl-D-Aspartate (NMDA), and non-NMDA receptors are selectively agonized by quisqualate, kainate and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) but not with NMDA.

Glutamate binding to non-NMDA receptor opens non-selective cation channels which are more permeable to sodium (Na^+) and potassium (K^+) ions than to Ca^{2+} ions (Mayer and Westbrook, 1987). In addition, the non-NMDA receptor gated currents differ from the NMDA receptors elicited ones. NMDA-gated currents typically have slower kinetics than AMPA- and kainate-gated channels. Glutamate binding onto an NMDA receptor opens a non-selective cation channel, resulting in a conductance increase, but the high conductance related with these receptors is more permeable to Ca^{2+} than to Na^+ ions (Mayer and Westbrook, 1987). Five subunits (NR1, NR2a, N2b, N2c, and N2d) of NMDA receptors have been cloned (Ishii et al., 1993). NMDA receptor subunits can co-assemble as homomers or heteromers (Moyner et al., 1992; Ishii et al., 1993). However, all functional NMDA receptors express the NR1 subunit (Moyner et al., 1992; Ishii et al., 1993).

NMDA receptors are structurally complex, with separate binding sites for glutamate, glycine, magnesium ions (Mg^{2+}), zinc ions (Zn^{2+}) and a polyamine recognition site. The glutamate, glycine, and magnesium binding sites are important for receptor activation and gating of the ion channel. The zinc and polyamine sites are not required for receptor activation, but rather affect the efficacy of the channel. The glutamate, glycine, and Mg^{2+} binding sites contribute to both, ligand-gated and voltage-gated properties of NMDA receptors. Mg^{2+} ions provide a voltage-dependent block of NMDA-gated channels (Nowak et al., 1984). NMDA receptors are ligand-gated solely because the binding of the ligand, glutamate is required to activate the channel. In addition, small concentrations of glycine must be present too, making them co-agonists to NMDA receptors (Kleckner and Dingledine, 1988).

9.4.2. Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are class C family GPCRs, also known as seven transmembrane receptors (7TM receptors) or heptahelical receptors (for review, see, Niswender and Conn, 2010). They are a protein family of transmembrane receptors that transduce an extracellular signal, a ligand binding, into an intracellular signal by G protein activation. The GPCRs are characterized by two distinctly separated topological domains: a large extracellular N-terminal domain, which contains a venus flytrap module (VFTM) for the orthosteric binding (Kunishima et al., 2000), and the 7TM helical segments with intracellular carboxyl-terminal domain that is involved in the receptor activation and G-protein coupling. The mGluRs have central roles in the brain as modulators of glutamatergic and other major neurotransmitter systems. They are involved in synaptic transmission, synaptic plasticity and ion channel activity. The mGluR activation has also role in internalization of iGluRs (Snyder et al., 2001).

Based on pharmacology, sequence homology and second messenger coupling the mGluRs have been divided into three groups, which contain eight subtypes. Group 1 receptors (mGluR1 and mGluR5) are coupled to the activation of phospholipase C (PLC) leading to the generation of

diacylglycerol (DAG) which activates protein kinase C (PKC) and IP_3 triggering the release of Ca^{2+} from intracellular stores. Group II (mGluR2 and -3) and group III (mGluR4, -6, -7 and -8) are negatively coupled to cyclic AMP production.

9.4.3. Metabotropic glutamate receptor 5

The mGluR5 is specifically expressed in the regions of active neurogenesis in the embryonic and postnatal brain and its expression has also been shown in embryonic stem (ES) cells and in NPCs (Di Giorgi Gerevini, et al., 2004; 2005; Cappuccio, et al., 2005; Melchiorri et al., 2007). Earlier studies have shown that group 1 mGluRs have developmental pattern of expression (Catania et al., 1994). Glutamate-stimulated phosphoinositide hydrolysis (PI) shows a developmental peak in the early postnatal life, which gradually decreases with age. Additionally, mGlu5Rs can generate oscillatory rises in $[Ca^{2+}]_i$ as a response to agonist stimulation in neuronal cells (Kawabata et al., 1996; 1998), and hence have a potential to regulate early steps of cell growth and development (Berridge et al., 2003). Therefore, suggesting a role for mGlu5Rs in the early brain development and in the basic cellular processes such as proliferation and differentiation (Di Giorgi Gerevini et al., 2004; 2005).

Indeed, it has been shown that an early endogenous activation of mGlu5Rs supports the survival and maturation of cerebellar granule cells (Copani et al., 1998) and Purkinje cells (Catania et al., 2001) and the self-renewal, survival and proliferation of embryonic NPCs (Di Giorgi-Gerevini et al., 2004; 2005; Cappuccio et al., 2005). The pharmacological blockade of mGlu5R activation with 2-methyl-6-(phenylethynyl)pyridine (MPEP) which is a selective non-competitive antagonist for mGluR5, and it has the ability to block mGlu5Rs independent of the ambient glutamate concentration (Gasparini et al., 1999; Varney et al., 1999), led to reduced NPC proliferation and survival, whereas the activation of mGlu5Rs substantially enhanced the cell proliferation (Di Giorgi-Gerevini et al. 2005). Similarly, a role for group 1 mGluRs in the control of survival, proliferation and as well differentiation has been shown in cultured adult mice NPCs derived from SVZ. Specifically, mGlu5Rs were implicated in the survival of neuronal-restricted precursors (Castiglione et al., 2008). In fact, it was shown that pharmacological blockade of mGlu5Rs with MPEP promoted the apoptotic cell death of progenitors undergoing differentiation specifically into neurons (Castiglione et al., 2008). Furthermore, previously by investigating intracellular Ca^{2+} responses to glutamate, two major functional cell types were distinguished during differentiation of NPCs which were derived from early prenatal human fetus or early postnatal mice brains (Castrén et al. 2005). In this study, type I cells showed a robust metabotropic Ca^{2+} elevation which was mediated exclusively via mGluR5, whereas type II cells showed highly elevated iGluR responses, concluding that these two cell types may represent two different stages of development during differentiation (Castrén et al., 2005).

The mGlu5Rs have been as well implicated in the regulation of glutamate-dependent development of somatosensory cortex in mice (Wijetunge et al., 2008). Furthermore, proper mGluR5 function plays a role in synaptic function and plasticity of mice thalamocortical pathway, and the altered dendritic morphology of cortical layer IV spiny stellate neurons in mGluR5 KO mice implicates mGluR5 in the dendritic morphogenesis of excitatory neurons (She et al., 2009). Additionally, with blockade with MPEP, mGlu5Rs has been shown to be involved in dendrite differentiation and excitatory synaptic transmission in NTERA2 human embryonic carcinoma cell-derived neurons (Park et al., 2007), indicating a role for mGluR5 signaling in the control of synaptic functions and dendritic morphology of neurons. Intriguingly, an important role for mGluR5 activation and its mediated downstream signaling pathway has been shown in FXS, a disease-causing mental retardation and associated with altered synaptic structures and functions (Bear et al., 2004).

10. Neurotrophins and their receptors in the CNS

Neurotrophins are a family of protein growth factors that through their activated receptors stimulate proliferation and survival of neuronal cells and take part in determining the fate of neural precursors in addition to axon and dendrite growth and patterning (for review, see Huang and Richardt, 2003; Park and Poo, 2013). Neurotrophins and their receptors also have an essential role in the regulation of the expression and activity of functionally important proteins, such as ion channels and neurotransmitter receptors in the synaptic sites. Particularly, BDNF (Barde et al., 1982) plays a significant regulatory role in the glutamatergic synapses (for review, see Carvalho et al., 2008). Neurotrophin protein growth factor family in mammalians includes: [1)] nerve growth factor (NGF), [2)] BDNF, and [3-4)] neurotrophins 3 and 4 (NT3 and NT4).

The neurotrophin functions are mediated through distinct types of cell surface receptors. Trk receptors are a high-affinity receptor tyrosine kinase sub-family for neurotrophins. The Trk receptor family consists of three receptor tyrosine kinases (called tropomyosin-related kinase A, -B or -C; TrkA, TrkB or TrkC, respectively). Each of these transmembrane Trk receptors can be activated with a varying affinity by binding of one to four of different neurotrophin ligands to its extracellular domain (for review, see Huang and Richardt, 2003). TrkB receptors are high-affinity catalytic receptors for BDNF that can be activated also by NT4 or to a lesser extent by NT3 (Klein et al., 1991; Squinto et al., 1991; Klein et al., 1992). TrkB mediates multiple effects of these neurotrophic factors including neuronal differentiation and cell survival.

As well low-affinity nerve growth factor receptors (LNGFRs), also called p75 neurotrophin receptors (p75NTRs) can be activated by neurotrophins (for review, see Underwood and Coulson, 2008). Each neurotrophin shows similar binding affinity for the p75NTR that is a member of the Fas/tumor necrosis factor receptor family (Johnson et al., 1986; Radeke et al., 1987; Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992; reviewed in Bothwell, 1995; Hempstead, 2002). Importantly, when Trk proteins and p75NTR are co-expressed, they form complexes, which may alter the signaling of both partners (Huang and Reichardt, 2003). Thus, the p75NTRs act as accomplices for Trk receptors by modulating ligand binding and neurotrophin responses, but also operate Trk-independently in the regulation of cell survival (Bothwell, 1995; Hempstead, 2002).

Trk receptors can also be activated in the absence of NT ligands, in response to GPCR activation. This transactivation of Trk receptors through GPCRs provides an alternative route for Trk signaling in the absence of neurotrophin and occurs via GPCR-ligands adenosine and neuropeptide PACAP (Lee and Chao, 2001; reviewed in Jeanneteau and Chao, 2006).

In conclusion, neurotrophin signaling via Trk generally mediates cell survival, differentiation or plasticity, whereas the p75NTR-mediated actions often stimulate pro-apoptotic pathways (Huang and Reichardt, 2003). Furthermore, different neurotrophins not only bind to their specific high-affinity Trk receptors but also bind with varying affinity to other Trk receptors and commonly bind to lower affinity p75NTRs to exert their variety of functions. However, Trk receptor-mediated functions can also be activated ligand-independently through GPCR-mediated transactivation.

10.1. BDNF expression and production

BDNF is a prototypic neurotrophin, which through its high-affinity TrkB receptor regulates diverse developmental events in the CNS in addition to connectivity of neurons (for reviews, see Yoshii and Constantine-Paton, 2010; Leal et al., 2014; 2015). BDNF mRNA is abundantly expressed in brain and the mature BDNF protein expression resembles the mRNA distribution (Nawa et al., 1995). The BDNF expression levels are low throughout fetal development, then increases after birth and subsequently decrease to adult levels (Maisonpierre et al., 1990). In the adult brain,

particularly high expression is seen in the cortex, hippocampus, amygdala, cerebellum and various hypothalamic nuclei's (reviewed in Lewin and Barde, 1996; Brigadski and Lessmann, 2020).

BDNF is first produced as a precursor or so-called pre-pro-BDNF protein, thereafter, following sequestration to ER, the pre-sequence is cleaved off. The remaining pro-BDNF is then further processed via Golgi machinery and packed into secretory vesicles. The pro-BDNF is cleaved intracellularly by either furin or pro-convertase enzymes and secreted as a mature peptide. Alternatively, the BDNF protein can be secreted as a pro-BDNF and cleaved extracellularly by tissue-type plasminogen activator (tPA)/plasmin proteolytic system or matrix metalloproteinases (MMPs) 3, 7 or 9 (Lee et al., 2001; Lessmann et al., 2003; Pang et al., 2004; Gray and Ellis, 2008;). These secreted mature and pro-BDNF forms have distinct biological actions upon release (Lee et al., 2001). The p75NTR has high affinity for proBDNF whereas TrkB binds mature BNDF, and it is thought that the mature form of BDNF plays a key role in different forms of synaptic plasticity; long-term-potentiation (LTP) through activation of TrkB receptors, whereas proBDNF induces long-term depression (LTD) through binding to p75NTR (Woo et al., 2005; Yang et al., 2014). The secretion of mature and pro-BDNF forms from a cell can be either constitutive or regulated depending on the cellular environment and furthermore, the efficiency of the proteolytic cleavage controls BDNF functions (Seidah et al., 1996; Mowla et al., 2001; Brigadski et al. 2005). Indeed, posttranslational modifications may possibly be a requirement for interactions of BDNF with chaperones or sorting receptors that guide differential vesicular targeting of BDNF primarily to vesicles of the regulated secretory pathway (reviewed in, Lessmann and Brigadski, 2009; Brigadski and Lessmann, 2020).

10.2. TrkB receptor expression

The expression of TrkB gene begins early during embryonic development and persists throughout adulthood. The transcription pattern of the rodent and human TrkB gene is complex with numerous mRNA transcripts (Klein et al., 1989; Klein et al., 1990; Middlemas et al., 1991; Stoilov et al., 2002). However, the TrkB locus codes for two major types of TrkB mRNAs which are expressed in the mammalian brain: [1)] TrkB.FL/TK$^+$ and [2)] truncated splice variants. Additionally, in human brain, also truncated isoform called TrkB.T-Shc which may act as a negative regulator of TrkB.TK$^+$ is expressed (Stoilov et al., 2002). The mRNAs encoding the different TrkB isoforms are abundantly expressed in rodent brain from E9.5 onwards, and the expression of these isoforms is differentially spatiotemporally regulated, in such a way that the full-length receptor mRNA and protein is the major form during early development, especially in the cortical layers, thalamus and hippocampus (peak at P1), and the truncated receptor mRNAs during later development (peak at P14) and adulthood (Klein et al., 1990; Kokaia et al., 1993; Allendoerfer et al., 1994; Escandon et al., 1994; Cabelli et al., 1996; Fryer et al., 1996; Drake et al., 1999).

10.3. BDNF/TrkB signaling pathways

The catalytic, full-length isoform of TrkB receptor (TrkB.FL/TK$^+$) is a typical tyrosine kinase receptor with extracellular transmembrane and cytoplasmic domains which transduces the BDNF ligand-binding receptor activation in parallel via mitogen-activated protein kinase (MAPK/Ras-Erk), phosphatidylinositol 3-kinase (PI3K/AKT- mammalian target of rapamycin (mTOR)), and PLCγ (DAG-IP$_3$) signaling pathways (for reviews, see Huang and Richardt, 2003 and Yoshii and Constantine-Paton, 2010; Leal et al., 2014). Upon BDNF ligand binding, TrkB receptors dimerize, and the cytoplasmic tyrosine kinase residues phosphorylate thus activating the receptor. Subsequently, the active tyrosine residues act as initiation sites to several intracellular signal

transduction cascades that eventually result in cellular responses and altered physiological functions.

Specifically, the BDNF/TrkB signaling activated MAPK and PI3K pathways play crucial roles in both; the translation and/or trafficking of synaptic activity induced proteins, whereas PLCγ regulates intracellular Ca^{2+} that can drive gene transcription via cAMP and PKC. Specifically, the TrkB-PI3K-AKT pathway activates translation via a signaling cascade driving mTOR, a major regulator of protein synthesis (Sarbassov et al., 2005). MAPK/Erk plays a critical role in protein-synthesis dependent plasticity by increasing phosphorylation of eukaryotic initiation factor 4E (eIF4E), 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 (Kelleher et al., 2004; Klann and Dever, 2004) and by activating cyclic AMP responsive element binding (CREB) transcription factor (Shaywitz and Greenberg, 1999). Whereas PLCγ pathway triggered elevation in Ca^{2+} increases Ca^{2+}-sensitive adenyl cyclase activity that is required for the formation of synaptic PSD-95-TrkB complexes (Ji et al., 2005) and is also involved in CREB-dependent transcription (Nguyen et al., 1994; Shaywitz and Greenberg, 1999). Indeed, BDNF via catalytic TrkB signaling has been implicated in several brain functions, such as neuronal cell survival and migration, neurite growth, regulation of balance between excitatory and inhibitory activity, and more precisely, glutamate-dependent spine and dendritic maturation, synapse formation, stabilization and potentiation (for reviews, see Yoshii and Constantine-Paton, 2009; Leal et al., 2014; 2015).

The truncated splice variants (TK-. T1/TrkB.T1, TK-. T2/TrkB.T2 and TrkB.T-Shc) possess the same extracellular domain, transmembrane domain, and first 12 intracellular amino acid sequences as TrkB.FL indicating equally competent ligand binding. However, in contrast to the catalytic TrkB, the C-terminal sequences are short, isoform-specific and lack the intracellular kinase domain (Klein et al., 1989; Klein et al., 1990; Middlemas et al., 1991). Therefore, truncated receptors play mainly a modulatory role in BDNF signaling but may also have regulatory actions independent of the catalytic TrkB.FL. In fact, the truncated receptors expressed in non-neuronal cells can act as ligand scavengers by binding and releasing BDNF and hence modifying BDNF signaling (Biffo et al., 1995; Rubio, 1997). The T1 form is predominantly expressed in the brain and when co-expressed in neurons, acts as a dominant-negative inhibitor of BDNF signaling by forming heterodimers with TrkB.TK- (Eide et al., 1996; Haapasalo et al., 2002) and thus restricting the availability of BDNF during development (Biffo et al., 1995). Furthermore, T1 has been reported to direct cortical NSCs to a glial cell fate by a novel signaling mechanism and this unique signaling cascade is involved in the regulation of cell morphology and calcium influx in glial cells (Cheng et al., 2007).

10.4. The role of BDNF signaling in NSCs/NPCs and the developing neocortex

BDNF plays an essential role in neuronal development and maturation in addition to plasticity (for review, see Mattson, 2008). BDNF signaling via TrkB and p75NTR activation regulates the *in vitro* survival and differentiation of NSCs and NPCs derived from the developing embryonic and postnatal brain (reviewed in, Barnabe-Heider and Miller). BDNF and nitric oxide form a paracrine positive feedback loop to inhibit NPC proliferation and promote cell differentiation in the mammalian brain (Cheng et al., 2003). BDNF signaling, specifically via activation of TrkB receptors play an important role during early neuronal differentiation (Takahashi et al., 1999; Benoit et al., 2001; Barnabe-Heider and Miller, 2003). Intriguingly, BDNF over-expression has been shown to increase neurogenesis, whereas inhibition of TrkB causes a delay in the generation of neurons, but not astrocytes and ultimately leads to disturbed postnatal localization of cortical neurons *in vivo* (Barnabe-Heider and Miller, 2003; Bartkowska et al., 2007). Since BDNF has been shown to increase the expression of bHLH proneural genes; *Mash1* and *Math1* it may through this regulation take part in guiding specifically neuronal differentiation of NSCs (Ito et al., 2003).

Additionally, BDNF has been implicated in the control NPC proliferation through the truncated TrkB.T1 isoform (Tervonen et al., 2006; Islam et al., 2009). The expression of TrkB.T1 in neocortical NSCs has been shown to direct cell fate towards glial fate through a novel Ca^{2+}-signaling dependent mechanism (Cheng et al., 2007). It has been also reported that with overexpression of TrkB.T1 in neurons, proliferation of adult SGZ progenitors is enhanced and this is accompanied by reduced survival of newborn granule neurons *in vivo* (Sairanen et al., 2005). The role of BDNF signaling through p75NTR activation has been studied in progenitors, too. Interestingly, it has been shown that the p75NTR expression defines BDNF-responsive neurogenic progenitors in the developing and adult SVZ (Young et al., 2007). Furthermore, the lack of p75NTR increases proliferation and nestin expression in the embryonic forebrain NPCs and BDNF signaling through p75NTR is required for neuronal differentiation *in vivo* (Hosomi et al., 2003). Additionally, it has been reported *in vitro* that sequential activation of p75NTR and TrkB receptors is involved in the dendritic development of SVZ derived differentiating NPCs (Gascon et al., 2005).

Crucial and diverse roles for BDNF signaling via TrkB in cortical development *in vivo* have been as well revealed (Gates et al., 2000; Ohmiya et al., 2002; Polleux et al., 2002; Medina et al., 2004; Lush et al., 2005; Fukumitsu et al., 2006; Chiaramello et al., 2007; Bartkowska et al., 2007). Neocortical neurons lacking functional TrkB receptors display abnormal delayed differentiation and migration and defects in the formation of dendritic structures with reduced neurite outgrowth (Gates et al., 2000). Administration of BDNF at E13, but not at E14, during neocortical development alters cell migration of newborn neurons and results in the differentiation of deeper layer neurons instead of superficial neurons demonstrating that BDNF affects specific progenitors at limited stages and suggesting the existence of a Reelin-independent mechanism to regulate cell migration (Ohmiya et al., 2002).

Furthermore, BDNF participates in the determination of neuronal laminar fate, since BDNF alters the position, gene-expression properties and projections of neurons destined to IV layer, to match neurons of deeper layers V and VI, whereas in the absence of BDNF some of these neurons become upper layer II/III neurons (Fukumitsu et al., 2006). Interestingly, it was also shown that shifts in the laminar fate of neurons are changed by BDNF only if their parent progenitor cells are exposed to it at certain timepoint, at the S-phase of the cell cycle, by accelerating the completion of S-phase and possibly by down-regulating Pax6 expression and consequently affecting INM. It has been also revealed that the expression of TrkB in thalamic axons is essential for appropriate timing of barrel cortex development, indicating a regulatory role for TrkB signaling in developmental maturation of the somatosensory cortex (Lush et al., 2005). TrkB receptor signaling through Shc/FRS2 adaptors and PLCgamma controls cortical stratification through the timing of neuronal migration and regulates differentiation of neocortical neurons and oligodendrocytes during development (Medina et al., 2004). NPC migration from the SVZ along the rostral migratory stream is promoted by local expression of both; BNDF and TrkB receptors and further, BDNF signaling leads to the activation of CREB through PI3K and MAPK pathways in migrating neurons (Chiaramello et al., 2007). BDNF plays a part as well in the regulation of tangential interneuron migration by acting as a motogenic factor for interneurons which are migrating into the developing neocortex from medial ganglionic eminence (Polleux et al., 2002).

10.5. BDNF/TrkB signaling in synapse functions and synaptic plasticity

BDNF is tightly implicated in the regulation of synaptogenesis as well as in synaptic function and plasticity in the developing and mature brain (for review, see Leal et al., 2014). BDNF modulates the development and function of synapses in various brain structures, including cortex and hippocampus (for reviews, see Lewin and Barde, 1996; Brigadski and Lessmann, 2020).

Furthermore, the functions of BDNF are controlled temporally by activity-dependent secretion. Increase in glutamatergic synapse activity stimulates the release of BDNF and the regulated BDNF secretion from neurons can be induced by a variety of stimuli such as high potassium, glutamate or neurotrophins themselves (Canossa et al., 1997). Both depolarization and neurotrophin-induced BDNF release depend on the increase in the intracellular Ca^{2+} concentration (Canossa et al., 1997). BDNF among other neurotrophins is a key regulator of activity-dependent modification of synapses which can lead to structural changes in dendritic structures and thus alterations in synaptic connectivity or in other words synaptic plasticity. In fact, it has been revealed that TrkB- or BDNF-deficient mice show LTP impairment (Korte et al., 1995; Minichiello et al., 1999; Pozzo-Miller et al., 1999; Xu et al., 2000). Accumulating evidence also suggests that BDNF affects both, early LTP (transcription and translation independent) and late LTP (transcription and de novo protein synthesis dependent) through pre- and post-synaptic mechanisms and that these are involved in learning and initiating activity-dependent modifications of developing neural circuits (for reviews, see Leal et al., 2014; 2015).

Nonetheless, local and synapse-specific modulation of BDNF is yet to be fully understood (Yoshii and Constantine-Paton, 2010; Leal et al., 2015). "Input-specificity" or "synapse-specificity" (meaning the dependency of sensory-induced electrochemical activity changes to modulate structural changes in dendritic spine structures) is crucial for synaptic plasticity since structural and functional modulation of synapses and consequently changes in dendritic structures, neurons and ultimately at neural network level, occurs only at synapses that experience changes in their activity. In this process, the local translation of specific mRNAs at synapses might be of particular importance for the regulation of protein expression within dendrites and growing axons and suggests that numerous components important for synaptic plasticity can be specifically synthesized and regulated by signaling events initiated in a specific synapse.

One general hypothesis arising from these observations suggest that neuronal activity enhances BDNF signaling by selectively modulating the TrkB receptors at active synapses, without affecting the TrkB receptors in currently less active synapses (for reviews, see Nagappan and Lu, 2005; Santos et al., 2010). This activity-dependent regulation of TrkB receptors on the cell surface provides one mechanism how BDNF signaling could be restricted to active neurons. Indeed, several mechanisms that could restrict BDNF modulation to active synapses, by controlling cellular responsiveness to BDNF with local and synapse-specific regulation of TrkB signaling, have been revealed. Subcellularly, the nuclear mRNA transcripts could be selectively transported to active dendrites and be translated locally. In fact, activity-dependent regulation of *TrkB* transcription and activity-dependent trafficking of *TrkB* mRNA into the dendrites has been demonstrated (Tongiorgi et al., 1997, Tongiorgi, 2008). Furthermore, TrkB.FL receptor and mRNA transcript expression in neurons appears to be tightly Ca^{2+}-dependently regulated and hence, the coordinated regulation of BDNF and TrkB by Ca^{2+} may play a role in activity-dependent survival and synaptic plasticity by enhancing BDNF signaling in electrically active neurons (Kingsbury et al., 2003). Indeed, the BDNF-TrkB induced changes in excitatory neurons results from increases in internal Ca^{2+} (Balkowiec and Katz, 2002). BDNF-induced TrkB phosphorylation leads to Ca^{2+} elevation triggered by PLCγ and as a result, increases Ca^{2+}-sensitive adenyl cyclase activity that in turn can activate cAMP facilitated trafficking of TrkB to dendritic spines, probably by promoting its interaction with synaptic scaffolding protein PSD-95 and thus contributing to dendritic spine formation (Ji et al., 2005).

As well mRNAs for *BDNF* transcript levels can be regulated by diverse neuronal activity. Induction of hippocampal LTP has been reported to upregulate *BDNF* mRNA both *in vitro* and *in vivo* conditions (Patterson et al., 1992; Castren et al., 1993). It has been also shown that depolarization

of hippocampal neurons leads to more dendritic distribution of mRNAs for *BDNF*, in addition to *TrkB* transcripts by a PI3K-dependent mechanism, and this is suggested to induce local protein synthesis in dendrites (Righi et al., 2000; Tongiorgi et al., 1997; 2008). Still, selective transport of *TrkB* mRNA into only active synapses has not been so far confirmed. Nevertheless, trafficking of *TrkB* mRNA into the dendrites upon membrane depolarization implies *TrkB* mRNA translation locally at dendrites and opens the possibility for local protein synthesis at active synapses.

Additionally, BDNF has been reported to be involved in the regulation of trafficking of several other mRNAs in dendrites such as: calcium-calmodulin-dependent kinase II (CaMKIIa), Homer2, AMPA receptor subunit GluA1, NMDA receptor subunit 1 and activity-regulated cytoskeletal protein (Arc) mRNAs (Aakalu et al., 2001; Ying et al., 2002; Schratt et al., 2004; Shiina et al., 2005). These together with modifications in the translation machinery at the synapse, in response to activity, contribute to local changes in the proteome and in the regulation of the post-synaptic responses. Thus, it can be concluded that, BDNF has an important regulatory role in the control of local protein synthesis machinery (reviewed by Santos et al., 2010).

In summary, BDNF-TrkB signaling is involved in transcription, translation, and trafficking of numerous proteins at various phases of synaptic development and has been implicated in several forms of synaptic plasticity. Synaptic activity drives BDNF actions via TrkB receptor activation and downstream PI3K-AKT, PLCγ and MAPK-ERK signaling pathways that regulate the assembling of TrkB with synaptic proteins, gene transcription, protein translation and dendritic trafficking (reviewed in, Yoshii and Constantine-Paton, 2010; Leal et al., 2014). BDNF-TrkB signaling can play a direct role in excitatory synaptic morphogenesis and there are also several mechanisms through which BDNF appears to facilitate spine formation (Amaral and Pozzo-Miller, 2007; Yoshii and Constantine-Paton, 2007; Carvalho et al., 2008). To conclude, BDNF signaling via TrkB regulates the formation and maintenance of dendrites, axons and synapses, therefore supporting the organization and maintenance of functional neuronal circuitry.

11. Overview of Fragile X Syndrome

Fragile X Syndrome (FXS) is the most common cause of inherited intellectual disability (for review, see Hagerman et al., 2017). This mental impairment can range from mild learning defects to more severe cognitive or intellectual disabilities. Symptoms include characteristic physical and behavioral features such as delay in speech and language development, hyperactivity, anxiety, deficits in social interactions, sensory abnormalities, mild facial dysmorphology including large ears, and macro-orchidism. FXS is the most known single gene cause of autism spectrum disorder (ASD) (for reviews, Santoro et al., 2012; Kaufmann et al., 2017). Furthermore, epilepsy is seen in 20% to 25% of individuals with FXS (Musumeci et al., 1999; Sabaratnam et al., 2001; Hagerman et al., 2005). Therefore, the multifaceted FXS can be perceived as a leading genetic model of several complex diseases. The prevalence of FXS is estimated to be approximately 1 in 5000 males and due to the random inactivation of the X chromosome 1 in 8000 females worldwide. FXS affects all populations with varying genetic backgrounds with similar occurrence (Pengarikano et al., 2007; Coffee et al., 2009).

FXS is a chromosome X-linked genetic disorder. At first, in the early 80s intellectual disability linked to fragile X-chromosome was termed as Martin-Bell syndrome (Richards et al., 1981), based on the earlier description made by Martin and Bell of a pedigree with an X-linked intellectual disability (Martin and Bell, 1943). The name fragile X stems from the observation that a portion of the X-chromosome from an individual with FXS appears broken or fragile (Lubs, 1969). This is due the methylation of the *FMR1* locus in the long (q) arm of the X chromosome at position 27.3 (Richards et al., 1981; Oberle et al., 1986).

FXS is caused by dysfunction of fragile X mental retardation 1 (*FMR1* also know as, FMRP translational regulator 1) gene typically due the expansion of the trinucleotide sequence CGG repeat in the 5' untranslated region of the *FMR1* gene on the X chromosome. An expansion over 200 repeats leads to promoter hypermethylation, causing transcriptional silencing of the *FMR1* gene and therefore the consequent loss of the encoded Fragile X Mental Retardation Protein (FMRP) results in FXS (Verkerk et al., 1991; Pieretti et al., 1991; Ashley et al., 1993b; O'Donnell and Warren, 2002). FMRP is an RNA-binding protein that associates with polyribosomes and acts as a negative regulator of translation, playing an important role in brain development, synaptic functions and neuronal plasticity by regulating the localization, stability and activity-dependent translation of specific mRNAs (for reviews, see Braat and Kooy, 2015; Contractor et al., 2015; Huber et al., 2015; Richter et al., 2015; Banerjee et al., 2018; Bagni and Zukin, 2019). However, recent accumulating evidence also directs towards new functions for FMRP. Several mechanisms or pathways by which FMRP influences nuclear gene expression and genome function have been uncovered, implicating FMRP function in DNA damage repair and maintenance of genome stability (Casingal et al., 2020; for review, see Dockendorff and Labrador, 2019). Also, other translational-independent synaptic functions for FMRP in neurons have been revealed. FMRP has been shown to directly interact with ion channels and modulate neuronal excitability and neurotransmitter release (Brown et al., 2010; Darnell et al., 2011; Deng et al., 2013; Ferron et al., 2014; Ferron, 2016; Yang et al., 2020; Ferron et al., 2020). Therefore, emphasizing the importance of understanding of the diverse functional roles of FMRP.

Cloning of the *FMR1* gene along with the fact that it is well-conserved between species allowed the creation of animal models to study the disease and especially, the generation of *Fmr1*-knockout (KO) mouse model accelerated research on the neurobiological basis underlying the pathophysiology of FXS (Verkerk et al., 1991; The Dutch-Belgian Fragile X Consortium, 1994). However, developmentally regulated inactivation of *FMR1* in FXS human embryos, a process, that is not fully recapitulated by *Fmr1*-KO mice strains along with variations in *FMR1*-related epigenetic measures, like CGG repeat number and the percent of methylation that correlate to *FMR1* mRNA and FMRP expression, in addition to sex differences that affect the clinical outcome FXS, have urged to study FMRP function also in human models (for review, see Zafarullah and Tassone, 2019; Telias, 2019).

11.1. The expression and function of FMRP

FMRP is expressed in all mammalian tissues except for striated muscles and especially enriched in neuronal tissue (Abitbol et al., 1993; Devys et al., 1993; Hinds et al., 1993; Feng et al., 1997). FMRP can be expressed throughout neuron structures; the soma, axon and dendrites, and even though FMRP has functional nuclear localization and export elements, it is primarily seen in the cytoplasmic department (Eberhart et al., 1996; Feng et al., 1997; Bakker et al., 2000; Antar et al., 2004; 2006; Price et al., 2006). Intriguingly, FMRP binds to a numerous different mRNA and microRNA targets in the brain including its own message, several hundred (859) are axonally and/or dendritically localized mRNAs (Darnell and Klann, 2013; Pasciuto and Bagni, 2014). Therefore, indicating a crucial and diverse regulatory role for this protein in the neuronal functions, and consequently meaning that one major key to the understanding, how FMRP regulates the nervous system and how the loss of FMRP in FXS affects, is to identify the relevant mRNA targets. Identified "key targets" of FMRP are many mRNAs encoding pre- and postsynaptic proteins, including*, mGluR5, Homer1, NMDARs, PSD-95, Shank1-3, PIKE, neuroligins, neurexins, SNAP-25, AP-2, bassoon, synapsin*, and calcium channels (Pasciuto and Bagni, 2014). Since the expression of FMRP is most prominent during early development in murine brain (Abitbol et al., 1993; Zang et al., 2013), and FMRP is expressed in the RGCs and immature neurons of the developing brain (Saffary and Xie, 2011; Pilaz et al., 2016) along with glial expression (Pacey et al., 2007; Gholizadeh et al., 2015), an important role for this protein specifically in neuronal development is indicated (Bhakar et al., 2012). In fact, it has been shown that FMRP plays important roles in synaptic plasticity and dendritic maturity and indeed appears to be involved in

the development of axons, formation, maturation and elimination of synapses and the development and wiring of neural circuits (for reviews, see Richter et al., 2015; Hagerman et al., 2017; Bagni and Zukin, 2019).

Even though a wide range of FMRP expression, numerous functions of FMRP seem to be strictly spatiotemporally and cell- specifically regulated. FMRP has a variety of functions at the molecular level, including regulation of local protein synthesis, translational regulation of a subset of mRNAs via several functional interaction sites, along with mRNA transport from the nucleus to dendritic synaptic sites, and stabilization of mRNAs (Dictenberg et al., 2008; Darnell et al., 2011; Darnell and Klann, 2013; Richter et al., 2015). It has been shown that FMRP encoded by the *FMR1* gene is an RNA-binding protein and compelling evidence suggests that it is responsible for regulating local protein synthesis of a substantial fraction of mRNAs within dendritic synapses of the brain (for reviews, see: Braat and Kooy, 2015; Contractor et al., 2015; Huber et al., 2015; Richter et al., 2015; Banerjee et al., 2018; Bagni and Zukin, 2019). Local translation of *Arc, Fmr1*, *αCaMKII, Map1b, PSD-95,* and $GABA_A$ receptors is regulated by FMRP in dendrites (Zalfa et al., 2003; 2007; Antar et al., 2004; Dictenberg et al., 2008). Various neurochemical pathways are disturbed when FMRP is lacking. FMRP is involved in several postsynaptic signaling cascades, including glutamate, acetylcholine, GABA, and dopamine receptor signaling (Volk et al., 2007; Wang et al., 2008; Osterweil et al., 2010; Kang et al., 2017). The absence of FMRP results in increased protein synthesis, leading to enhanced signaling in several intracellular pathways, including the mGluR5, ERK/MAPK, mTOR, PI3K and AKT inhibited glycogen synthase kinase 3 beta (Gsk3β) (Bagni and Zukin, 2019). There is up-regulation of the mGluR5 pathway leading to enhanced LTD, down-regulation of GABA pathways (Lozano et al., 2014), and dysregulation of dopamine and cholinergic pathways (Hagerman et al., 2014; Hare et al., 2014). Nevertheless, recent studies have also uncovered other signaling pathways or mechanisms by which FMRP effects nuclear gene expression and genome function, including the possibility that FMRP directly participates in the DNA damage response and the maintenance of genome stability (for review, see Dockendorff and Labrador, 2019).

11.2. *Fmr1* gene and Fragile X mental retardation protein structure

There are few functional highly conserved structural domains in the *FMR1* gene encoded protein product, however not all functions are yet fully understood (Dockendorff and Labrador, 2019). The polymorphic CGG trinucleotide repeat region is located in the 5' UTR adjacent to the first exon of the *FMR1* gene and the translation initiation sequence is located downstream the repetitive region (Ashley et al., 1993a). Additionally, the *FMR1* gene contains a (cytosine-phosphate-guanine) CpG-island upstream from the CGG trinucleotide sequence (Eichler et al., 1993). There are four transcription factor-binding sites in the *FMR1* gene promoter, including: a palindrome, two GC-like boxes, and an overlapping E-box-cAMP response element site. Studies investigating protein binding to these sites, have identified six factors that are most actively involved in *FMR1* transcriptional activity: upstream stimulatory factor 1 and 2, nuclear respiratory factor 1 and 2, specificity protein 1 and cAMP response element binding protein (CREB) (Kumari et al., 2005; Smith et al., 2006).

The RNA interacting domains that include two middle located K-homology domains (KH1 and KH2) and a C-terminal arginine-glycine-glycine (RGG) box allow FMRP to bind and regulate the translation of a specific subset of mRNA targets involved in synaptic plasticity (Brown et al., 2001; Darnell et al., 2011). Strictly controlled activity-dependent local protein synthesis is crucial for many forms of synaptic plasticity, and in the absence of FMRP, defects in these protein synthesis-dependent synaptic plasticity pathways contribute to the pathophysiology of FXS (for reviews, see Braat and Kooy, 2015; Huber et al., 2015; Richter et al., 2015; Bagni and Zukin, 2019). The nuclear localization signal (NLS) and nuclear export signal (NES) of FMRP indicates that this protein can shuttle between nucleus and cytoplasm, even though its reported predominant localization is in the cytoplasm (Fridell et al., 1996; Sittler et al., 1996; Feng et al., 1997). It has been well documented,

that the amino terminus can bind RNA, despite having no recognizable functional motifs (Adinolfi et al., 1999; 2003; Ramos et al., 2006; Yan et al., 2011; Hu et al., 2015). Nevertheless, this has been mainly understudied and there are still several unresolved questions about its exact functions (Dockendorff and Labrador, 2019). A clear nuclear function for FMRP had not been able to be specified until recently, when nuclear FMRP was found to interact with chromatin and modulate the DNA damage response via its newly recognized tandem amino terminal Agenet domains and new KH motif (Alpatov et al., 2014; Myrick et al., 2015b). As well translation-independent FMRP function in the presynaptic space by modulating action potential duration through calcium-activated potassium (BK) channel β4 subunits through this motif has been discovered (Deng et al., 2013; Myrick et al., 2015a). The amino terminal is recognized as the site for most of FMRP's protein–protein interactions, including NUFIP1, 82-FIP and cytoplasmic FMRP-interacting protein CYFIP1/2 (Bardoni et al., 1999; Schenck et al., 2001; Bardoni et al., 2003), though the biological role of these protein interactions with FMRP remains not completely understood.

11.3. Causes of FXS

11.3.1. Inheritance and transmission

FXS is an X-chromosome-linked dominant genetic disorder caused by mutation of the *FMR1* gene with variable expressivity (for review, see Healy et al., 2011). X-linked dominant inheritance implies that because certain gene is located on the sex-related X-chromosome, only one copy of the allele is sufficient to cause a disorder when inherited from a parent who has the mutated gene. Since FXS is caused by single gene mutation in the X-chromosome and males normally have just one copy of X- with Y-chromosome and females have two X-chromosomes, the syndrome is more common and generally more severe in males than in females. Most females with FXS experience symptoms to a lesser degree because of one normal allele and thus an increased probability of having a working *FMR1* allele. Due to X-inactivation, only one X-chromosome is active in each cell and thus the variable X-activation ratio (or the percentage of cells with the normal X, as the active X) controls how much FMRP is produced and determines the intellectual level and physical involvement caused by the fragile X mutation (de Vries et al., 1996; Hagerman, 2002; Healy et al., 2011). Therefore, meaning that the symptoms could be also as severe in females as in some males with FXS. Also because of X-linked inheritance, FXS males cannot transmit the mutation to their sons, but will transmit a premutation to all their daughters since men contribute an X-chromosome to all their daughters, and their children are of risk to be affected with FXS. Females carrying a copy of the mutation have a 50% chance to transmit it to their children and a son who inherits the *FMR1* gene mutation is at high risk of intellectual disability. Also, a premutation can develop into a full mutation in a generation, see next chapter.

11.3.2. Genetic cause

Typically, in FXS, an expansion of a single CGG trinucleotide gene sequence (over 200 repeats) in the 5’ UTR of the *FMR1* gene on the X-chromosome results in full mutation which causes the repression or silencing of the *FMR1* gene and ultimately leads to a failure to express the functional protein; FMRP, which is required for normal neural development and synaptic plasticity (for review, see Hagerman et al., 2017). The length of the repeated CGG sequence varies from person to another, through mechanisms that have yet to be elucidated, such a way that 6-44 repeats is considered normal, whereas 45-54 repeats is intermediate or grey area, and 55 to 200 repetitions is considered as premutation and like previously mentioned, over 200 repeats is considered a full mutation and results in FXS (Verkerk et al., 1991; Pengarikano, 2007). The *FMR1* CGG trinucleotide repeat sequence is typically interrupted by an AGG trinucleotide sequence in around every nine to ten repeat units. If the loss of these interrupting sequences increases, the instability of the trinucleotide repeat region is affected and in some premutation cases may lead to full mutation in a pedigree (Eichler et al., 1993; Yrigollen et al., 2014; Nolin et al., 2015). The mechanism responsible for the repeat instability is largely unknown (Zhao et al., 2019). The

premutation can lead as well to conditions like premature ovarian failure in females and the onset of Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) in adult males (for reviews, see Loesch and Hagerman, 2012; Hall and Berry-Kravis, 2018; Salcedo-Arellano et al., 2020).

In general, if the *FMR1* allele has 200-4000 repeats of the CGG repeating codon in the promoter region, it results in a hypermethylation of that portion of the DNA, effectively transcriptionally silencing the expression of the *FMR1* gene coded protein; FMRP (Nolin et al., 2003). Mechanistically, the repeated CGG sequence and its upstream CpG-island are hypermethylated together with the surrounding sequence region, causing a repression or silencing of the *FMR1* gene (Pieretti et al., 1991; Ashley et al., 1993a and 1993b; Eichler et al., 1993). This methylation of the *FMR1* gene prevents direct transcription factor binding and induces chromatin condensation which impairs the binding of transcriptional machinery and ultimately leads to a lack or deficit of functional FMRP (Kumari et al., 2005; Smith et al., 2006; Penagarikano et al., 2007).

Although a vast majority of FXS cases are caused by the CGG trinucleotide repeat mutation in the *FMR1* gene (O'Donnell and Warren, 2002), as well other causes can lead to loss of functional FMRP and consequently result in a manifestation of FXS (reviewed in, Sitzmann et al., 2018). Recent studies suggest that intragenic *FMR1* variants, although much less frequent than CGG expansions, are a significant mutational mechanism leading to FXS (reviewed by Quartier et al., 2017). FXS has been diagnosed in few cases where chromosomal deletions or missense loss-of-function point mutations were identified in the promoter or coding region of *FMR1* gene (De Boulle et al., 1993; Siomi et al., 1994; Lugenbeel et al., 1995; Handt et al., 2014; Myrick et al., 2014; Okray et al., 2015; Quartier et al., 2017; Sitzmann et al., 2018). A point mutation may lead to the expression of non-functional protein, thus offering a chance to characterize functional domains of certain protein. A case of FXS with a severe phenotype was reported, where a missense point mutation was seen in the nuclear ribonucleoprotein KH2 domain of FMRP at amino acid site 304, caused by substitution of isoleucine (I) to asparagine (N) (I304N) (De Boulle et al., 1993; Siomi et al., 1994; Feng et al., 1997). This mutation has indeed provided valuable information of the molecular binding partners of FMRP and highlights this binding domain importance in the pathophysiology of FXS (De Boulle et al., 1993; Feng et al., 1997; Myrick et al., 2014).

To summarize these findings, the absence or loss of functional FMRP causes the variable phenotype seen in FXS.

11.4. The behavioral and physical phenotype of patients with FXS

Signs and symptoms in FXS vary and even between affected relatives in FXS families. Specifically, in FXS females even with full mutation the phenotype is more variable, due to X-inactivation and thus depending on the X activation ratio and FMRP level (de Vries et al., 1996; Hagerman, 2002). In fact, the level of intellectual ability and physical features of FXS individuals correlates positively with FMRP level in the blood (Loesch et al., 2004; Tassone et al., 1999). One major cognitive characteristic of patients with FXS is mental retardation, with IQ ranging from 20 to 70 in men (Fisch et al., 2002). Approximately 25% of females with full mutation have IQ above 70, whereas 40% have borderline IQ range from 70-85 and 35% have normal range, however, executive function deficits, learning and attention problems and impulsivity are common despite IQ level (de Vries et al., 1996; Hagerman, 2002). Most affected cognitive skills in FXS are speech, working or so-called short-term memory and visuo-spatial abilities (Cornish et al., 2008; Lightbody and Reiss, 2009). Indeed, the CGG length has been significantly correlated with the central executive and the visual-spatial memory. Very commonly, a developmental delay in speech, which often results in learning problems, is the first symptom observed in children with FXS along with social avoidance and furthermore, their curve of development can differ from what is typical for a particular age (Hall et al., 2008).

A variety of non-cognitive symptoms or behavioral features of FXS include attention deficit, hyperactivity, impulsivity, mood instability, irritability and hypersensitivity to visual, auditory,

tactile, and olfactory (sensory) stimuli and autistic-like behavior (for reviews, see O'Donnell and Warren, 2002; Hagerman et al., 2017). As well aggression occurs in approximately 30% of males and is seen most frequently in adolescence. FXS is characterized by social anxiety and withdrawal, including gaze avoidance, shyness and prolonged time to initiate social interaction. Social anxiety in individuals with FXS is reportedly related to challenges with face encoding (Holsen et al., 2008). Perseveration or repetitive behaviors (hand-flapping, hand-biting, self-talk) along with gaze aversion and tactile defensiveness, which are as well common behavioral characteristic for autistic individuals, are frequently seen in FXS individuals (for review, see Bagni et al., 2012). Indeed, the prevalence of autism in individuals with FXS is reported to be around 20%-40% and about 40%-60% of male FXS individuals and 20% of female patients meet the criteria for ASD and approximately one-third of FXS children are diagnosed with autism (Hagerman et al., 2005; Kaufmann et al., 2017). Specifically, children with FXS may echo certain activity repeatedly, and in speech; cluttered speech and self-talk using different tones and pitches are often observed (Hanson et al., 1986). Interestingly, when both autism and FXS are present, a severe language deficit and lower IQ is observed with more stereotyped and aggressive behaviors as compared to children with only FXS (Hagerman et al., 2002; Bailey et al., 1998; Hatton et al., 1999; McDuffie et al., 2010; Thurman et al., 2015; Kaufmann et al., 2017).

Aside from intellectual disabilities, prominent physical characteristics of FXS include an altered facial morphology with elongated face, in such a manner that prominent jaws, forehead and large or protruding ears accompany a long and narrow face (Chudley and Hagerman, 1987; Hagerman, 2002). Common physical characteristic in FXS males is macroorchidism after puberty (enlarged testicles) (Turner et al., 1980; Butler et al., 1993). Other common physical features in FXS include a high arched palate, flat feet, low muscle tone, hypotonia, mitral valve prolapse, and hyperflexible finger joints along with double-jointed thumbs, soft skin and a variety of skeletal problems (Hagerman et al., 1983; Hagerman, 2002; Erickson et al., 2017). A variety of these physical features are considered to stem from defects in connective tissues and cellular proliferation (Laxova, 1994; Penagarikano et al., 2007). Additionally, a neuroendocrine dysfunction and particularly, hypothalamus dysfunction have been reported in FXS individuals (Hessl et al., 2004). Even though the understanding of all the molecular mechanisms involved is still lacking, neuroendocrine dysfunction may be related to at least some part with unusual overgrowth patterns that are seen especially in childhood in FXS. These include macrencephaly, increased birth weight and height, and increased stature (Chiu et al., 2007). Additionally, an unbalanced neural activity between inhibitory and excitatory circuits is indicated in FXS (Schaefer et al., 2017) and believed to underlie many of the clinical manifestations of this disorder. Epileptic seizures have been reported to occur around 20% of patients with FXS (Musumeci et al., 1999; Berry-Kravis et al., 2010). As well, dysregulation of sympathetic and parasympathetic peripheral nervous system function has been observed in children and adolescents with FXS (Hall et al., 2009).

See **Figure 7** for phenotypic FXS patient.

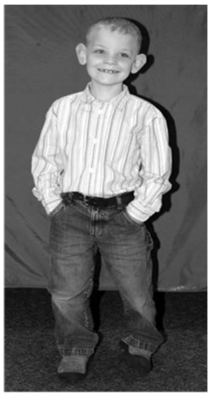

Figure 7. *Phenotype in Fragile X syndrome.*

In the picture, a young boy with fragile X syndrome and phenotypic physical appearance is seen. Picture representation reproduced with permission from Penagarikano et al., 2007.

11.5. Neurobiology of FXS

11.5.1 Neuroanatomical phenotype

Previous studies on postmortem FXS brains have indicated that the loss of FMRP does not affect the overall brain structure and gross assemblies appear normal (Rudelli et al., 1985; Hinton et al., 1991; Wisniewski et al., 1991; Irwin et al., 2001). However, ventricular enlargement and mild cortical atrophy was observed already in the early studies (Wisniewski et al., 1991). Technical advances in brain imaging have shown that the sizes of few specific brain structures certainly are affected in FXS, characterized by enlargement in the lateral ventricles and caudate nuclei and a reduction in cerebellar vermis (reviewed in Salcedo-Arellano et al., 2020).

Structural MRI studies have consistently shown patients with FXS to have enlarged caudate nuclei and lateral ventricles (Reiss et al., 1995a; Eliez et al., 2001; Gothelf et al., 2008; Hoeft et al., 2008; Hazlett et al., 2009; 2012; Hallahan et al., 2011; Sandoval et al., 2018). Caudate nucleus is a structure which is linked to memory and learning, and additionally the controlling of repetitive movements, a characteristic frequently displayed in FXS patients (Hall et al., 2008). A functional link to abnormality in this brain region has been shown, with negative correlation between caudate size and IQ (Reiss et al., 1995b; Gothelf et al., 2008; Hallahan et al., 2011), and further by positive correlation between aberrant behavior (measured as Autism Behavior Checklist) and caudate size (Gothelf et al., 2008). Whereas, the cerebellar vermis, a structure, closely related to executive function, visuo-spatial processing, language and learning (De Smet et al., 2013), is consistently reduced in FXS individuals (Reiss et al., 1991; Mostofsky et al., 1998; Lee et al., 2007; Hoeft et al., 2008; Gothelf et al., 2008). In fact, the cerebellar vermis size is a predictor for lowered IQ and positively correlated with the loss of FMRP expression (Mostofsky et al., 1998; Gothelf et al., 2008). Alterations in caudate nucleus and cerebellar vermis appear already at first born year and persist into adulthood in FXS (Lee et al., 2007; Gothelf et al., 2008).

In addition to regional volume alterations in caudate nucleus and cerebellar vermis, also hippocampus, amygdala, fusiform gyrus and insula may be affected in FXS (reviewed in, Lightbody and Reiss, 2009). Hippocampus is an important structure for learning and memory, and it contains high levels of FMRP mRNA during human fetal development (Abitbol et al., 1993). Interestingly, hippocampal volumes were observed to be enlarged in children with FXS (Reiss et al., 1994; Kates et al., 1997), but in adults this increase in the size of the hippocampus was not indicated (Jäkälä et al., 1997). Subsequent studies have reported contrasting results (Hoeft et al., 2008; Hazlett et al., 2009). Therefore, further studies are still needed to resolve if significant age-dependent hippocampal volume changes occur and play a part in the pathogenesis of FXS. Additionally, it has been reported that some FXS children have reduced size of amygdala which is a structure that takes part to social behavior and emotion processing (Kates et al., 1997; Gothelf et al., 2008; Hazlett et al., 2009). Furthermore, a bit surprisingly these findings of reduced amygdala size in young FXS male individuals seem not to be associated with autism (Hazlett et al., 2009). The fusiform gyrus, which is important structure in face processing, is enlarged in young FXS individuals, whereas the insula which plays a role in emotion regulation and processing, was shown to be smaller in size (Hoeft et al., 2008).

Minor neuroanatomical alterations have been revealed in other FXS brain structures. There appears to be a moderate and region-specific alteration in cortical lobe grey matter volume, with a modest increase in the parietal and occipital lobes, and moderate reductions in temporal and frontal lobes (Gothelf et al., 2008; Hallahan et al., 2011; Sandoval et al., 2018). White matter volumetric alterations have also been detected. Including an increase in parietal and temporal white matter (Lee et al., 2007) and septal fornix (Sandoval et al., 2018) and brainstem-hippocampus tract and

cingulate-corpus callosum tract volumes (Hallahan et al., 2011). Whereas decreases have been shown in frontal lobes (Hallahan et al., 2011) and in cerebellar white matter (Sandoval et al., 2018). Interestingly, periventricular heterotopia has been found in two unrelated FXS patients, suggesting neuronal migration defects in FXS (Moro et al., 2006). Furthermore, individuals with FXS show decreased activation in the prefrontal regions of the brain, regions which are strongly associated with social cognition (Holsen et al., 2008).

11.5.2. Defects in neuronal ultrastructure

In neurons, FMRP can be expressed in various structures; the soma, axon, dendrites (Eberhart et al., 1996; Feng et al., 1997a; Bakker et al., 2000; Antar et al., 2004; 2006; Price et al., 2006). Particularly high expression of FMRP can be found in dendritic spines and post-synaptic densities of excitatory neurons. In fact, dendritic spines; the protrusions of dendritic structures which are crucial for proper synapse formation and neuronal communication, are dysmorphic in various brain regions of FXS individuals and its mouse model and generally exhibit an immature phenotype with an increased density of abnormally long, thin and tortuous spines, which appear to be differentially developmentally regulated in different brain regions (Galvez and Greenough, 2005; Grossman et al., 2006; 2010; He and Portera-Cailliau, 2013). See **Figure 8** of altered dendritic spine structures in neurons of FXS.

During development, synapses are often produced in numbers that exceed those that will ultimately survive; therefore, synapse maturation and pruning, which requires sensory experience, are important steps for establishing more lasting synaptic arrangements (Rakic et al., 1986; Grutzendler et al., 2002; Zuo et al., 2005a; 2005b). Since the absence of functional FMRP leads to alterations in dendritic structures and deficiencies in spine number, morphology and higher turnover, a role for FMRP in the regulation of synapse formation, maintenance and/or elimination has been proposed. However, to date it still is not exactly clear which of these processes are particularly affected and there are still several controversies regarding the exact spine defects in FXS. In the light of current knowledge, it seems that different combinations are affected and that there are differences in distinct brain regions that may be developmentally regulated (reviewed in, Portera-Cailliau, 2012; He and Portera-Cailliau, 2013). Therefore, the real problem might be a defect in activity-dependent spine maturation and plasticity.

It has been hypothesized that the absence of FMRP leads to a deficit in synaptic pruning which consequently results in surplus of synapses (Weiler and Greenough, 1999; Bagni and Greenough, 2005; Antar et al., 2006). It has been shown that postsynaptic expression of FMRP negatively regulates synapse number in hippocampal pyramidal neurons (Pfeiffer and Huber, 2007). In fact, dendritic pruning defects have been also indicated in the somatosensory barrel cortex of mouse model for FXS. Normally during development when the immature spiny stellate cells extend their dendrites into barrels the septal-oriented protrusions are eliminated whereas, in the absence of FMRP a failure to prune these septal-oriented dendrites is observed (Galvez et al., 2003). In addition, the rates of spine formation and elimination are significantly higher in the primary somatosensory cortex of both young and adult *Fmr1*-KO mice when compared to controls (Pan et al., 2010). Intriguingly, they also showed that the formation of new spines and the elimination of existing ones, were less sensitive to modulation by sensory experience in transgenic mice. Very recent paper also found reduced astrocyte participation in the tripartite synapse and less mature post-synaptic densities in the CA1 region of *Fmr1*-KO hippocampi due altered synaptic pruning during development, indicating delayed synaptic maturation and important role for astrocyte signaling in controlling synaptic pruning (Jawaid et al., 2018).

Collectively, these results indicate that the lack of FMRP leads to ongoing overproduction of transient spines and suggest that the developing synaptic circuits may not be finely tuned by sensory stimuli in FXS.

It has been also shown that dendritic spine density is higher in the dentate gyrus of *Fmr1*-KO mice across development and although as well more immature morphological phenotype was shown compared to wild type, gradual spine maturation development, rather than impairments during specific phases of spine maturation was seen (Grossman et al., 2010). Additionally, the hippocampal spine length showed a complex developmental pattern which differed from other brain regions examined (Grossman et al., 2010). Therefore, as combined these findings indicate dynamic brain region- and/or cell-type-specific dendritic spine maturation defects during the formation and maintenance of functional synaptic contacts in the absence of FMRP and suggest an important dynamic regulation by FMRP and other brain region-specific proteins.

Interestingly, it was shown in mouse brain with shRNA-ko study that FMRP is required cell autonomously for proper synaptic morphology (Scotto-Lomassese et al., 2011), confirming the important role for FMRP in both; spinogenesis besides dendritogenesis and necessity for activity-dependent dendritic remodeling and consequently in synaptic plasticity. Furthermore, the increased dendritic spine density along with altered morphology and therefore resulting augmented excitatory neuronal activity have been proposed to play a role in epileptic seizure susceptibility in FXS (Incorpora et al., 2002) and might lead to an exaggerated response to sensory stimuli due to hyperconnectivity. Potential consequences of failure of spines to exhibit normal plasticity due to delayed maturation, overproduction or pruning failure, would also include sensory integration defects and problems with learning and memory. Additionally, dendritic spine abnormalities have also been observed in other syndromes with intellectual disability, including Down and Rett's Syndromes (Takashima et al., 1981; Kaufmann and Moser; 2000; Dierssen and Ramakers, 2006). These findings highlight the importance of dendritic spine abnormalities in the alterations of synaptic functions along with development and maintenance of neuronal circuits and suggest that these defects may underlie the cognitive dysfunctions in these diseases.

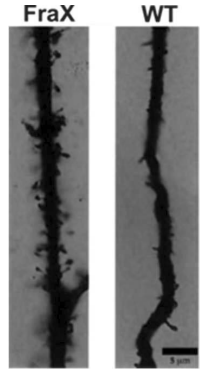

Figure 8. Dendritic spine structures in neurons from an individual with fragile X syndrome have an immature appearance when compared to wild type dendritic structures.

Image reproduced with permission from Irwin et al., 2001.

11.5.3. The mGluR signaling and synaptic plasticity in FXS

Synaptic plasticity or the ability of neurons to tirelessly strengthen or weaken their discrete synaptic connections in a response to changing activity patterns is a central molecular mechanism for learning and memory. Neural activity can be mediated by glutamate or BDNF via activation of their receptors and may eventually lead to activity-dependent protein synthesis in the cell bodies and dendritic spines of neurons that on turn play a crucial role on synaptic plasticity related events. Forms of synaptic plasticity; LTP and LTD contribute pre- and/or post-synaptically to the creation, maintenance and elimination of synapses and to the regulation of synaptic strength. A strong connection between FMRP and mGluR5-dependent LTD has been shown (for reviews, see Bear et al., 2004; Ronesi and Huber, 2008; Santoro et al., 2012).

Normally mGluRI-dependent postsynaptic LTD depends on the dendritic protein synthesis and is required for the persistent internalization of AMPA receptors from the cell surface. However, in *Fmr1*-KO mice mGluRI-dependent LTD is enhanced and therefore AMPA receptor internalization

increased, and this occurs independently of protein synthesis in the hippocampus (Huber et al., 2002; Hou et al., 2006; Nosyreva and Huber, 2006). Also, various other studies have consistently shown correlation of augmented mGluRI-dependent LTD to the loss of FMRP (Todd et al., 2003; Antar et al., 2004; Aschrafi et al., 2005; Desai et al., 2006; Huang et al., 2015). The mGluR theory of FXS suggests that FMRP acts as a negative regulator of translation, downstream of activated Gp1 mGluRs at the postsynaptic sites, and in FXS; in the absence of FMRP, uncontrolled protein synthesis leads to excess AMPA receptor internalization and thus exaggerated LTD (Bear et al., 2004). Furthermore, because of exaggerated mGluR signaling, the mGluR theory of FXS suggests, that many synaptic events that underlie the phenotypic cognitive impairment in FXS may be directly attributed to the alterations in mGluRI-mediated signaling and therefore mGluR antagonists could be a useful therapy for FXS. Indeed, many studies support an important role for mGluR5 signaling in the absence of FMRP and its selective blockade or suppressed function has been shown beneficial in FXS animal models (Vanderklish and Edelman, 2002; McBride et al., 2005; Yan et al., 2005; Nakamoto et al., 2007; Dölen et al., 2007; De Vrij et al., 2008; Guo et al., 2016). However, human clinical trials targeting directly mGluR5 unfortunately failed (Berry-Kravis et al., 2016) and therefore emphasize the understanding of the molecular mechanism behind increased mGluRI signaling that is not yet fully elucidated. Importantly, increased mGluRI-dependent LTD might be a common consequence of intellectual disability and not the cause of it, since it has been shown to exist in some other mouse models of intellectual disability and ASDs, regardless of the *Fmr1*-KO mutation (for review, see Telias, 2019).

One mechanism linking exaggerated mGluR-LTD to overactivated mTor signaling in FXS, is PI3 kinase enhancer PIKE which upon activation of mGluR1/5-Homer complex, in response to synaptic stimulation, engages PI3K/AKT/mTOR signaling (Sharma et al., 2010; Ronesi et al., 2012; Gross et al., 2015). Yet, another exciting mechanism, linking mGluR5 signaling, might be through matrix metalloproteinases (MMP), since it has been reported that MMP9 which is excessively activated via mGluR5 in FXS animal models is inhibited by minocycline (Bilousova et al., 2009). Moreover, core deficits in a mouse model of FXS are ameliorated by selectively normalizing hyperactivation of ERK/mTOR signaling, eIF4e phosphorylation and the increased expression of MMP9 with metformin (Gantois et al., 2017).

Additionally, mGluR-mediated LTP defects have been reported in *Fmr1*-KO mice along with altered excitability, and delayed synaptic maturation (Contractor et al., 2015). The mGluR-dependent LTP seems to occur in widespread areas of the brain, including neocortex, hippocampus and striatum. Furthermore, mGluR-dependent forms of LTP have been shown to be diverse, involving activation of mGluR1 or mGluR5 and can be of AMPAR-mediated transmission or of NMDAR-mediated transmission (for review, see Anwyl, 2009). In the hippocampus of *Fmr1*-KO mouse model, AMPA receptor subunit GluA1-dependent synaptic plasticity is disturbed (Hu et al., 2008; Soden and Chen, 2010; Lim et al., 2014). In contrast, reduced LTP and decreased levels of GluR1-containing AMPA receptors in the cortex of *Fmr1*-KO mice, but normal LTP in the hippocampus and normal levels of GluR1 in hippocampus have been reported (Li et al., 2002). Also, developmentally altered iGluRs and plasticity changes in the hippocampus of *Fmr1*-KO mice have been reported (Pilpel et al., 2009). Whereas, in the visual neocortical layer 5 of WT mice slices, LTP depends primarily on mGluR5 activation and in *Fmr1*-KO mice this mGluR5-mediated synaptic plasticity seems to be absent (Wilson and Cox, 2007). Interestingly, it was also reported that in the prefrontal cortex of *Fmr1*-KO mice the spike-timing-dependent LTP is not so much absent, but rather, the threshold for its induction is increased and compromised calcium signaling in dendrites and spines seem to be involved in defects of neocortical LTP (Meredith et al., 2007).

Reportedly, NMDA receptor dependent LTP is significantly reduced in *Fmr1*-KO mice (Zhao et al. 2005; Meredith et al., 2007; Hu et al., 2008; Shang et al., 2009; Seese et al., 2012), due to a selective deficiency of signal transduction between Ras and PI_3K/PKB that also impairs GluA1-dependent plasticity in *Fmr1*-knockout mice (Hu et al., 2008). Moreover, with compounds

activating serotonin or dopamine, pharmacological rescue by enhancing Ras–PI_3K/PKB signaling input, GluA1-dependent synaptic plasticity, and learning in *Fmr1*-KO mice, is achieved (Lim et al., 2014). Recently it has been also shown that dysregulated NMDA receptor signaling is the underlying cause of the enhanced mGluR-LTD in *Fmr1*-KO that is developmentally regulated (Toft et al., 2016) and by inhibition of GluN2A NMDA receptors, plasticity deficits (mGluR-LTD and LTP) in the *Fmr1*-KO mice can be restored (Lundbye et al., 2018). Therefore, these findings underline the developmental complexity and regional regulation of receptor signaling dynamics and forms of plasticity defects.

11.5.4. FXS and BDNF signaling

Connections between FMRP function and BDNF signaling has been suggested (Castrén and Castrén, 2014). Indeed, it has been observed that BDNF takes part in regulating the *Fmr1* mRNA expression, since cultured hippocampal neurons display a transient decrease in *Fmr1* mRNA levels after BDNF administration (Castrén et al., 2002). Likewise, transgenic mice overexpressing TrkB.FL receptor have decreased hippocampal expression of *Fmr1* mRNA and FMRP, suggesting a part for FMRP in BDNF-induced synaptic plasticity. In addition, TrkB receptor expression is shown to be increased in parvalbumin positive GABAergic neocortical neurons in adult *Fmr1*-KO mice (Selby et al., 2007), and in the sensory cortex of neonates, during a critical period of synaptogenesis and circuit formation, maturation of fast-spiking interneurons was found to be delayed in *Fmr1*-KO mice and could be rescued by chronic delivery of a TrkB agonist (Nomura et al., 2017). Furthermore, BDNF infusion can restore severely impaired LTP elicited by threshold levels of theta burst afferent stimulation in the hippocampal field cornu ammonis 1 (CA1) in young adult *Fmr1*-KO mice (Lauterborn et al., 2007).

BDNF via activation of TrkB receptors takes part to local protein synthesis in neurons and among other important roles, activates pathways involved in spine cytoskeletal reorganization and morphology (reviewed in, Leal et al., 2014). Whereas, in the absence of functional FMRP, defects are interestingly seen in both, local protein synthesis and spine morphology. BDNF activates the translation of two dendritic FMRP target mRNAs that encode *Arc/Arg3.1* and *αCaMKII* (Aakalu et al., 2001; Yin et al., 2002; Zalfa et al., 2003; Schratt et al., 2004). Another reported enthralling link between BDNF-signaling and FXS is a finding that FMRP-mediated repression of translation requires an interaction with CYFIP1 which also binds and inhibits the translation eukaryotic initiation factor 4E (eIF4E) (Napoli et al., 2008). In the brain, CYFIP1 forms a complex with specific FMRP-target mRNAs and reduced levels of CYFIP1 cause an increase in the synthesis of MAP1b, αCaMKII, Arc and amyloid precursor protein (APP), whose mRNAs are identified to be regulated by FMRP (Bagni and Greenough, 2005; Hou et al., 2006; Westmark and Malter, 2007). Translational repression is regulated in an activity-dependent manner since BDNF or DHPG stimulation of neurons releases CYFIP1 from eIF4E and from bound RNAs at synapses, and this BDNF and glutamate-mediated synaptic activity leads to the alleviation of translation repression, thereby resulting in protein synthesis. Therefore, the translational repression activity of FMRP is mediated, at least in part, by CYFIP1 (Napoli et al., 2008). Indeed, reducing the eIF4E interactions restores the balance between protein synthesis and actin dynamics in a mouse model for *Fmr1*-KO (Santini et al., 2017). Furthermore, BDNF activates mTOR-mediated mRNA translation to regulate synaptic increase in AMPA receptor subunit GluR1 expression which is required for memory formation (Slipczuk et al., 2009). Moreover, dysregulation of mTOR pathway has been shown in the FXS mouse model and humans, where mTOR phosphorylation and activity were elevated along with its upstream targets (Gross et al., 2010; Sharma et al., 2010; Ronesi et al., 2012; Hoeffer et al., 2012; Casingal et al., 2020; for review, see Borrie et al., 2017).

As combined, these BDNF linked-actions can be mechanistically connected to deficiencies seen in the absence of FMRP, in addition to overactivation of group I mGluRs and thus increased activity-dependent protein synthesis and increased AMPA receptor internalization. Consequently, alterations in BDNF signaling via TrkB could significantly contribute to defects seen in FXS.

11.5.5. FXS, neural progenitors and neuronal circuit formation

The correct formation and function of neuronal circuits relies partially on to highly orchestrated progressive genesis of RG NSCs and NPCs where functional neuronal cells are produced with exact quantities at precise time and location during development (Kriegstein and Alvarez-Buylla, 2009; Sousa-Nunes et al., 2010). Defects in this process have been implicated in the absence of FMRP by various research groups (for reviews, see Callan and Zarnescu, 2011; Contractor et al., 2015). Several studies link the loss of FMRP to some aspect of abnormality in NSC or NPC function or/and defect in early cortical formation (for review, see Li and Zhao, 2014). Abnormal expression pattern of stem cell specific genes, but normal neurogenesis in cortical progenitors of 14-weeks fetal human FXS brain at very early developmental stage has been reported (Bhattacharyya et al., 2008). The loss of FMRP led to altered expression of several genes from different categories including transcription factors (Bhattacharyya et al., 2008), indicating that various regulatory signaling pathways may be affected during brain development in FXS. Indeed, ribosome profiling and transcriptomic analysis of aNSC revealed many mitosis and neurogenesis genes that are dysregulated primarily at the mRNA level, while numerous synaptic genes are mostly dysregulated at the translation level, indicating that FMRP controls diverse transcriptional and posttranscriptional gene expression programs critical for neural differentiation (Liu et al., 2018). Alterations in the balance between neurons and glia at different developmental stages and over proliferation of progenitor cell types and defects in the establishment of neuronal networks have been also shown (Castrén et al., 2005; Callan et al., 2010; Luo et al., 2010; Saffary and Xie, 2011; Gonçalves et al., 2013; La Fata et al., 2014). Furthermore, in neurons differentiated from patient derived embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), unusual gene expression profiles during cortical development along with morphological and functional abnormalities have been shown when the function of FMRP is distorted (Urbach et al., 2010; Sheridan et al., 2011; Telias et al., 2013; Doers et al., 2014; Telias et al., 2015a; Telias et al., 2015b; Halevy et al., 2015; Boland et al., 2017; Sunamura et al., 2018; Achuta et al., 2017; Danesi et al., 2018). As combined, these studies suggest that the function of FMRP may be strongly dependent on the developmental timing stage and that the changes in cell proliferation and fate determination control along with migration regulation contributes to circuitry defects seen in the absence of FMRP.

Indeed, early postnatal neuronal circuit formation and plasticity has been investigated in the absence of FMRP. Plasticity defects in the neocortex of *Fmr1*-KO mice were revealed with intracellular recordings in neocortical slices at early postnatal development, while they found that the intrinsic plasticity was normal, synaptic plasticity was altered in such a way that LTD was robust, but LTP was entirely absent (Desai et al., 2006). Subsequently, neuronal circuit and plasticity defects in the developing somatosensory cortex of *Fmr1*-KO mice were observed (Bureau et al., 2008). In this study, a major ascending projection connecting layer IV to layer III was altered in several ways in 2-week-old *Fmr1*-KO mice; the connection strength was low, and layer IV cell axons were spatially diffuse, indicating that FMRP has a role in shaping sensory cortical circuits during developmental critical period. Furthermore, it has been shown that the prenatal critical period plasticity is disrupted in the barrel cortex of *Fmr1*-KO mice. In excitatory thalamocortical synapses of the somatosensory cortex, the absence of FMRP resulted in a dysregulation of glutamatergic signaling maturation in such a way that the fraction of silent synapses persisting to later developmental period was increased and there was a temporal delay in the window for synaptic plasticity (Harlow, et al., 2010). Taken together, these findings suggest that FMRP is required for normal neuronal circuit formation and developmental progression of synaptic maturation (for review, see Contractor et al., 2015).

In summary, these findings highlight the importance of FMRP related functions not only in the early postnatal brain development and synaptic functions but already before synaptogenesis during cortical formation and thus urge additional considerations for the mechanisms underlying FXS.

11.6. Modelling FXS

The cloning of the *FMR1* gene (Heitz et al, 1991; Verkerk et al., 1991) fast-tracked the FXS research. Among the fact, that the *FMR1* gene is highly conserved amongst species (Verkerk et al., 1991), although the size of the human CGG repeat is longer than in other mammals (Deelen et al., 1994). The expression level and the location of the murine Fmrp in different tissues is remarkably similar to the level and location seen in humans (Abitbol et al., 1993; Devys et al., 1993; Hinds et al., 1993). This has also allowed the generation of animal models to study neurodevelopmental diseases such as FXS. A transgenic mouse model: an *Fmr1*-KO mouse, has been created to mimic the human syndrome by inserting a neomycin cassette into exon 5 of the mouse *Fmr1* gene by homologous recombination which results in the loss of FMRP in all tissues (Bakker, 1994; The Dutch-Belgian Fragile X Consortium, 1994).

Although, it is challenging to model human intellectual disabilities in mice, the *Fmr1*-KO mouse recapitulates various aspects of the human FXS disorder, including hyperactivity, macroorchidism, anxiety and deficits in learning and memory (Bakker, 1994; Paradee et al., 1999; Spencer et al., 2005; Brennan et al., 2006). As well susceptibility to audiogenic seizures has been shown (Musumeci et al., 2000; Chen and Toth, 2001). Furthermore, the mouse model shares the main features seen in the brain of human FXS individuals as impairments of synaptic plasticity in dendritic structures and slight abnormalities in different cortical layer structures and brain tissue size abnormalities, yet the principal brain structures being normal (Kooy et al., 1996; Comery et al., 1997; Ellegood et al., 2010). Additionally, dopaminergic dysfunction has been indicated in FXS (Roberts et al., 2005) and studies with *Fmr1*-KO suggest specific abnormalities in forebrain D1 signaling and dopamine release (Ventura et al., 2004; Fulks et al., 2010). Also defects in serotonin synthesis in the brains of young children with autism have been reported (Chugani, 2002; Hanson and Hagerman, 2014) and correlates to findings in *Fmr1*-KO mice model, providing evidence that serotonin could be beneficial in treating various dysregulated pathways in FXS (Costa et al., 2012; Lim et al., 2014; Hanson and Hagerman, 2014). Structural abnormalities in the GABAergic inhibitory neocortical circuits of the adult *Fmr1*-KO mice have also been reported (Selby et al., 2007) along with other alterations in GABAergic system (Gruss and Braun, 2004; El Idrissi et al., 2005; D'Hulst et al., 2006; Gantois et al., 2006). However, the formation and complexity of the brain structures is extended in humans compared to mice. Considerably, the human brain is more dependent on the role of interneurons and astrocytes and so FXS mouse models may not amply reveal discrepancies in these systems (Hansen et al., 2010; Lui et al., 2011; Molofsky and Deneen, 2015). For instance, human interneuron development occurs over a prolonged time period and integrates unique migration and maturation mechanisms to generate more numerous and more complex interneurons (Hansen et al., 2013; Marin, 2013). To this note, some human FXS structural abnormalities, such as striatal, cerebellar or cortical lobe volumes are not either recapitulated in the *FMR1*-KO mouse (Kooy et al., 1999; Ellegood et al., 2010; Lai et al., 2016).

The increased spine density along with immature and/or elongated spine phenotype observed in FXS patients can be seen in the *Fmr1*-KO mouse model in various regions, including neocortex and hippocampus (Galvez and Greenough, 2005; Grossman et al., 2006; 2010; Meredith et al., 2007; Pan et al., 2010; Jawaid et al., 2018). However, the presence and amount of the spine alterations in the *Fmr1*-KO mice vary according to brain region, developmental age and genetic background and interestingly, there appears to be a developmental regulation of the spine phenotype which suggests a dynamic role for FMRP in regulating dendritic spine shape and synapse formation (for reviews, Bassel and Warren, 2008; Santoro et al., 2012; He and Portera-Cailliau, 2013). Taken together, despite some discrepancies, all these resemblances make *Fmr1*-KO mice valid animal model to gain insight into the physiological function of *FMR1*, FMRP and the pathogenesis of FXS.

Additionally, a *Drosophila* model, with null alleles of the *Drosophila Fmr1* (*dFmr1*) gene has been developed to study FXS (Zhang et al., 2001; Schenck et al., 2002, reviewed in Drozd et al., 2018). The *dFmr1* gene in *Drosophila melanogaster* is structurally and functionally well-conserved ortholog of the human *FMR1* gene (Wan et al., 2000). The protein product of *dFmr1* gene; dFMRP, is highly homologuous to the mammalian FMRP and contains identical protein domains, including the RGG box and the two KH domain, thus reflecting well neuroarchitectural and behavioral defects seen in the human syndrome (reviewed in Drozd et al., 2018).

However, it is important to note and consider when choosing different experimental approaches, that there is, species and timing dependencies of FMRP functions. The clinical outcome of FXS is affected in addition to sex differences, by variations in *FMR1*-related measures, like CGG repeat number and the percent of methylation that correlate to *FMR1* mRNA and FMRP expression (for review, see Zafarullah and Tassone, 2019). Moreover, the final human FXS phenotype may be modulated in a complex manner by several environmental factors, other epigenetic modulations and gene polymorphisms. FMRP may essentially affect gene expression during neurogenesis via translational control of epigenetic and transcriptional regulators (Sheridan et al., 2011; Korb et al., 2017). The epigenetic silencing of the *FMR1* gene that leads to methylation of the repeats and the *FMR1* promoter, chromatin condensation, and a loss of FMRP expression that causes FXS occurs only in human and is not mimicked by the engineered mouse models (Brouwer et al., 2007). Meaning that the developmentally regulated inactivation of *FMR1* in FXS human embryos starts with full expression of *FMR1* in the pluripotent state, even with an existing full mutation (Malcov et al., 2007). Consequently, the expression of *FMR1* in FXS humans progressively diminishes until it is completely lost in mature neurons (Willemsen et al., 2002). Importantly, this process is not recapitulated by *Fmr1*-KO mice strains, where the absence of functional FMRP is present throughout development (Verkerk et al., 1991; Mientjes et al., 2006). Intriguingly, this developmentally regulated inactivation of FMRP can be recapitulated by using human embryonic stem cells (hESCs) (Telias et al., 2015), therefore, emphasizing the advantage of their use in future mechanistic studies. Also induced pluripotent stem cell methodologies offer one versatile solution for modelling FXS (for reviews, see Bhattacharyya and Zhao, 2016; Telias 2019).

To conclude, animal models to study FXS provide valuable information in elucidating the molecular, physiological and behavioral phenotypes caused by a deficit in the FMRP function but in parallel with human based models, further specifics of the underlaying molecular mechanisms and more detailed picture of the affected functions is achieved.

11.7. Treatment for FXS patients and future aspects

Although a variety of medication to treat the symptoms of FXS patients is available, currently there is no cure for FXS. However, there is hope that further understanding of its underlying molecular mechanisms would lead to new therapies. At present, symptoms of the patients with FXS can be treated with a combination of behavioral and physiological therapy and assisted with special education. Pharmaceutical treatments include stimulants, 2-adrenergic agonists, selective serotonin reuptake inhibitors (SSRIs) or other serotonergic anxiolytic therapies, anticonvulsants, and atypical antipsychotics (for reviews, see Berry-Kravis and Potanos, 2004; Tranfaglia, 2011; Protic et al., 2019). Rising knowledge of FMRP function in neuronal development and synaptic plasticity enable possibilities for new pharmacological interventions in FXS (for reviews, see: Ligsay and Hagerman et al., 2016; Erickson et al., 2017; Berry-Kravis et al., 2018; Zafarullah and Tassone, 2019).

EXPERIMENTAL SECTION

3. AIMS OF THE STUDY

The overall aim of this study was to elucidate the cellular mechanisms that direct the differentiation and migration of neuronal progenitor cells to their correct position in the developing brain and clarify their role in the pathophysiology of FXS. Particularly, how do the dynamics of Ca^{2+} signaling induced by neurotransmitter and trophic receptor activation impact correct neocortical formation, neuronal-radial glial interactions and synaptic plasticity changes of dendritic structures, were investigated.

The specific aims were as follows:

I. Given that the cell fate specification and its link to the functional properties of NPCs during differentiation were not well-understood, we set out to explore and characterize the neurotransmitter evoked calcium dynamics in differentiating NPCs by using the neurosphere mouse model, in combination with Ca^{2+}-imaging and immunocytochemistry. It was of particular interest to elucidate if changes in receptor/ion channel activity are related to certain cell fate.

II. Our previous findings that blocking mGluR5 and/or TRPC3 signaling causes a drastic reduction in RG process growth (Jansson et al., 2013; Louhivuori et al., 2015) led us to investigate more in detail radial glial-neuronal interactions and the regulation of RG process growth in differentiating mouse neurosphere cultures. Specifically, it was of interest to study the role of NRG/ErbB receptors in the mGluR5/TRPC3-mediated maintenance of RG process growth and in mediating the migratory behavior of cortical progenitors and RG scaffold formation.

III. The important role of BDNF and its TrkB receptor signaling in various cell functions during neuronal development and clues of its potential role in the pathophysiology of FXS (Castren et al., 2002, Lauterborn et al., 2007 and Selby et al., 2007) led us to study *in vitro* and *in vivo* the effects of BDNF/TrkB signaling on NPC differentiation and to mature neurons along with dendritic targeting of *Bdnf* mRNA in the absence of FMRP in the mouse model for FXS.

IV. Our research groups previous finding of altered oscillatory mGluR5-related calcium responses in differentiating NPCs derived from human FXS fetal brain and FXS mouse model (Castren et al., 2005) drove us to study *in vivo* the effects of the absence of functional FMRP on glutamatergic neurogenesis and on the embryonic and early postnatal neocortex formation in FXS.

4. MATERIALS AND METHODS

All used methods are listed in **Table 1**.

Method	Publication
Fetal human brain tissue samples	IV
BrdU injections to analyze proliferation of cells	IV
In utero electroporation	IV
Pilocarpine study	III
Culturing of neurospheres	I, II, III
Differentiation of progenitors	I, II, III
Calcium-imaging	I, II, III
Immunocytochemistry	I, II, III
Immunohistochemistry	III, IV
Microscopy	I, II, III, IV
Nonradioactive *in situ* hybridization	III
Primary cultures of hippocampal neurons	III
RNA interference and transfection efficiency control study	III
Western blotting	III
Enzyme-linked immunoassay	III
Time-lapse imaging	II
RNA isolation, cDNA synthesis, and PCR	II

4.1. Fetal human brain tissue samples and processing for immunostaining (IV)

Postmortem human fetal tissue was obtained in accordance with the guidelines of National Institutes of Health, the government of Finland, and the local Ethics Committee of the Kuopio University Hospital. All investigations were conducted according to the principles of the Declaration of Helsinki. Full informed consents were obtained. A mutation in the *FMR1* gene was detected by polymerase chain reaction (PCR) and Southern analysis. The brain of an 18-week-old FXS fetus with methylated repeat expansion of 276–300 trinucleotide repeats in the 5′UTR of the *FMR1* gene, and two age-matched control fetus brains were fixed in 10% paraformaldehyde (PFA), embedded in paraffin, cut into 7 μm thick coronal sections and further processed for immunohistochemistry (IHC).

4.2. Animals (I, II, III, IV)

Postnatal day 6-7 (P6-7) old CD-2 male mice were used in studies which aimed to shed light on the early postnatal differentiation of NPCs *in vitro* (I). Male *Fmr1*-KO mice and/or their WT littermates with FVB background at embryonic and early postnatal stages and adult age were used as indicated separately in each experiment (II, III, IV). WT NMRI mice were used in *in utero* electroporation and transgene study (IV).

The inbred of FVB *Fmr1*-KO mice and their genotyping by tail-PCR has been earlier described (The Dutch-Belgian Fragile X Consortium, 1994) (III, IV). All animals were housed in standard laboratory conditions (12 h light and dark cycles at 23 °C and where food and water were kept freely available) in a qualified animal facility in accordance with guidelines of National Institutes of Health. All animal experiments were performed corresponding to the guidelines of The Society for Neuroscience and European Economic Community Council Directive. The studies were

approved by the Experimental Animal Ethics Committee of the National Laboratory Animal Center, Finland.

4.2.1. BrdU injections to analyze proliferation of cells (IV)

To mark cells that were actively replicating their DNA in the developing neocortex, 5-bromo-2-deoxyuridine (BrdU), which incorporates into newly synthesized DNA by substituting thymidine during the S phase of the cell cycle was used. A cumulative BrdU labeling (Nowakowski et al., 1989; Takahashi et al., 1995; Haubensak et al., 2004) of embryonic day (E13) or E14 mouse embryos (FVB background) was performed by repeated intraperitoneal (i.p.) injections (3 h intervals for 12 h, 4x50 mg/kg of BrdU; Sigma-Aldrich, Germany) into pregnant heterozygous (+/-) *Fmr1* mice.

4.2.2. *In utero* electroporation and transgene delivery into the mouse brain (IV)

The *Fmr1* gene was mutated by inserting a neomycin cassette into the exon 5 and the transgene delivery into the brain was accomplished with *in utero* electroporation. *In utero* electroporation was performed on pregnant female WT NMRI mice carrying E14 old litter similarly as described previously in detail (Tabata and Nakajima, 2001). The expression plasmids: encoding a fusion protein of FMRP with I304N mutation with enhanced green fluorescence protein (FMRPmt-eGFP) (1.3 µg/µl) and WT FMRP with eGFP (FMRP-eGFP) (1.3 µg/µl) under SV40 promoter, have been described previously (Castrén et al., 2001). In addition, an expression plasmid encoding eGFP under CMV promoter (1 µg/µl) was used as a control (p eGFP-F, Clontech, CA, USA).

4.2.3 Pilocarpine study (III)

Pilocarpine, the non-selective cholinergic muscarinic receptor agonist can be used to induce sustained seizures in rodents (Turski et al., 1989) and leads to activity-dependent accumulation of *Bdnf* mRNA and protein in hippocampal dendrites (Tongiorgi et al., 2006).

2-months-old male *Fmr1*-KO and their WT littermate mice were prepared according to an established protocol to induce sustained seizures by pilocarpine (Turski et al., 1983). Accordingly, the mice were injected i.p. with pilocarpine hydrochloride (300 mg/kg: Sigma-Aldrich, Germany) or controls with phosphate buffered saline (PBS) pH 7.4. In addition, methyl scopolamine (Sigma-Aldrich) was injected (1 mg/kg; i.p.) to reduce peripheral cholinergic effects 30 minutes (min) prior to pilocarpine or PBS-control treatment. All pilocarpine-treated mice developed within the first hour after injection discontinuous seizures. The mice were sacrificed 3 h after pilocarpine injection, at the time of highest pan-*Bdnf* mRNA accumulation in neurons (Tongiorgi et al., 2004) and further processed for *in situ* hybridization study.

4.3. Mice brain tissue and processing (III, IV)

Postnatal and adult mice were anesthetized with carbon dioxide (CO_2) and sacrificed by cervical dislocation before brain tissue extraction except for *in situ* hybridization study. For IHC studies during cortical development, the pups were quickly removed from the uteri of pregnant *Fmr1* +/- female mice and sacrificed by decapitation at E16 (IV), E17 (III, IV) or E18 (IV). Then the brains were freshly collected and processed for either paraffin (III, IV) or frozen (IV) sectioning based IHC. The brains collected from P5 or P7 day old male *Fmr1*-KO mice along with their WT littermates were processed for paraffin-embedded immunofluorescence-based stereology (IV).

All the brains collected for paraffin- or frozen-sectioning based IHC were fixed in 4% PFA overnight (o/n) and washed twice in PBS after fixation. For frozen sectioning, the brains were cryoprotected in 30% sucrose solution o/n at 4 °C and afterward mounted into Tissue-Tek (Sakura Finetek, Zoeterwoude, Netherlands), frozen on dry ice and cut into 20 µm coronal sections and

finally thaw-mounted onto object glasses with MICROM HM 550 cryostat (MICROM International GmbH, Walldorf, Germany) (IV). Whereas for paraffin-based sectioning, the fixed brains were dehydrated through increasing alcohol series: 70% ethanol for 2 h, 96% ethanol for 2 h and 100% ethanol o/n at 4 °C. Subsequently, the brains were treated twice for 2 h with xylene (Riedel-de Haën, Germany) before immersion in paraffin o/n at 60 °C. Then the brains were molded into paraffin and cut to 20 µm thick coronal sections on object glasses with a rotary microtome MICROM HM 355 S (MICROM international GmbH) (III, IV).

For nonradioactive *in situ* hybridization study, the 2-month-old male *Fmr1*-KO and WT littermate mice were transcardially perfused with 4% PFA under ketamine (100 mg/kg: i.p.) anesthesia. Right after, the brains were removed and kept in 4% PFA with 20% sucrose at 4 °C for at least 5 days before sectioning to 40 µm thick free floating coronal sections (Tongiorgi et al., 2004) (III).

For Western blotting and enzyme-linked immunoassay (ELISA) studies, the brain tissue samples of hippocampi and prefrontal and motor cortexes were freshly collected from 2–4-month-old male *Fmr1*-KO mice and their WT littermates (III). Samples were frozen on dry ice, homogenized and processed in a lysis buffer as previously described (Castrén et al., 2002).

4.4. Extraction of neural progenitors from mice brains (I, II, III)

Neural progenitors were extracted from mice brains and grown as free floating neurosphere cultures to study the fate determination and migration of NPCs *in vitro* during embryonic and early postnatal development as previously described (Castrén et al., 2005).

P6-7 old CD-2 male mice were sacrificed quickly by decapitation (I). Whereas the pregnant +/- *Fmr1* mice were anesthetized with CO_2, sacrificed by cervical dislocation and right after, the E14 pups were quickly removed from the uteri and sacrificed by decapitation (II, III). All brains were removed and kept on ice in cold 1% bovine serum albumin (BSA)-PBS solution supplemented with 1M glucose until further processing (I, II, III). The small brain tissue pieces were dissected under light microscope at P6-7 (I) or E14 (II, III) from the cortical wall of the lateral ventricles into a 1% BSA-PBS solution supplemented with 1M glucose. See **Figure 9**. Cell tissue samples were then dissociated (30 min at 37 °C) with DNAse, trypsin (1.33 mg/ml), hyaluronidase (0.7 mg/ml) and kynurenic acid (0.2 mg/ml) dissolved in Hank´s balanced salt solution (HBSS) supplemented with 2 mM glucose. Next, the cells were centrifuged (at 200 g for 5 min), resuspended in 0.9 M sucrose in 0.5x HBSS, and centrifuged again (for 10 min at 750 g). Then the cells were resuspended in 2 ml of Earl's balanced salt solution (EBSS) and centrifuged through a gradient of the same salt solution supplemented with 4% BSA and 15 mM HEPES (at 200 g for 7 min).

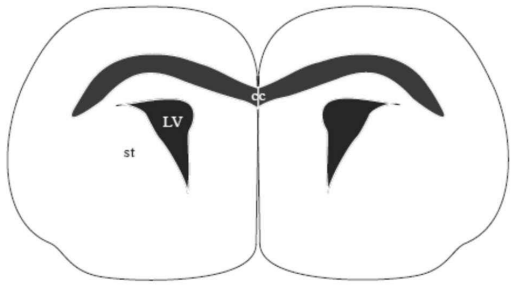

Figure 9. *Coronal view of postnatal mice brain showing the location of lateral ventricle.* The neural stem and progenitor cells were extracted from the cortical side of the thin wall of ventricular zone which surrounds the lateral ventricles. Abbreviations: cc=corpus callosum, LV=lateral ventricle and st=striatum.

4.5. Culturing of neurospheres (I, II, III)

The dissociated cells were plated in Dulbecco's modified Eagle's medium Nutrient Mix F-12 (DMEM-F12; Gibco, Life Technologies, Invitrogen) culture medium supplemented with 2 mM L-glutamine, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/ml Penicillin, 100 mg/ml Streptomycin, B27 supplement (2 %) (serum-free antioxidant cocktail with vitamin A, and insulin; Gibco, Life Technologies), 20 ng/ml epidermal growth factor (EGF; Gibco, Life Technologies), and 10 ng/ml fibroblast growth factor (FGF-2, PeproTech EC Ltd, London, UK). The cells were then grown as free-floating neurosphere cultures in the 5% CO_2-humidified incubator. Mitogens, (20 ng/ml EGF and 10 ng/ml FGF-2) were added to the medium every third day and one half of the growing medium was refreshed every 3 to 4 days. The neurosphere cultures were passaged every 7-9 day mechanically with glass capillary pipet with (I) or without papain (II, III) (0.5 mg/ml, Sigma-Aldrich, Taufkirchen, Germany) and regrown back to neurospheres from single cells at cell density of 100.000 cells/ml.

4.6. Differentiation of neurospheres (I, II, III)

For NPC differentiation studies 20 to 30 round neurospheres (diameter around 225 µm) with nearly translucent centers were collected under light microscope from the free-floating neurosphere cultures with a 10 µm pipette. The collected neurospheres were then placed on cover slips coated with 10 µg/ml poly-DL-ornithine (Sigma-Aldrich, Taufkirchen, Germany) and differentiated in DMEM-F12-based culturing medium without the mitogens (EGF and FGF-2) for 5h to 20 days as indicated separately in each experiment. One third of the DMEM-F12-based culturing medium was changed every third day during the differentiation of neurospheres. To study the differentiation mechanisms of progenitors' antagonists or agonists were added as indicated separately in each respective experiment (I, II, III).

The mGluR5 antagonist, MPEP (2-methyl-6-(phenylethynyl)pyridine hydrochloride) (1-3 µM) and mGluR1 specific blocker; LY367385 (3 µM) were both from Tocris Bioscience, Bristol, UK (I, II). NGR3 (Neuregulin3), inhibitor of the ErbB1/4 receptor tyrosine kinase activity, afatinib (4 µM) and ErbB1 receptor specific blocker, gefitinib, pyr3, a selective blocker for transient receptor potential channel 3 (TRPC3) and nonselective TRPC cation channels blocking, pyr2 were all purchased from Sigma-Aldrich or Tocris Bioscience (II). SKF96365, that blocks some receptor operated channels, Gd^{3+} lanthanide, a selective orai channel blocker, 2-aminoethoxydiphenylborate (2-APB; 1 mM), a blocker of store operated Orai channels and Ilomastat (1 µM), a pan inhibitor of MMP/ADAM proteases were all acquired from Sigma-Aldrich or Tocris Bioscience (II).

4.7. Calcium-imaging of differentiated neurosphere derived cells (I, II, III)

Intracellular Ca^{2+}-imaging allows the analysis of calcium-dependent receptor activation mechanisms and reveal functional receptor responses to various neurotransmitter or growth factor stimulations in differentiating neuronal cells.

For the measurements, the differentiated cells were incubated at 37 °C for 20 min with 4 µM fura-2-acetoxymethyl-ester (fura-2AM; Molecular Probes, Life technologies) in a HEPES buffered Na^+ medium consisting of: 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose, 20 mM HEPES and 1 mM probenecid (pH adjusted to 7.4 with NaOH). Following incubation, the coverslips were placed on the bottom of a thermostat-controlled holder on the stage of an inverted Nikon microscope perfusion chamber (37 °C). Warmed (37 °C) Na^+-Ringer solution (with or without stimulants) was continuously perfused through the chamber at a rate of 2 ml/min during recordings. Around 50 single cells near the edge of the differentiated neurospheres were simultaneously recorded using 340 and 380 nm light excitation filter changer

under the control of an InCytIM-2 system (Intracellular Imaging corp., Cincinnati, OH, USA) and dichroic mirror (DM430, Nikon). The light emission was measured through a 510 nm barrier filter with an integrating charge-coupled device camera (COHU Inc, Poway, CA, USA). The cells were excited by alternating wavelengths of 340 and 380 nm and an image of 340 nm/380 nm ratio was taken every second. The collected data was analyzed with the InCyt 4.5 software (Intracellular Imaging Inc.) and further processed with Microsoft Excel and Microcal Software OriginPro 6.0, 6.1 or 7.5 (OriginLabCorp, Northampton, USA, www.originlab.com).

The differentiating cells were challenged when differently specified in each experiment with various substances known to elevate intracellular Ca^{2+}.
Among these were: angiotensin II (AngII; 1 μM; I), orexinA (Ox-A; 100 Nm; I), substance P (SP; 1 μM or 100 nM; I), neurotensin (NT; 10 μM; I), the muscarinic agonist oxotremorine (OxoM; 10 μM; I, II), acetylcholine (Ach; 100 μM; I, II), adenosine triphosphate (ATP; 10 μM or 100 μM; I and III), noradrenaline (NE; 10 μM; I), glutamate (5 μM or 100 μM) in the absence and in the presence of extracellular Ca^{2+} in the Na^{+}-Ringer solution (I, II), NMDA (50 μM; I and III) without $MgCl_2$ in the Na^{+}-Ringer solution and high potassium chloride (70 mM K^{+} using iso-osmotical replacement of NaCl with KCl; I) and DHPG (10 mM, 3,5-dihydroxyphenylglycine, II), EFG domain of NRG1 (100 ng/ml, III), EGF (20 ng/ml, II), Kainate (10 μM: II) and brain derived neurotrophic factor (BDNF; 20 ng/ml; III). All purchased either from Sigma-Aldrich, Germany or Tocris Bioscience.

4.8. Immunocytochemistry of differentiated neurosphere derived cells (I, II, III)

Immunocytochemistry (ICC) was used to examine the expression of certain receptor proteins of interest and their cell type/spatial localization. All used immunomarkers are listed in **Table 2.**

Cells on the cover slips were fixed (10 min at RT) with 4% PFA in PBS, pH 7.4, and rinsed with PBS (2× 5min). Next the cells were permeabilized with freezer cold methanol (20 min) and rinsed (2× 5 min) with PBS. To prevent the non-specific binding of the antibodies, the cells were treated with 20% normal goat or horse serum (NGS; HS; Chemicon International, Temecula, CA, USA) in PBS (20 min in RT, I, III) or 10% normal donkey serum (NDS)/1%BSA/0.1% Triton X-100/PBS (45 min in RT, II). Right after, the cells were incubated with primary antibodies diluted in 5% NGS or HS in PBS or 1% donkey serum in 1% NDS/1% BSA/0.1% TritonX-100/PBS (for 1 hr at 37 ˚C or o/n at 4 ˚C) and rinsed with PBS (3× 5 min). Thereafter, the cells were stained with appropriate secondary antibody (at 37 ˚C in dark for 45 min) and rinsed again with PBS (3× 5 min). For double or triple staining of the cells, incubations with another primary and suitable secondary antibody/antibodies and subsequent PBS washes were performed. In most of the experiments the nuclei of the cells were counterstained with 4',6-diaminodino-2-phenylindole (DAPI; 0,1μg/ml; Boehringer Mannheim Biochemica, Germany). Finally, the PBS rinse step was repeated (3× 5 min), and the dried cover slips were mounted to glass slides with Gel Mount™ Aqueous Mounting Medium (Sigma-Aldrich, St Louis, Missouri, USA; I) or with Dapi-containing Vectashield mounting media (Vector Laboratories, Inc. USA; II, III) and the cover slip edges were sealed with nailpolish. The cells were viewed and pictured with suitable filters using either epifluorescence microscope Olympus AX-70 or with confocal imaging LSM 5 Pascal system (Zeiss). The specificity of the antibody staining, and background fluorescence was controlled by omitting either the primary or secondary antibody.

4.9. Immunohistochemistry (III, IV)

The cellular expression of protein of interest was studied by immunostaining of histological brain sections. All used immunomarkers are listed in **table 2**.

Frozen sections were incubated in a 1:1 ethanol/chloroform solution to remove fat from the brain tissue and after rehydrated through descending alcohol series as paraffin sections (100%, 96% and 70% ethanol and pure water, 5 min each; IV). Whereas, for paraffin based IHC the sections were first deparaffinized with xylene (3 times for 10 min; Riedel-de Haën) and then rehydrated with descending alcohol series (III, IV). For antigen retrieval, the paraffin sections were boiled for in 10 mM citrate buffer (pH 6.0, 15 min) and then rinsed with water and PBS. Background blocking and permeabilization was performed with 0.5% Triton X-100 in 20% normal goat serum or horse or rabbit serum in PBS depending on the secondary antibodies used (1 h at RT). Incubation with the primary antibody (o/n at 4 °C) was followed by washes with PBS (3× 5 min). Then the sections were incubated with the secondary antibody diluted in PBS (1 h at RT in dark) and after rinsed with PBS (3× 5 min). The cell nuclei of the brain sections were counterstained with DAPI (0,1 ug/ml, Boehringer Mannheim Biochemica, Germany). The sections were mounted with Gel Mount™ Aqueous Mounting Medium (Sigma-Aldrich). The sections were visualized and imaged with a LSM 5 Pascal system (Zeiss). The specificity of the antibodies was controlled by immunostaining adjacent brain section glass slides with omitting either the primary or secondary antibody and imaging the samples with similar light excitation values as in known control experiments.

Nissl staining detects Nissl bodies or large granules of rough ER (with free ribosomes) which are the sites of protein synthesis in neurons by using aniline stain to label extranuclear RNA granules purple-blue and is commonly used for identifying the basic neuronal structure of the brain (III, IV). For Nissl staining, after rehydration the brain sections were incubated in a cresyl violet solution (Sigma) (1 to 5 min) and washed with water x2. The sections were dehydrated by sinking into 70% ethanol (5 min), in 96% ethanol (10 min) and 100% ethanol (2x5 min) and cleared by immersing to xylene (2× 5 min). The sections were mounted with DePex medium (BDH Laboratory Supplies, England).

In the colorimetric detection of rabbit anti-ER81 (1:5000, Abcam, IV), biotinylated goat anti-rabbit IgG (1:150, Vector Laboratories, CA, USA) was used as secondary antibody, and the sections were stained according to the manufacturer's instructions with Vectastain ABC kit and DAB-substrate kit for peroxidase/nickel (both from Vector Laboratories). The sections were counterstained with the most common nuclear counterstain, hematoxylin**,** that produces blue Hematein via an oxidation reaction with nuclear histones causing cell nuclei's to show blue.

The detection of BrdU labeled cells was performed according to manufacturer's instructions with a commercial kit (Amersham, GE Healthcare, Life Sciences, RPN20, IV) as previously described (Hienola et al., 2004). The nuclease containing mouse monoclonal anti-BrdU antibody was mixed with the aqueous cell proliferation labeling reagent and then brain sections were incubated (at 4 ºC o/n) and standard IHC staining protocol was normally proceeded after.

For detection of proliferating cells in human fetal brain tissue (IV), the sections were autoclaved in 10 mM citrate buffer (pH 6.0, 10 min at 120 °C) and then left in the buffer to cool down (60 min in RT). Polyclonal antibody against Ki67 nuclear antigen (1:100, DAKO, Glostrup, Denmark) and the streptavidin-alkaline phosphatase system for immunodetection (Histomark Kit, KLP, USA) were used. The incubation with primary antibody was carried out o/n at 4 °C. Reaction product, the streptavidin–biotin-complex, was visualized using Vector-Red (Vector Labs, CA, USA).

Table 2. Primary and secondary antibodies used in the immunostainings.

Primary antibody	Dilution	Method	Provider	Publication
anti-BDNF (rabbit)	1:500	ICC	Chemicon	III
anti-BLBP (rabbit)	1:500, 1:1000	ICC, IHC	Chemicon	I, II, III, IV
anti-BrdU-kit (mouse)	*	IHC	Amersham/GE Healthcare	IV
nuclei staining with DAPI	*	ICC, IHC	Boehringer Mannheim Biochemica/ Vector Laboratories, Inc.	I, II, III, IV
Anti-EAAT1 (rabbit)	1:500	ICC	Abcam	I, II, III
anti-ER81 (rabbit)	1:5000	IHC	Abcam	IV
anti-ErbB4 (mouse)	1:100	ICC	Abcam, as a gift from Klaus Elenius	II
anti-GFAP (rabbit)	1:250	ICC	Sigma-Aldrich	I
anti-GFP (rabbit)	1:500	IHC	Nordic Biosite AB, Sweden	IV
anti-GLAST (guinea pig)	1:400	ICC, IHC	Chemicon	I, II, III, IV
anti-HER4/ErbB4 (clone E200) (rabbit)	1:100	ICC	Merck Millipore, Germany	II
anti-Ki67 (goat)	1:200	IHC	Santa Cruz Biotechnology	IV
anti-nestin (clone rat-401) (mouse)	1:200-400	IHC	DSHB, University of Iowa, USA	IV
anti-NeuN (mouse)	1:400	ICC	Chemicon	I, II, III
anti-MAP2 (rabbit)	1:500	ICC	Chemicon	I, II, III
anti-mGluR5 (rabbit)	1:250, 1:500	ICC	Chemicon	I
anti-Tbr1 (rabbit)	1:1000	IHC	Chemicon	IV
anti-Tbr2 (rabbit)	1:2000	IHC	Chemicon	IV
anti-TrkB.FL (goat or rabbit)	1:150, 1:250	ICC, IHC	Santa Cruz Biotechnology	III
anti-βIII-tubulin (mouse)	1:500	ICC	Babco, Richmond, CA, USA	I, II, III
Cy3-conjugated anti-mouse IgG	1:500	ICC	Jackson ImmunoResearch, West Grove, PA, USA	I, II, III
Cy3-conjugated anti-guinea pig IgG	1:500	ICC	Jackson ImmunoResearch, West Grove, PA, USA	I
Cy2-conjugated goat anti-guinea pig	1:200	ICC	Chemicon	IV
AMCA-conjugated goat anti-rabbit IgG	1:250	ICC	Jackson ImmunoResearch, West Grove, PA, USA	I
Alexa Fluor 488, 546 or 568- conjugated goat or donkey anti-rabbit, anti-mouse or anti-guinea pig or anti-goat or anti-donkey IgG	1:400, 1:500, 1:1000	ICC, IHC	Molecular Probes, Life Technologies, Invitrogen, Ltd USA	I, II, III, IV

* Diluted according to manufacturer's instructions

The primary antibodies were visualized by utilizing the following fluorescent secondary antibodies that were diluted in PBS at the following working concentrations: Cy3-conjugated anti-mouse IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA) or Cy3-conjugated anti-guinea pig IgG (1:500), Cy2-conjugated goat anti-guinea pig (1:200, Chemicon) and AMCA-conjugated goat anti-rabbit IgG (1:250; Jackson ImmunoResearch) or Alexa Fluor 488, 546 or 568-conjugated goat or donkey anti-rabbit, anti-mouse or anti-guinea pig or anti-goat or anti-donkey IgG (1:400; 1:500; 1:1000; Molecular Probes, Life Technologies, Invitrogen, Ltd USA).

4.10. Brightfield, epifluorescence and laser scanning confocal imaging (I, II, III, IV)

Immunostainings were viewed and imaged with an epifluorescence microscope Olympus AX-70 (I) or Axioplan 2 microscope system (Zeiss, Jena, Germany) connected to a high-resolution AxioCam camera (Zeiss) and AxioVision 4.1 (Zeiss) software (I, II, III, IV). LSM5 Pascal laser scanning confocal microscopy system (Zeiss) with water immersion objectives (10x/0.45 W C-Apochromat, or 25x or 40x) were used for imaging of differentiated NPC cultures (I, II, III) and brain sections (III, IV). For stereological analyses, high resolution images from ER81 and hematoxylin-stained brain sections were obtained with a digital MicroFire S99808 camera (Optronics, CA, USA) attached to Olympus BX51 epifluorescence microscope (Olympus) with a 60x oil objective (Olympus) and Stereo Investigator software (MicroBrightField, Inc., Vermont, USA) (IV). In all these studies the fluorophores were excited with an air-cooled argon-krypton, helium-neon gas or 405/UV lasers (Omnichrome; Melles Griot, Carlsbad, CA, USA) through proper excitation nanometer (nm) filter-cubes (350/ultraviolet, 450/blue, 550/green, 600/yellow and 650/red) with emission bands of 405-450 (blue), 488 (green), 546 (orange) or 568 (red) nm and the help of short and long bandpass filters. Crosstalk between these different channels and background noise was eliminated with sequential scanning of different fluorophores and frame averaging. In confocal microscopy, stacks of images taken at 0.85 µm (cell cultures) or 3 µm (brain sections) intervals on z-dimension were compiled to compose maximum intensity projection images. Image stack sizes in z-dimension were approximately: 13 µm for differentiating neurospheres (I, II, III) and 23 µm for brain sections (III, IV).

All the acquired images were further processed with LSM Image Browser Software (Zeiss) or ImageJ and image analysis was done with Image-Pro Plus version 6 or 6.1 (Media Cybernetics, Inc., Silver Spring, MD, USA) or ImageJ.

4.11. Nonradioactive *in situ* hybridization (III)

Nonradioactive *in situ* hybridization was used to examine the expression and subcellular localization of *Bdnf* mRNA *in vivo* in pyramidal neurons of cortical layer 5 and hippocampal CA1 area and *in vitro* in hippocampal neuron cultures.

In situ hybridization on free-floating, 40 µm thick mouse coronal sections cut at the level of dorsal hippocampus was performed as previously described (Tongiorgi et al., 2004). All *in situ* hybridizations on pilocarpine-treated brain sections were carried out sequentially with brain sections of control animals. Hippocampal neuron cultures were analyzed by *in situ* hybridization after 48 h of silencing of *Fmr1* specific small interfering RNAs (siRNAs), either in normal growing medium conditions or after depolarization for 3 h with 10 mM KCl as previously described in detail (Tongiorgi et al., 1997).

4.12. Primary cultures of hippocampal neurons (III)

Primary cultures of hippocampal neurons provide an established *in vitro* model to study the effects of pharmacological substances on neurons and were used to verify the *in vivo* studies of subcellular localization of *Bdnf* mRNA.

Rat hippocampal neuron cultures were prepared at P0-1 as described in detail by (Tongiorgi et al., 1997).

4.13. RNA interference and transfection efficiency control study (III)

RNA interference (RNAi) was used to study the effects of reduced FMRP expression to the subcellular localization of *Bdnf* mRNA in cultured hippocampal neurons.

Fmr1 siRNAs (Sequence #1 GGUUUAUUCCAGAGCAAAUtt corresponding nucleotide (nt) 460–478 in exon 4 and #2 GCAUGUGAUGCUACGUAUAtt nt 554–572 in exon 5; GenBank NM 008031.2) and negative control siRNA were all from Ambion, Life technologies. Transfection of cultured hippocampal neurons was performed after 11 days *in vitro* using 30 nM of *Fmr1* or control siRNA mixture with 1 µl of siPORT NeoFX (Ambion, Life technologies) per well, following manufacturer's instructions. To test the efficiency of siRNA duplexes to reduce FMRP expression, rat pheochromocytoma cells (PC12) were grown for 24 h and then transfected with siRNAs using Lipofectamine 2000 (Invitrogen, Life technologies). At 48 h post-transfection, the cells were harvested and immunoblotted with an anti-FMRP antibody (1:1000; Abcam, UK) in Western analysis. The detection was performed by chemiluminescence using the ECL++kit (Amersham, GE Healthcare Life Sciences).

4.14. Western blotting (III)

Western blotting is a commonly used analytical technique in molecular biology to reveal proteins of interest in a tissue extract sample. The expression of the TrkB and BDNF proteins was studied from samples of proliferative cortical neurosphere cultures and mice brain lysates of hippocampi and prefrontal and motor cortexes with Western analysis. Also, FMRP was investigated in siRNA studies.

The obtained samples were homogenized in a lysis buffer (20 min at 4 °C) and centrifuged (13000 rpm for 15 min at 4 °C). Bio-Rad DC protein assay was used to determine the total protein concentration of the supernatant samples and afterward volumes according 60 µg of total protein were loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page) which separates proteins according to their electrophoretic mobility. The protein extracts (60 µg) were electrophoresed on (7.5% or 15%) SDS-polyacrylamide minigels and transferred to polyvinylidene fluoride (PVDF) membranes (Schleicher & Schuell) (for 1 h at 400 mA). The membranes were washed (10 min) in Tris-buffered saline (TBS; 0.1 M Tris, 0.15 M NaCl; pH 7.4) and blocked in 5% non-fat dry milk, in TBS with 0.1% Tween 20 (TBS-T) (1.5 h at RT in gentle agitation). Then the membranes were probed against target proteins, through the incubation with the primary mouse TrkB (1:2000; BD Transduction Laboratories™) or polyclonal rabbit BDNF (1:200; Chemicon) (o/n at 4 °C in gentle agitation). To remove unbound primary antibody the membranes were washed in TBS-T and after incubated with the secondary goat anti-mouse or goat anti-rabbit horseradish peroxidase (HRP)-linked antibody (1:5000) (1.5 h at RT in gentle agitation). Protein detection was performed using the ECL++ kit according to manufacturer's instructions (Amersham, GE Healthcare Life Sciences). The membranes were exposed to X-ray film (Fuji), scanned with Epson Perfection V750 Pro (Epson, America, inc.) or imaged with luminescent image analyzer Las-3000 (Fuji FILM, Japan) and analyzed with ImageJ (NIH).

The specificity of the Western blot results was confirmed by negative controls and with the assistance of known molecular weight size markers. Ponceau S staining was used to control the lane loading of the samples, uniformity and overall effectiveness of the protein transfer from the gel to the membrane. As well tubulin protein antibody detection was utilized after membrane stripping for protein amount normalization.

4.15. Enzyme-linked immunoassay (ELISA) (III)

Through the utilization of an enzyme-linked monoclonal antibody specific for mature human BDNF whose primary structure is conserved among all mammalian species the quantitative sandwich ELISA technique allows the determination of BDNF levels in various sample types and across species.

The BDNF expression was determined in proliferative cortical neurosphere cultures, mice brain tissue samples using BDNF ELISA (Quantikine human BDNF kit, R&D Systems, Abingdon, UK) following manufacturer's guidelines. Cortical neurosphere culture and brain tissue samples of hippocampi and prefrontal and motor cortexes were homogenized and processed in a lysis buffer as previously described (Castrén et al., 2002). The total protein concentration of the tissue and cell samples was determined using Biorad DC protein assay. To determine the measured BDNF concentration, the absorbance values of sample duplicates at 450 nm were averaged and the background value was reduced (absorbance measured at 540 nm) along with reduction of zero value. The BDNF concentrations were calculated as pg/ml according to the standard curve, corrected for dilution factor and total protein concentration. Used dilution factor was 2.5 for the mice brain tissue lysates (Calibrator Diluent RD5K) and no dilution was used for the cell culture samples. The specificity of the ELISA results was confirmed by using lysis buffer and cell medium without protein extract as negative controls. Standard BDNF sample duplicates were included to every ELISA plate to control and standardize the measured values.

4.16. Phase-contrast microscopy with Cell-IQ (II)

Time-lapse imaging of differentiating neurosphere cultures was performed in an enclosed cell-culturing instrument combining phase-contrast microscopy, automation and environmental control (Cell-IQ® system, Chip-Man Technologies Ltd, Finland). The imaging system enables through integrated gas flow-controlled incubator, precision movement stages (x and y axes: – 1 mm; z axis: –0.4 mm) and automated optics module with analysis software, a continuous monitoring of cells in two 6-well plates in an integrated plate holder. The obtained image series and cellular movement were further analyzed with ImageJ software and the data computed with Excel (Microsoft, Redmond, USA) and Microcal Software OriginPro 6.0 or 6.1 (USA, www.originlab.com)

4.17. RNA isolation, cDNA synthesis, and PCR of neurosphere samples (II)

Total RNA was isolated from proliferating neurosphere cultures and after differentiation of 1, 3 and 5 days using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Concentration of the RNA was quantified using a NanoDrop ND8000 spectrophotometer (Thermo Fisher Scientific, USA). Transcriptor High-Fidelity cDNA Synthesis Kit (Roche Applied Science, Switzerland) and random hexamer primers were used for cDNA synthesis. The resulting cDNA was amplified in the T100 thermal cycler (BIO-RAD, USA) with the primers for neuregulins (NRG1, NRG2, and NRG3) and ErbB3 and ErbB4 (see **article II, table 1**). As a positive control for the PCR reactions, an adult mouse whole brain cDNA (Takara Clontech, CA, USA) was used. The obtained PCR products were run on 1.5% agarose gel with ethidium bromide and the images were captured with the MultiImageTM light cabinet (Alpha Innotech Corporation, CA, USA).

4.18. Data Analysis

4.18.1. Statistical analysis of the immunostaining data (I, II, III, IV)

Image stack processing of the images obtained with a LSM 5 Pascal confocal system (Zeiss, Inc.) was done with LSM Image Browser (Zeiss, Inc.) or ImageJ. All image analysis was performed with Image-Pro Plus version 6.0 or 6.1 (Media Cybernetics, Inc., Silver Spring, MD, USA) or with CorelDRAW Graphics Suite X3 (Corel Cooperation, Canada). The brightness and contrast were adjusted for cell counting in each experiment. The cell counting analyses were performed semi-quantitatively from either fluorescent-stained maximum projection images of differentiated neurospheres (10x, 25x or 40x magnification) (I, II, III) or from confocal or brightfield images (10x or 25x magnification) of immunostained brain sections (III, IV). For the experiments, 2-13 sections from each 3-6 male mice per group were blindly analyzed, then averaged and pooled

together. For semiquantitative analyses of the brain sections, the number of cells expressing the immunostaining was counted from the developing neocortex as described previously with minor modifications (Fukumitsu et al., 2006). Cell counting analysis was performed with "auto count" function with defined cell size limits and threshold values with defined counting frame sizes or cortical sector dimensions. Some of the obtained results were also confirmed with hand-counting. The TrkB.FL (III) and BrdU, Tbr2, Tbr1 (IV) positive cells were analyzed from an area of the dorsomedial neocortical wall above the medial part of lateral ventricle that corresponds to the primary somatosensory cortex. Nissl- and DAPI staining was used for the layer determination in the developing cortex (III, IV). The number of TrkB.FL expressing cells was analyzed in addition to intermediate zone (IZ) and cortical plate (CxP) of the somatosensory neocortex as well from hippocampus (HC), globus pallidus (GP) and lateral dorsal/posterior thalamic nucleus (ldt/lpt). The coronal somatosensory neocortical level at E16 and E17 was approximately 860 µm and 1.3 mm posterior to the rostral border of olfactory bulbs (Jacobowitz and Abbott, 1998).

After *in utero* electroporation, the stained cells were counted from a cortical sector with mediolateral dimension of 395 µm (IV). In Nissl-stain analysis, all positive cells were counted in a cortical sector ranging from pial surface to lateral ventricle with mediolateral dimension of 430 µm at E16 and in with mediolateral dimension of 540 µm at P5 (IV). In human fetal brain sections, Ki67-positive cells were analyzed from images of neocortical sector with mediolateral dimension of 410 µm (IV). Stereological analysis of the ER81 immunostained P5 brain sections was performed with the Stereo Investigator software (MicroBrightField, Inc., Vermont, USA) attached to MicroFire S99808 digital camera (Optronics) and Olympus BX51 (Olympus) epifluorescence microscope with a 60×oil objective (Olympus). Stereological assessment was done on 9-12 coronal serial sections of somatosensory cortex per brain, ranging around 1.18 mm to -0.94 mm from Bregma according to mouse brain atlas (Paxinos and Franklin, 2001).

Prior to statistical testing the normality of the data distributions was tested. Independent two population Student's *t*-test was used in the statistical analysis of confocal imaging data. Statistical significance was set at level ns, not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. All the confocal imaging data are represented as means ± standard error of mean (SEM).

4.18.2. Other statistical data analysis (I, II, III, IV)

The calcium-imaging data and along with ELISA and PCR data were analyzed, and all statistical comparisons were made with Microsoft Excel (Microscoft, Redmond, WA, USA) and Microcal Software OriginPro 6.0 or 6.1 (USA, www.originlab.com) (I, II, III, IV). Cell-IQ acquired time lapse image series (II) were analyzed with ImageJ (Schneider et al., 2012) and for cell movements analysis with its plugin, MJtracker (Meijering et al., 2012) and the data quantified with Excel (Microsoft, USA) and Origin 6.0 (OriginLabCorp). Western blot analysis was performed with ImageJ (III). *In situ* hybridization data was quantified as previously described (Tongiorgi et al., 1997; 2004). Statistical data analysis was performed with Sigma Stat 3.2 software (Systat Software, Inc.) and graph elaborations with Sigma Plot 11 software (Systat Software, Inc.). Image processing and graphical illustrations were done with CorelDRAW Graphics Suite X3 (Corel Cooperation, Ontario, Canada). Prior to statistical testing the normality of the data distributions was tested. Statistical significance among sample groups was evaluated by performing one-way analysis of variance (ANOVA) and followed by Dunn's *post-hoc* comparison (III) or Tukey *post hoc* tests (II). Student's nonpaired *t*-test was used in experiments with two groups (I, II, III). The statistical significance was set at level ns, not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and the values are represented as means ± (standard error of mean) SEM or ± standard deviation (SD) (I, II, III, IV, respectively).

5. RESULTS

5.1. Characterization of differentiated mice-derived neurosphere cultures (I)

Several lines of evidence indicate that neurotransmitters acting on GPCRs, or ionotropic receptors impose a considerable impact on neuronal plasticity and development together with growth factors (reviewed in, Hagg, 2009; Padamsey et al., 2018). Spontaneous changes in both, local and global $[Ca^{2+}]_i$ have been shown to play essential roles in the differentiation of NPCs (Ciccolini et al., 2003) and may determine their neurotransmitter phenotype (Spitzer et al., 2004; Rosenberg and Spitzer, 2011). Thus, receptor-mediated changes in $[Ca^{2+}]_i$ may represent a common target for factors and signals that regulate NPC differentiation and acquisition to specific neuronal phenotypes. However, the mechanisms involved in determining the differentiation of NPCs into mature functional neurons have not been completely resolved and specifically, the information concerning neurotransmitter responsiveness of NPCs in relation to cell maturation, specification and differentiation during brain development is still lacking. Therefore, we set out to explore these processes more closely in differentiated neurosphere cultures derived from postnatal mouse brains. Specifically, it was of interest to characterize Ca^{2+}-mediated responses thoroughly and reveal if subpopulations of cells can be discovered based on neurotransmitter receptor responsiveness during differentiation.

5.1.1. Characterization of NPCs based on their functional calcium responses to various neurotransmitters (I)

To gain insight into the dynamics of neurotransmitter responsiveness, cortical progenitors were extracted from P6 CD-2 mice brains and grown as free-floating neurosphere cultures in the presence of mitogens EGF and FGF-2 and afterward differentiated 1 to 8 days by the withdrawal of the mitogens and then studied with Ca^{2+}-imaging. The NPCs were challenged with neuropeptides and neurotransmitters angiotensin II (AngII), neurotensin, orexin-A (Ox-A), substance P (SP), ATP, acetylcholine (Ach), norepinephrine (NE), oxotremorine (OxoM), as well as with 70 mM of K^+ to assess the presence of functional VGCCs. Our results showed that during the first day of differentiation, NPCs were surprisingly homogenous in the Ca^{2+} responses with a majority (60–80%) of the cells displaying changes in $[Ca^{2+}]_i$ to ATP, NE, Ach and the muscarinic receptor agonist, OxoM (**Article I**: Figure 1A). Only SP of the tested neuropeptides (neurotensin, Ang II and Ox-A) elicited a Ca^{2+} response in a significant proportion of measured cells (10–50% in each experiment). Interestingly, the percentages of cells responding to Ach and OxoM showed a declining trend during the first four days of differentiation, while the response percentages to ATP, SP and NE remained fairly constant, with only a minor decrease seen in SP and NE at the fourth day of differentiation (**Article I**: Figure 1C).

To reveal if any specific neurotransmitter responding subset populations of cells could be seen during the early differentiation period, correlations between different responses were analyzed. The data showed that the majority of SP responding cells responded to NE (>80%) and that there was a positive correlation between the magnitude of SP and NE responses (**Article I**: Figure 2A). While, between the magnitudes of SP and Ach or OxoM responses, a weak negative correlation was seen (SP vs. OxoM shown in **Article I**: Figure 2B) implying the presence of two different cellular subpopulations (SP/NE and Ach/OxoM) already at the early stages of differentiation in NPC cultures derived from postnatal mice brains. Furthermore, it was found that ATP, NE and SP responses did not significantly correlate to depolarization induced with 70 mM K^+ but instead Ach responses were positively correlated with K^+ responses at differentiation day 4 (**Article I**: Figure 2C).

5.1.2. Identification of different subpopulations based on their functional calcium responses to glutamate, cell positioning and morphology (I)

To assess the presence of metabotropic and ionotropic glutamate responses, the differentiated cells were challenged with glutamate stimulation in the absence of Ca^{2+} (mGlu) and with Ca^{2+} in the solution (iGlu). Majority of cells gave mGlu Ca^{2+}-responses to glutamate stimulation, especially at first days of differentiation. As group I mGluR (mGluRs1 and 5) are coupled to Gq_{α} and activate the classical IP3 hydrolysis and Ca^{2+} release pathway, whereas Groups II and III mGluRs act via inhibitory G-proteins (G_i/G_o) and their subsequent AC activity and PKA signaling pathways (see section 9.4.). We decided to use specific pharmalogical blockers of GroupI mGluR1 and 5 to test their involvement in the mGlu Ca^{2+}-responses seen in differentiated NPCs. We discovered that the selective mGluR5 antagonist; MPEP (1-3 μM) totally blocked the glutamate response in Ca^{2+}-free conditions whereas, mGluR1 specific blocker; LY367385 (3 μM) did not have any significant effect (**Article I:** Figure 3B and C). Therefore, the results obtained together with glutamate stimulation in Ca^{2+}-free conditions and MPEP or LY367385 show that the mGlu responses were mainly mediated via the mGluR5 receptor subtype.

5.1.2.1. Early differentiation stages (1-4 days)

Based on the glutamate responses recorded, two main populations could be distinguished: mGlu and iGlu responding cells (I, Figures 4, 5, 6 and 7). Cells that gave a prompt glutamate response when Ca^{2+} was reintroduced, responded also to 70 mM K^+ with a robust Ca^{2+} elevation and furthermore, did not show immense glutamate responses in the absence of Ca^{2+}, demonstrating that iGluR activation and not mGluR activation is the main pathway of calcium entry (**Article I:** Figure 4A). Interestingly, none of the immense iGlu/K^+ responding cells showed a response to SP, whereas mGlu responses were seen in all SP responding cells. Moreover, the dynamics of the mGlu responses displayed a trend similar to the previously described responses to NE and SP during the early differentiation of NPCs. It is of interest to note, that during the first two days of differentiation there were only few cells responding with robust iGluR activation and/or high K^+ stimulation, rather these responses appeared during the later phases of the differentiation (**Article I:** Figure 4B). While cells showing mGlu responses could be observed already during the first day of differentiation and their proportion was progressively reduced towards the later phases of differentiation period at the outer edges of differentiated neurospheres (**Article I:** Figure 4B). Furthermore, a positive correlation between iGlu and high K^+ responses (calcium rise above 200 nM) was seen (**Article I:** Figure 5A). Conversely, the correlation between mGlu responses and high K^+ responses showed a weak negative trend (**Article I:** Figure 5B). In addition, the correlation between mGlu and iGlu responses showed a negative trend, suggesting that the two types of glutamate responses were expressed in different cell stages (**Article I:** Figure 5C). In summary, these results imply that these mGlu and iGlu responses represent different cell populations most likely at different stages of neuronal maturation.

Due to these exciting findings of mGlu and iGlu cell populations, we proceeded to analyze the spatial distribution of individual cells with respect to their glutamate responsiveness at different time points of differentiation and then combined fluorescent based immunostainings (neuronal versus RG and glial markers) to identify the phenotype and/or specify the maturation stage of these Ca^{2+}-measured cell populations. The cells showing robust calcium responses to mGluR activation were denoted to *type I cells*, while cells showing only iGlu responses were named as *type II cells*. By analyzing the locations of glutamate responding cells from the fluorescence images obtained with fura-2, it was found that at D1, all cells regardless of their spatial distribution with respect to their mother neurosphere were type I cells (**Article I:** Figure 6A and B). While at D3, type I cells were most clearly identified at a region very close to the neurosphere and type II cells could be

observed at the outer periphery of the migration area and some of these cells, especially in the inner area of migration showed immunopositivity to RG markers (GLAST or BLBP) after immunostaining (**Article I:** Figure 6C, D and E). Further analysis of neurospheres with approximately equal numbers of type I and type II cells after 3 days of differentiation revealed that cells showing a certain type of response appeared in clusters. The likelihood of I or II cell types to have a similar cell type as neighbor was two times higher than the probability of being next to a different type of cell (**Article I:** Figure 6F). Further supporting the discovery that mGlu and/or iGlu responding cells during differentiation likely represent different cell populations on different spatial locations.

5.1.2.2. Late differentiation stages (5-8 days)

After 5 to 6 days of differentiation, Ca^{2+}-imaging measurements were performed and then the differentiated neurospheres were immunostained against neural and RG markers (**Article I:** Figures 7, 8, and 9). At first, individual cells were localized and identified by comparing the fura-2 images from the site of the measurements to the obtained images of the immunostained cells (**Article I:** Figure 7A and C). At later days of differentiation many of the type I or type II cells in the differentiated neurospheres were found positive for neural Tuj-1 immunostaining (**Article I:** Figure 7A). Especially the most migrated cells and some cell processes were found highly positive for Tuj-1. As expected from the calcium-measurements, cells at the edge of the neurosphere showed immunoreactivity to mGluR5 antibody and at this location, many of these cells were Tuj-1 negative. However, developmental timing-dependent changes in the activity of mGluR5 signaling pathway in specific subpopulations of cells were implied, since unexpectedly, also some cells in the periphery of the differentiated neurospheres showed immunoreactivity to mGluR5 even if none or very small mGlu responses were seen in calcium-measurements (**Article I:** Figure 7A, B and C).

Fascinatingly, at later stages of neurosphere differentiation a clear spatial pattern for glutamate responsiveness had emerged (**Article I:** Figure 7A, B, C and D). The fura-2 images of the measured areas were analyzed, and cells located based on their responsiveness to glutamate (I, Figure 7C). Cells at the inner edge of the differentiated neurospheres remained responsive to mGluR activation, but the mGlu responses were progressively lost at the outer layers of the migrated cells (**Article I:** Figure 7A, B, C and D). The statistical analysis of data from 5D differentiated neurosphere experiments clearly showed that type I cells reside at the edge and while both type I and II cells can be seen in the middle layer of migration; the outer periphery contains almost solely type II cells (**Article I:** Figure 7D). Taken together, it appears that cells expressing mGluR5 (and other metabotropic receptors) represent an early developmental stage of a cell population, which subsequently progresses towards a more neural phenotype.

With immunostaining of differentiated neurospheres the observed subpopulations of cells could be further characterized. The mGluR5 immunoreactivity was seen in the neurosphere, around its boundaries and in some cells that had migrated out from neurospheres (**Article I:** Figure 8A). The mGluR5 was co-expressed with some neural markers; however, not all mGluR5 cells co-expressed Tuj-1 or NeuN (**Article I:** Figure 7A and 8A, B and D). Specifically, mGluR5 staining could be seen in some processes of Tuj-1 immunostained cells (I, Figure 8B). Only inside the neurospheres edges the mGluR5 staining appeared to partially overlap with the RG marker, GLAST that showed the strongest staining near the edges of the differentiated neurospheres (**Article I:** Figure 8C). As expected, GLAST overlapped with another RG marker, BLBP. The neural markers (NeuN and Tuj-1) did not show almost any overlap with BLBP (**Article I:** Figure 8E and F). It is of interest to note that none of the tested neuronal/RG markers could be used for exact identification of these

detected cell populations, although the cells monitored in Ca^{2+} imaging experiments could later be identified by immunostaining. The cells denoted type I stained with markers for both RG and neural lineage, depending on differentiation time and location relative to the neurosphere.

To analyze other neurotransmitter responsiveness of NPCs with relation to glutamate responses and cell phenotype, cells at the periphery of the differentiated neurosphere were measured with Ca^{2+}-imaging and afterwards immunostained against Tuj-1 at D8. A picture from Tuj-1-positive cells overlayed with a negative image of the respective fura-2 fluorescence clearly shows that the cells had gained a more neuronal morphology (**Article I:** Figure 9A). When the functional calcium responses were analyzed at this timepoint (D8), it was seen that the migrated cells had largely lost their responses to SP, NE, Ach/Oxo-M, ATP and mGlu, but instead robust iGlu and NMDA responses, as well as high K^+ depolarization was seen (**Article I:** Figure 9B). In three separate experiments only one of the cells that had migrated out from the neurosphere gave immense mGlu response and none responded to SP or NE. Only few of the migrated cells responded to Ach and ATP. Conversely, all these cells gave high iGlu responses and most responded to NMDA. In addition, a highly elevated response to K^+ was seen in a majority (77%) of the monitored cells. As combined, these findings further confirm our discovery of loss of metabotropic responses with the gain of ionotropic responses towards more neural phenotype as differentiation proceeds, see also **Figure 9** on page 67 for overview of neurosphere findings.

5.2. Mechanisms of cell migration and process growth regulation in differentiating neurosphere cultures (II)

Based on prior findings, suggesting that early electrical activity (Spitzer, 1994; Rosenberg and Spitzer, 2011) and neurotransmitter functions (Emerit et al., 1992; Nguyen et al., 2001; Hagg, 2009) are of prime importance in neuronal differentiation it was of interest to study more in depth the molecular mechanisms involved in mediating the migratory patterns of NPCs (**Article II**).

We have previously characterized two different morphological types of cells emanating from the neurospheres based on their morphology, immunostaining together with migration and movement speed analysis with time-lapse imaging: fast-type (neural) cells and slow-type (radial glial) cells (Louhivuori et al., 2013; Louhivuori et al., 2015). The fast-type cells are on morphology, smaller in diameter with either bi- or multipolar extensions and migrated significantly further distances than the slow-type cells, whereas the slow-type cells are larger in diameter and lack clear small-diameter extensions in line with our previous findings of different cell populations based on their glutamate responsiveness and immunostainings in **article 1** *type 1 cells* (mGlu) and *type 2 cells* (iGlu). These findings combined confirm a scenario where two different major types of cell populations reside in the differentiating neurospheres, slow-type 1 mGlu-responding RG cells (*type 1*) and fast-type 2 iGlu-responding neural ones (*type 2*). These prior data were utilized in the experimental set up of **article II**. In **Figure 10**, on page 67, is combined illustration of the main findings of differentiated neurosphere cultures.

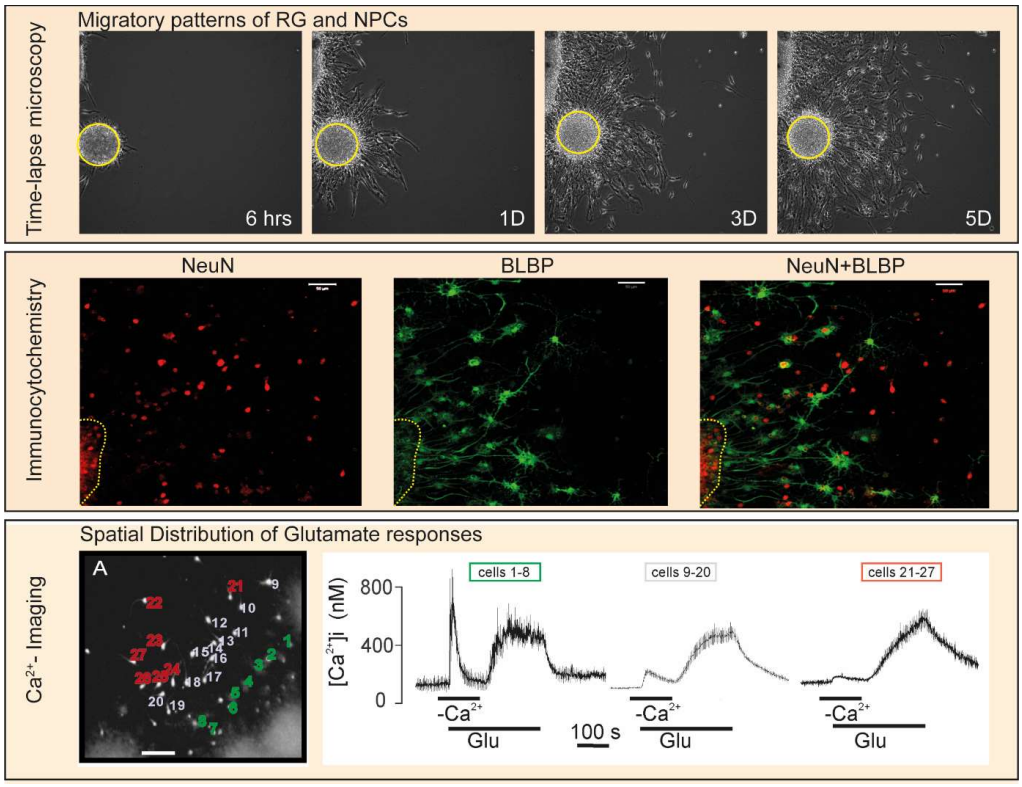

Figure 10. *Combined illustration of calcium-imaging based glutamate responding cell populations in differentiating neurospheres and their spatial locations and cell types correlated based on their appearance, migrational movement and immunostainings.*

Taken together, these results demonstrate that mGluR5 responding cells (*type I cells*, slow-type) are mostly located near the differentiated neurospheres with some immunoreactivity for RG markers (BLPB/GLAST), whereas iGluR responsponding cells (*type II cells*, fast-type) are migrated further with more mature neural phenotype as showed by enhanced neural (Tuj-1, NeuN, Map-2) immunostaining. Furthermore, it was revealed that during the differentiation of neurosphere cultures, the amount of mGlu responding cells decreases while the amount of iGlu responding increases as the migration ensues, and NPCs mature to gain more neural phenotype. Ca^{2+}-measurements with Fura-2AM show the spatial location of glutamate responding cells at differentiation day 5. Time-lapse imaging after 6 hours, 1, 3 or 5 days, yellow circle represents center of the neurosphere. Scale bars on immunocytochemistry 50 µm. Ca^{2+}-imaging picture adapted and modified from original published Article I.

5.2.1. The regulation of radial glial process growth by glutamate via mGluR5/TRPC3 and neuregulin/ErbB4 (II)

Due to the essential role of mGluR5 in neurogenesis (Di Giorgi Gerevini et al., 2004; 2005; Baskys et al., 2005; Cappuccio et al., 2005) and its abundant expression in RG like cells (**article I**) we wanted to study the mechanisms involved in its actions.

Blocking of mGluR5 with MPEP has previously been shown to distort the RG processes (Jansson et al., 2013). In addition, mGluR5 in association with its downstream signaling mediated by canonical transient receptor potential TRPC channels play an integral role in the interaction between neuronal and RG cells (Louhivuori et al., 2015). Disrupting the signaling complex of TRPC3/mGluR5 leads to disturbances in the migratory behavior of neuronal cells and reduces the

contact-based interaction between neural and glial cells *in vitro* (Louhivuori et al., 2015). Since neuregulins are known to play a major role in neuronal migration and RG integrity (Anton et al., 1997; Rio et al., 1997; Lopez-Bendito et al., 2006) and it has been reported that, both NRG and ErbB4 receptors are expressed in RGCs of SVZ during embryonic development and, that NRG knock-down disturbs RG growth (Anton et al., 2004, Li et al., 2012). Likewise, selective knock-down of RG ErbB4 disturbs neuronal migration and correct cell placement (Schmid et al., 2003) Therefore, we aimed to elucidate the role of NRG/ErbB receptors in mGluR5/TRPC3 mediated maintenance of RG process growth and were keen to examine the molecular mechanisms of their interaction in more detail. Our proposed RG process growth mechanism is illustrated in **Figure 11** on page 60.

5.2.1.1. ErbB and neuregulin expression in differentiating NPCs

Firstly, we investigated the mRNA expression for neuregulins 1, 2 and 3 and their receptors ErbB3 and ErbB4 in proliferating and differentiating NPCs, as well as in adult mouse whole brain cDNA by using PCR (**Article II:** Figure 1). We were only able to detect ErbB4 receptor expression and a clear band for NRG3 ligand (**Article II:** Figure 1A and B.) However, we could not rule out NRG1 expression since it was below our detection limit and appeared occasionally in our gels.

5.2.1.2. Effect of Nrg/ErbB4 on radial glia processes

Since we had previously established a method for analyzing the migratory behavior of differentiating NPCs using time-lapse microscopy (Louhivuori et al., 2013; Louhivuori et al., 2015), we applied this method to investigate the role of ErbB4 using its pharmacological blocker, afatinib, which is also known to block ErbB1. Blocking ErbB1/4 receptors with afatinib drastically reduced RG growth, suggesting that ErbB1/4 receptors play an important role in RG maintenance. This was further supported by results gained with its ligand, NRG that in turn increased RG growth (**Article II:** Figure 2A and B). Because afatinib blocks both, ErbB1 and 4, we decided to use gefitinib, a specific blocker of ErbB1, to assess its role in RG maintenance. Surprisingly, blocking ErbB1 with gefitinib drastically reduced RG growth and this disruption was rescued by the ErbB4 ligand, neuregulin, suggesting that also ErbB1 takes part in regulating RG process growth (**Article II:** Figure 2C).

Since we have shown that MPEP, a specific blocker of mGluR5, also disturbs RG growth (Louhivuori et al., 2015) it was of interest to see, what happens if ErbB4 is stimulated with neuregulin in the presence of MPEP. Astonishingly, NRG was able to rescue the RG process growth disturbed by MPEP (**Article II:** Figure 2C, D and E). This would suggest a scenario, where ErbB4 works downstream of mGluR5. The mGluR5 is coupled to activation of a nonselective cation channel, TRPC3 (Kim et al., 2003; Berg, 2007; Louhivuori et al., 2015). Inhibiting TRPC3 with pyr3 (the selective blocker of TRPC3) resulted in a reduction of RG growth and similarly to the blockage of ErbB, this disruption was rescued by the ErbB4 ligand, neuregulin (**Article II:** Figure 2C).

5.2.1.3. GPCR activation of Nrg/ErbB4

Our previously shown robust Ca^{2+} responses to G_q-protein coupled muscarinic receptor stimulation with Ach and OxoM (**Article I** and Castren et al., 2005) led us to assess whether muscarinic receptor stimulation can replace mGluR5 activation with respect to RG process elongation. The differentiated cells at D1 were stimulated with mGluR5 agonist, DHPG and muscarinic receptor agonist, OxoM and both caused $[Ca^{2+}]_i$ rise and the responses were highly linearly correlated (Pearson's r 0.82) (**Article II:** Figure 3A and 3B). The obtained data proposes that G_q-coupled

muscarinic receptors recruit similar signaling components as mGluR5 to direct RG growth since in MPEP-treated cells, OxoM was able to cause a significant increase in process extension, to the same length as in control cells, and furthermore, this effect was blocked by pyr3, afatinib (ErbB1/4 blocker) and gefitinib (ErbB1 blocker) (**Article II:** Figure 3C). However, Pyr3+MPEP (TRPC3 blocker+mGlu5R blocker) treatment with OxoM, prevented the OxoM rescue, signifying that both G_q coupled receptors utilize this nonselective cation channel to maintain and promote RG growth. To further elucidate the role of NRG and ErbB receptors in RG growth, we investigated the effect of Ilomastat, a pan inhibitor of MMP/ADAM proteases which have been reported to allow interaction of NRG with ErbB receptors (Mei and Xiong, 2008) and block GPCR mediated transactivation of ErbB receptors through proteolysis (Santiskulvong & Rozengurt, 2003). Matrix metalloproteinase inhibition caused by Ilomastat significantly reduced RG process lengths and furthermore, administration of NRG in Ilomastat-treated neurospheres restored RG process lengths to control levels (**Article II:** Figure 3D). Interestingly, OxoM which was able to restore RG process growth in the presence of MPEP, failed to do so in the presence of additional Ilomastat (MMP inhibitor) (**Article II:** Figure 3D), further supporting that both of G_q-coupled receptors are employing the same downstream mechanisms.

5.2.1.4. Interaction of neuronal cells with radial glia promotes radial glial process growth

Since neuronal cells play a determinant role in regulating and maintaining the function of RG cells as migratory guides (Feng & Heintz, 1995; Hunter & Hatten, 1995) we were keen to evaluate the effects of neuronal contacts to RG growth. Interactions with neuronal cells promoted RG process extension (**Article II:** Figure 4A and B), while RG cells that lacked neuronal contacts displayed more stunted process growth (**Article II:** Figure 4C and D). These finding highlights chemoattractant impact of neurons to RG process extension growth.

To investigate whether ErbB4 receptor activation could induce $[Ca^{2+}]_i$ changes in cortical NPCs, the cells were challenged with DHPG, NRG and EGF (ErbB1 r) either on proliferating conditions, or after D1 or at D5. Stimulating ErbB4 receptors with the NRG caused no detectable rise in $[Ca^{2+}]_i$ in any of the proliferating cells (Figure 5A and B). However, elevated calcium responses to EGF and DHPG were seen in proliferating cells and after differentiation (**Article II:** Figure 5A and B). At D1 27.764.7% of the cells also displayed a calcium response to NRG (**Article II:** Figure 5A and 5B). Nearly all the cells (>98%) that gave NRG responses responded also to DHPG stimulation. However, there was a small population of cells that responded to kainate stimulation but not to DHPG (< 2%) and these cells were positioned very close to the mother neurosphere. EGF responses were highest at D1. At D5, no detectable change in $[Ca^{2+}]_i$ to ErbB4 stimulation with NRG was found, but calcium responses to EGF and DHPG were seen (**Article II:** Figure 5A and B). The DHPG responses showed declining amplitudes when the differentiation ensues fitting in line with our previous findings of mGlu responsiveness shown in **Article I**. To analyze ErbB activation with the stimulation of NRG induced calcium responses, pharmacological blockers were used at D1. By using various pharmacological blockers, we showed that NRG calcium response is partly dependent on ErbB1 and likely mediated by $PLC_{\beta\gamma}$ –signaling pathway (**Article II:** Figure 6 and 7).

5.2.1.5 Acute Embryonic E14.5 brain slices display similar functional responses to mGluR5 and NRG stimulation

To examine if the *in vitro* findings correlate to the situation *in vivo,* experiments were also performed with neocortical E14.5 brain sections using the cell permeant Ca^{2+} probe, fluo-4. The

averaged effect of DHPG, NRG and kainate stimulation on $[Ca^{2+}]_i$ in the embryonic cortical slabs is shown (**Article II:** Figure 8A and C). Fluorescence responses to application of NRG were seen in about 6% of all measured cells, all cells that responded to NRG also responded to DHPG or kainate. Cells responding to both, DHPG and kainate were the most abundant group, accounting for over 60% of the responding cells (**Article II:** Figure 8B). These results combined indicate that the same signal pathways as studied above in neurosphere model of differentiating progenitors and in **article I** also function "*in vivo*" in brain slices.

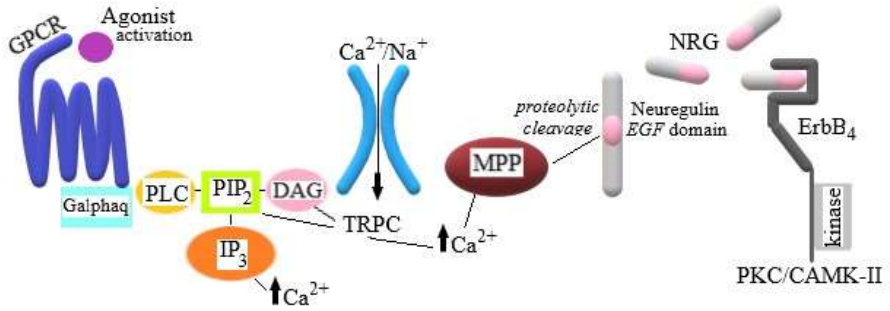

Figure 11. *Simplified schematic diagram illustrating the discovered mechanism of GPCR activation of Nrg/ErbB4 promoting radial glial process growth*
Agonist stimulation (Glu or OxoM) of G_q-coupled protein receptor (G_qCPR) activation leads to PLC mediated DAG production that in turn activates TRPC channels in radial glial (RG) cells. The consequent rise in intracellular calcium induces matix metalloproteases (MMPs) to proteolytically cleave cell membrane bound neuregulin (NRG) which in turn, on its soluble form, activates ErbB4 receptors and its subsequent signaling cascades to induce process growth of RG. Nearby neurons can also release NRG to promote RG expansion trough the activation of ErbB4 and work as a chemoattractants to induce RG process elongation.

5.3 Alterations of TrkB/BDNF-signaling in the absence of FMRP in a mouse model for FXS (III)

BDNF/TrkB signaling plays important roles in neuronal maturation and differentiation during brain development, and it is implicated in the regulation of synaptogenesis as well as in modulating synaptic function and plasticity (reviewed in, Huang and Reichardt, 2001; Lessmann et al., 2003; Mattson, 2008; Leal et al., 2014). Various prior studies have shown that BDNF/TrkB signaling is essential for normal cortical development (Gates et al., 2000; Polleux et al., 2002; Medina et al., 2004; Fukumitsu et al., 2006; Bartkowska et al., 2007). We sought to investigate TrkB/BDNF expression and signaling in progenitors during development and in alterations of neuronal maturation leading to circuit abnormalities and consequently impaired synaptic plasticity in the brain of *Fmr1*-KO mice.

5.3.1. The effects of various neurotransmitter stimuli and BDNF to $[Ca^{2+}]_i$ responses in differentiating NPCs in the absence of FMRP (III)

Previously shown impaired differentiation of NPCs in FXS and its mouse model (Castrén et al., 2005) led us to examine the neurotransmitter and growth factor responsiveness in NPCs with relation to neuronal maturation and differentiation in the absence of FMRP more in detail. We wanted to elucidate the role of BDNF signaling in NPCs with functional Ca^{2+}-imaging, since not only does BDNF via TrkB signaling have a role in neuronal differentiation and migration (reviewed in Mattson, 2008; Leal et al., 2014) but BDNF can also elicit Ca^{2+} increases in neurons which may

trigger cytoskeletal rearrangements essential for dendritic spine formation, remodeling and consequently synaptic plasticity (Berninger and Poo, 1996; Zhang and Poo, 2002). All these processes have been shown to be somehow disturbed in FXS (reviewed in, Bhakar et al., 2012, Li and Zhao, 2014, Richter et al., 2015, Zafarullah and Tassone, 2019).

Using cortical neurosphere cultures propagated from E14 male mice lacking FMRP, our first approach was to assess spatial distribution of RG and neural progenitors in neurospheres by characterizing the differentiating cell populations with ICC at D5 of differentiation. The differentiated NPCs showed immunopositivity to neural and RG markers (**Article III**: Figure 1A). An intensively immunostained layer of morphologically flat-like cells were identified as RG-positive, located around the edge of the neurospheres. Neural cells which had strong immunofluorescence for either Tuj-1, NeuN or Map-2, had small round cell bodies with thin processes and were mostly found migrated away from the RG layer (**Article III:** Figure 1A). Nonetheless, some of the neural cells marked cells were located within the RG layer. These results agree with results obtained from differentiated postnatal WT neurosphere cultures (**Article I:** Figures 7, 8 and 9). For overview of neurosphere differentation, see also **Figure 10** on page 67). Next, to reveal if subpopulations of cells can be found based on their neurotransmitter or growth factor responses in embryonic neurosphere cultures and whether there are differences in the absence of FMRP when compared to WT, Ca^{2+}-imaging measurements were performed. Based on the cell's functional $[Ca^{2+}]_i$ responses to BDNF, ATP, and NMDA and their spatial locations, three distinct cell populations could be distinguished: i) BDNF and ATP-responding, ii) ATP-responding and iii) NMDA-responding cells (**Article III:** Figure 2B and C). The number of BDNF responding cells did not significantly differ between WT and FMRP-lacking neurospheres (**Article III:** Figure 2D). However, the amplitude of responses to BDNF was significantly augmented (1.3-fold) in the absence of FMRP when compared to WT (**Article III:** Figure 2E). Interestingly, the differentiated NPCs with a BDNF $[Ca^{2+}]_i$ response gave as well, without an exception, an $[Ca^{2+}]_i$ response to ATP.

The majority of ATP responding cells were located at the edge of the differentiated neurospheres in both WT and transgenic cell cultures (WT: 78±6.6% vs. *Fmr1*-KO: 75±5.6%, spatial location is shown in **Article III:** Figure 2B and C). Interestingly, these ATP cells were located in the same positions as cells responding to mGluR5 activation in P6-cultures (**Article I:** Figure 7). Indeed, at D5 and D7 with additional Ca^{2+}-imaging measurements combined to ICC, we have confirmed that almost all ATP responding cells also in E14-cultures, respond to mGluR5 activation and are located near the edge of the neurospheres in RG immunopositive layer (representing a more immature cell phenotype) (unpublished data, Louhivuori et al.,). We observed no significant differences in the fraction of ATP responding cells between the transgenic and WT cultures after 5 days of differentiation (WT: 45±3.82% vs. *Fmr1*-KO: 52±4.93%) (**Article III:** Figure 2F). However, the fraction of ATP responding cells was significantly increased (17%) at D7 in neurospheres lacking FMRP vs. WT, suggesting temporal changes in ATP-responsive cell population during differentiation and possibly delayed maturation of ATP-mGluR5 responding cells.

We decided to study more thoroughly the ATP responses in differentiated WT and transgenic neurosphere cultures due these prior findings and since ATP signaling and Ca^{2+} transients have been shown to be essential for the proper formation of the SVZ (Kriegstein and Noctor, 2004; Weissman et al., 2004) and for the migration of Tbr2-positive cells (Liu et al., 2008). Interestingly, we found that the amplitude of the intracellular Ca^{2+} responses to ATP was significantly elevated in all FMRP-lacking cells when compared to WT controls; both in the group of cells that gave also an intracellular BDNF Ca^{2+} response (referred to as BDNF-responsive) and a cell group that did

not respond to BDNF with an $[Ca^{2+}]_i$ response (referred to as BDNF non-responsive) (**Article III:** Figure 2G). No significant difference was seen in the amplitudes of ATP-induced Ca^{2+} responses between BDNF-responsive and BDNF non-responsive WT cells (**Article III:** Figure 2G). However, in differentiated cells derived from the *Fmr1*-KO male mice, the amplitudes of ATP-induced Ca^{2+} responses were significantly higher in BDNF-responsive cells when compared to BDNF non-responsive cells (**Article III:** Figure 2G), suggesting that BDNF-mediated cellular mechanisms are involved in aberrant ATP responses in a subpopulation of differentiating NPCs lacking FMRP.

The third found subpopulation of cells was NMDA responding cells. Most cells that gave a response to NMDA did not respond to ATP nor did any of them respond to BDNF with $[Ca^{2+}]_i$ response (**Article III:** Figure 2B and C). The total number of NMDA-responsive cells did not significantly differ between transgenic and WT cultures at D5 (8.25±2.16% WT vs. 7.13±1.37% *Fmr1*-KO). When the spatial location of NMDA cells was investigated, we found that majority of the NMDA-responsive cells were migrated out from the neurospheres (90.3±3.6% WT vs. 83.7±7.3% *Fmr1*-KO), and only a small proportion (10.4±3.99% WT vs. 16.3±7.3% *Fmr1*- KO) of cells with NMDA response were found at the near edge of the differentiated neurospheres (**Article III:** Figure 2B and C). These results in combination with the immunostaining results suggest a more mature neural phenotype for NMDA responding cells.

5.3.2. TrkB and BDNF expression in NPCs lacking FMRP (III)

TrkB receptors convey the actions of BDNF and play an important role in neuronal maturation and plasticity (reviewed in, Leal et al., 2014). Reportedly, BDNF/TrkB signaling is vital for normal cortical development (Gates et al., 2000; Polleux et al., 2002; Medina et al., 2004; Fukumitsu et al., 2006; Bartkowska et al., 2007). Catalytically competent TrkB.FL receptors are expressed in progenitors (Tervonen et al. 2006) and in the brain (Klein et al., 1990; Kokaia et al., 1993; Allendoerfer et al., 1994; Escandon et al., 1994; Fryer et al., 1996; Drake et al., 1999). Furthermore, both BDNF via TrkB signaling and the lack of FMRP have been shown to promote neuronal differentiation of NPCs (Barnabe-Heider and Miller, 2003; Castrén et al., 2005; Tervonen et al. 2006), suggesting that BDNF and FMRP may play a role in the control of common intracellular pathways that regulate early neuronal fate determination. Therefore, we decided to examine the catalytic TrkB and BDNF expression in NPC cultures, which were propagated from the wall of the lateral ventricles of male *Fmr1*-KO pups and their WT littermates at E14 and grown as free-floating neurosphere cultures.

We found that the TrkB.FL expression was significantly increased (127%) in proliferating FMRP-lacking neurospheres as shown by Western Blot (**Article III:** Figure 1A). Furthermore, using a catalytic TrkB-specific antibody we revealed with immunostaining technique that TrkB.FL expression was significantly increased in 7 days differentiated neurospheres lacking FMRP (58%) when compared to WT neurospheres (32%) (**Article III:** Figure 1B and C). Since the number of analyzed DAPI immunostained cells was similar in WT and FMRP lacking NPCs, the data suggested that the changes seen, represent TrkB+ subpopulation specific differences in the fate determination of differentiating NPCs. To confirm this, we performed clonal analysis of proliferation and found that the primary cortical progenitors derived from WT and *Fmr1*-KO mouse embryos formed neurospheres in an identical manner. The proliferation or survival of transgenic primary progenitors did not significantly differ from WT controls.

Next, we used ELISA detection and ICC to assess the BDNF protein expression in FMRP-deficient NPCs. We found that the portion of cells with BDNF expression was significantly reduced in *Fmr1*-

KO NPCs when compared to WT cells after differentiation for 7 or 14 days (**Article III:** Figure 6A and B). However, the total amounts of BDNF protein in proliferative cortical NPC cultures derived from *Fmr1*-KO and WT mice were similar in BDNF ELISA (**Article III:** Figure 6C) and in Western analysis, suggesting a distorted differentiation or survival of differentiating NPCs in the BDNF expressing cell fraction in the absence of FMRP.

5.3.3 Alterations of TrkB.FL expression in the developing cortex of *Fmr1*-KO mice (III)

An elevated TrkB immunoreactivity has been discovered in cortical parvalbumin expressing cells in the brains of adult *Fmr1*-KO mice (Selby et al., 2007). Our findings combined to the prior abnormalities seen in the brain development and plasticity of *Fmr1*-KO mice (Huber et al., 2002; Li et al., 2002; Koekkoek et al., 2005; Zhao et al., 2005; Desai et al., 2006; Volk et al., 2006, 2007; Meredith et al., 2007; Bureau et al., 2008; Harlow, et al., 2010, Lau et al., 2010) led us to investigate the contribution of TrkB to cortical neuronal maturation in the absence of FMRP.

TrkB expression was investigated with TrkB.FL-specific antibody in the developing brain of *Fmr1*-KO mice and their WT littermates with immunofluorescent-based IHC. In coronal neocortical brain sections at E17, the catalytic TrkB expression was primarily recognized in the IZ and CP and only a faint cellular staining was observed in the SVZ (**Article III:** Figure 3A). Semiquantitative analysis of the maximum projection confocal images revealed that the number of TrkB.FL-positive cells was significantly increased in the IZ, whereas significantly reduced in the CP of the embryonic neocortex in *Fmr1*-KO mice when compared to their WT littermates (**Article III:** Figure 3C). We did not detect any significant changes in the TrkB.FL expression in the HC, globus pallidus (GP) or in the in the ldt/lpt of *Fmr1*-KO mice when compared to WT controls at this developmental stage (III, Figure 3C, D, E, and F). These findings suggest region-specific spatial differences of TrkB expression during brain development in the absence of FMRP.

5.3.4 BDNF expression in the brain of *Fmr1*-KO mice (III)

The *in vitro* alterations revealed in BDNF and TrkB.FL expression in differentiating FMRP-lacking neurosphere cultures (**Article III:** Figures 1, 2B, C, D, E G and 6) as well as the regional alterations in the expression pattern of TrkB.FL-positive cells in the developing cortex of *Fmr1*-KO mice (**Article III:** Figure 3), along with suggested function for FMRP in BDNF-induced plasticity (Castrén et al., 2002; Lauterborn et al., 2007) led us to further examine the BDNF protein expression in FMRP lacking neuronal tissue *in vivo* with ELISA detection.

We found that the expression profile for BDNF varied in the different brain areas investigated, in such a way that the expression level of BDNF was significantly reduced in the motor cortex but increased in the hippocampus and no significant differences were seen in the prefrontal cortex of adult *Fmr1*-KO mice when compared to WT control samples (**Article III:** Figure 6C). These findings suggested that alterations in the local expression pattern of BDNF might contribute to the regional plasticity differences previously observed in the brains of *Fmr1*-KO mice (reviewed in, Pfeiffer and Huber, 2009).

5.3.5. Dendritic targeting of *Bdnf* mRNA in the brain of *Fmr1*-KO mice and cultured neurons (III)

Dendritic structures in the absence of FMRP are altered (Bagni and Greenough, 2005) but the mechanisms and factors affecting the immature spine structures are not yet revealed. Synaptic activity can regulate the number of dendritic proteins by modulating the local translation of the corresponding mRNA (Steward, 1994, 1997; Schumann, 1997). Since there is evidence that TrkB

is associated with signals that control the dendritic targeting of *Bdnf* mRNA (Righi et al., 2000), we investigated whether the revealed alterations of the TrkB and BDNF expression in neurons lacking FMRP were linked to possible changes in the subcellular expression of *Bdnf* mRNA.

Activity-dependent targeting of *Bdnf* mRNA to dendrites has been revealed previously, both *in vitro* and *in vivo* (Tongiorgi et al., 1997; Tongiorgi, 2008). Furthermore, dendritic targeting of *Bdnf* mRNA has been shown to increase after pilocarpine-induced seizures (Tongiorgi et al., 2004). Therefore, we decided to also investigate the effects of induced neuronal activity on the subcellular *Bdnf* mRNA expression in FMRP-lacking neurons after neuronal activation initiated by a muscarine Ach receptor agonist, pilocarpine.

We analyzed the subcellular expression of *Bdnf* mRNA in the cortical layer V and hippocampal region CA1 neurons of *Fmr1*-KO and WT mice with *in situ* hybridization. An intense *in situ* staining for *Bdnf* mRNA was seen in almost all pyramidal neurons of layer V in the visual cortex under saline-treated control conditions, with more pronounced dendritic staining after pilocarpine treatment (**Article III:** Figure 4A). In the hippocampal CA1 area, the *in situ* signal was maximal in sparse large neurons located in the pyramidal layer, also with more pronounced dendritic staining after pilocarpine treatment (**Article III:** Figure 4B). The *Bdnf* mRNA transcripts were localized both in the soma and dendrites, but in neurons of the cortical layer V and hippocampal CA1 region of *Fmr1*-KO mice the labeling in dendrites was more pronounced when compared to the levels in neurons of WT mice (**Article III:** Figure 4A and B).

Similarly, to the saline-injected control conditions, after pilocarpine treatment, the *Bdnf* mRNA transcripts were mainly localized in the soma and proximal dendrites (**Article III:** Figure 4A and B). However, pilocarpine treatment enhanced the expression of *Bdnf* mRNA in more distal dendrites and the difference between the dendritic *Bdnf* mRNA expression in *Fmr1*-KO and WT neurons was further augmented after pilocarpine treatment (**Article III:** Figure 4A and B). Furthermore, we observed that the *Bdnf* mRNA expression was significantly increased in the most proximal dendrites in cortical neurons but not in the hippocampal neurons of pilocarpine-treated *Fmr1*-KO mice (**Article III:** Figure 4A and 4B) suggesting regional differences in the regulation of subcellular *Bdnf* mRNA expression.

To further confirm that a reduction of FMRP indeed affects the dendritic localization of *Bdnf* mRNA, we utilized specific siRNA (si-Fmr1) to knockdown FMRP expression in cultured hippocampal neurons *in vitro*. The si-Fmr1 was previously shown to diminish FMRP expression to the background level in ≈ 70% of dendrites after 4 days of transfection (Nakamoto et al, 2007). Similar reduction in FMRP expression 48 hours after transfection were obtained in our control experiments (**Article III:** Supplemental Figure 2). Similarly, to the *in situ* hybridization results obtained with brain sections, we found that the *Bdnf* mRNA was localized in the soma and proximal dendrites under basal conditions and became localized also into the distal dendrites upon cell depolarization with 10 mM KCl for 3 h (**Article III:** Figure 5A). Likewise, the basal expression of *Bdnf* mRNA was significantly increased in the distal dendrites of neurons when the FMRP was silenced (**Article III:** Figure 5B). On the contrary to the *in vivo* results, the dendritic targeting of *Bdnf* mRNA was not affected in FMRP-silenced neurons after KCl-induced neuronal depolarization (**Article III:** Figure 5B). These results suggest that in the absence of FMRP, subcellular activity-induced dendritic targeting of *Bdnf* mRNA expression may be differentially regulated after pilocarpine treatment *in vivo* and KCl-induced neuronal depolarization *in vitro*.

5.4. Alterations of neuronal maturation and differentiation in the brain of FXS mouse model (IV)

Based on our previous *in vitro* findings and prior literature on the alterations of neuronal development in FXS and its mouse model (Castrén et al., 2005; Bureau et al., 2008) we sought to investigate NPC maturation and differentiation *in vivo* during cortical development in the brain *Fmr1*-KO male mice.

5.4.1. Formation of neocortex in the presence of the dominant negative form of FMRP (IV)

To gain insight into the functional role of FMRP during development, it was of interest to examine how the interference of FMRP function (by a transient transfection of FMRP with a substitution of Ile→Asn at the amino acid position 304 of the KH2 domain) affects neocortical formation. FMRP with the I304N mutation sequesters WT FMRP and its target mRNAs into translationally inactive granules, indicating a dominant-negative function for the mutated protein (Laggerbauer et al., 2001; Wang et al., 2008) and reportedly, causes an extremely severe form of FXS (De Boulle et al., 1993; Feng et al., 1997; Schrier et al., 2004; Darnell et al., 2005). The expression plasmid containing the fusion protein for FMRPmt-EGFP, FMRP-EGFP (Castrén et al., 2001) or enhanced green fluorescence protein (EGFP) control was injected into the lateral ventricles of WT mouse embryos at E14 with *in utero* electroporation, and the expression was investigated three days later with IHC.

After transfection of the FMRPmt-EGFP, a significant accumulation of EGFP-positive cells in the cortical regions next to the ventricles (VZ and SVZ) was found, whereas in the superficial layers (IZ, CP and MZ) the relative number of EGFP-positive cells was reduced when compared to the relative number of EGFP-positive cells after transfection of the control plasmid encoding EGFP alone (**Article IV:** Figure 1A and B). Similar results were found when the relative number of FMRPmt-EGFP expressing cells was compared to the relative number of FMRP-EGFP expressing cells (**Article IV:** Figure 1A and B). In addition, the doublepositivity for EGFP and nestin, a type VI intermediate filament protein marker of NSCs, was investigated in the VZ and SVZ. The relative number of nestin/EGFP double-positive cells was significantly increased in both; VZ and SVZ after transfection of the expression plasmid for FMRPmt-EGFP when compared to the number of nestin/EGFP double-positive cells after transfection of the control plasmid encoding EGFP alone (**Article IV:** Figure 1C, D and E). Together, these results suggest an increased production of progenitors and impairment in the migration of newborn cells during neocortical development when the function of FMRP is compromised with a missense point mutation, I304N, in the KH2 domain.

5.4.2. Layer formation in the developing neocortex of *Fmr1*-KO mice and FXS fetus (IV)

FMRP seems to play important roles in the development and wiring of neural circuits, formation and function of synapses and thus synaptic plasticity (for reviews, see: Contractor et al., 2015; Richter et al., 2015; Banerjee et al., 2018; Bagni and Zukin, 2019). Furthermore, developmental alterations in neocortical formation in the absence of FMRP have been previously revealed (Castrén et al., 2005; Bureau et al., 2008). Therefore, we set forth to investigate the layer formation of neocortex in the *Fmr1*-KO male mice at late neurogenic phase with Nissl-staining which detects large granular Nissl bodies found in the cytoplasm of neurons.

The cortical layer analysis of Nissl-stained coronal brain sections at E16 (**Article IV:** Supplemental Figure 2A and B) revealed that the neuron cell density in the CP of the *Fmr1*-KO mice was significantly increased when compared to the neocortical sections of WT control brains (**Article IV**: Figure 2A and B). However, neither the thickness nor the total cell number in the *Fmr1*-KO mice neocortex did significantly differ from the WT controls at this developmental timepoint (**Article IV:** Supplemental Figure 3A and B). Since according to literature, postmitotic deeper layer neurons are localized in the CP at E16 of mouse neocortical development (Price and Thurlow, 1988; Desai and McConnell, 2000), alterations of cell density in the CP at the time of late neurogenesis suggests that specifically the neuronal lamination in the deeper layers of the neocortex could be affected in the absence of FMRP.

In addition, in line with the finding of increased cell density in the CP of *Fmr1*-KO mice and formerly shown increased expression density of BrdU-positive cells in the SVZ of *Fmr1*-KO mice (Castrén et al., 2005) it is of interest to acknowledge here, even though only a single human fetal FXS case and two age-matched controls were available for this study, we found that the cell density in the CP was higher in the 18-week-old FXS brain when compared to the age-matched WT controls (**Article IV:** Supplemental Figure 1). Moreover, the number of Ki67-positive dividing cells was increased in the VZ and SVZ of human fetal FXS brain (**Article IV:** Supplemental Figure 1) further suggesting aberrations in the neuronal lamination of deeper neocortical layers in the absence of FMRP.

5.4.3. Differentiation of glutamatergic cells in the developing neocortex of *Fmr1*-KO mice (IV)

Glutamate plays a central role in the control of neurogenesis (for reviews, see: Brazel et al., 2005; Mattson, 2008; Nakamichi et al., 2009). Glutamatergic neuron diversity in the neocortex is generated directly from RG NSCs and indirectly through IPCs in a spatiotemporally controlled manner (Noctor et al., 2001, 2004, 2007; Haubensak et al., 2004; Miyata et al., 2004; Wu et al., 2005). Neural IPCs or basal progenitors proliferate from one to three mitotic cycles at non-surface positions within the dorsal VZ and SVZ, and express Tbr2 when they are already committed to glutamatergic fate (Englund et al., 2005). Earlier observations of altered GluR mediated Ca^{2+}-signaling in FMRP-deficient differentiating NPCs *in vitro* and enlarged newborn cell population in the embryonic SVZ of *Fmr1*-KO mice *in vivo* (Castrén et al., 2005) led us to assess more closely the role of glutamatergic neurogenesis on neocortical development in *Fmr1*-KO male mice brains. We set to examine BrdU/Tbr2 double-positivity and the cell expression pattern of Tbr2 and Tbr1 during late phases of neurogenesis, at E16-E18.

A cell cohort of newly proliferated neocortical cells was labeled with multiple BrdU injections at E13 and the double positivity for BrdU and Tbr2 was studied after three days of differentiation with IHC. BrdU is incorporated into to the newly synthesized DNA by substituting thymidine during the S phase of the cell cycle and thus serving as a marker for cells that were recently actively replicating their DNA (Haubensak et al., 2004). The number of Tbr2/BrdU double-positive cells was significantly increased in the VZ and SVZ of the *Fmr1*-KO mouse brain at E16 when compared to WT control brains (II, Figure 2C, D, and F). However, the total number of BrdU-positive cells remained similar between cortical sections of WT and *Fmr1*-KO mice, nor we did not detect significant changes in the overall number of Tbr2-positive cells in the VZ or SVZ at E16, thus suggesting that particularly the production of Tbr2-expressing nIPCs was augmented in the absence of FMRP. Therefore, we decided to further examine the expression Tbr2-positive cells in later timepoint of neocortical development. We found that the density of Tbr2-positive cells was

significantly increased in the SVZ of *Fmr1*-KO mice brains at E17 when compared to WT controls (**Article IV:** Figure 3A and B).

Since Tbr1 expression sequentially trail the expression of Tbr2 in differentiating nIPCs and it is expressed in almost all postmitotic glutamatergic neurons and hence can be utilized as their specific marker (Bulfone et al., 1995; Hevner et al., 2006; Englund et al., 2005). We reasoned to study its expression at E18 when only neurons of deep cortical layers have completed their migration. The density of Tbr1-positive cells was significantly increased in the infragranular layers of *Fmr1*-KO mice neocortex at E18 when compared to WT controls (**Article IV:** Figure 5A and B), further indicating aberrations in the differentiation of glutamatergic cells in the developing neocortex of *Fmr1*-KO mice. Interestingly, Tbr1 expression is reportedly specifically important for the developing neurons of deeper neocortical layers (Hevner et al., 2001). Since the postmitotic deeper layer neurons are located in the CP at E16 (Price and Thurlow, 1988; Desai and McConnell, 2000), the increased cell density seen at that developmental stage in the CP of *Fmr1*-KO mice (**Article IV:** Figure 2A and B) may indeed contribute to the alteration seen in Tbr1 cell expression pattern at E18 and moreover, suggest that both of these findings relate to defects in the development of deeper neocortical layers.

5.4.4. Radial glial cells in the developing neocortex of the *Fmr1*-KO mice (IV)

We investigated the expression of RG lineage cytoplasmic markers for GLAST and BLBP since alterations in the expression pattern of glutamatergic lineage cells, and specifically in the generation of Tbr2-positive nIPCs in the embryonic neocortex of *Fmr1*-KO mice might involve aberrancies as well in the production or fate determination of RG NSCs. However, we did not observe significant changes in the expression of BLBP nor GLAST at E16 (**Article IV:** Figure 4A, B and C). As combined, these results imply that particularly aberrant production of glutamatergic nIPCs leads to disturbed differentiation of glutamatergic neurons during late embryonic neocortical neurogenesis in a mouse model for FXS.

5.4.5. Early postnatal defects of the cortical layer 5 cells in *Fmr1*-KO mouse (IV)

The last steps of mouse neocortical development take place during early postnatal phase in the form of synaptogenesis and the arrangement of neuronal network structures which are then maintained and shaped throughout life with a formation of new synaptic connections and by removing unnecessary ones in an activity-dependent manner (McConnell, 1988; Marin and Rubenstein, 2003; Guillemot et al., 2006). Given that particularly differentiation of cells devoted to glutamatergic lineage (↑Tbr+/BrdU+ at E16 in VZ+SVZ, ↑Tbr2+ at E17 in SVZ and ↑Tbr1+ at E18 in IGZ) was disturbed in the developing neocortex of *Fmr1*-KO mice, and defects in the development of deeper neocortical layers associated according to classical view of mouse neocortical development (*Mouse Brain Atlas,* Paxinos and Franklin, 2001). That led us investigate possible alterations in the cortical neuron lamination and positioning at early postnatal stage in *Fmr1*-KO mice with IHC-staining of coronal cortical brain sections.

The overall cell density of Nissl-stained sections was significantly increased in the anterior somatosensory neocortex of *Fmr1*-KO mice at P5 (II, Figure 6A, B and C). Moreover, we observed that specifically the cell density in the neocortical layer V was augmented with no alterations in the relative thickness, whereas the relative thickness of the layer VI was slightly but significantly decreased in the neocortex of *Fmr1*-KO mice when compared to WT controls (**Article IV:** Figure 6D and E). We further investigated the observed increase in the cell density of the neocortical layer V, with a layer V cell specific marker; a transcription factor ER81 (De Launoit et al., 1997; Yoneshima et al., 2006). Since ER81 is a known downstream target of Pax6 which has been

identified to be sequentially expressed before glutamatergic cell lineage markers; Ngn2, Tbr2, and Tbr1 in the developing cortical progenitors (Englund et al., 2005; Tuoc and Stoykova, 2008) a direct link between ER81 expressing neurons and glutamatergic progenitors was provided. Excitingly, we found that the cell density of ER81-positive cells was significantly increased in the P5 and P7 neocortex of *Fmr1*-KO when compared to WT controls (**Article IV:** Figure 6F and G, Supplemental Figure 4A and B).

6. DISCUSSION and IMPLICATIONS

6.1. Dynamic subpopulations of progenitors during early neurosphere differentiation (I)

Prior studies have shown that $[Ca^{2+}]_i$ signaling via ionotropic receptors or metabotropic GPCRs are potential regulators of NPC proliferation and differentiation and have been implicated in the control of neurogenesis (Emerit et al., 1992; Nguyen et al., 2001; Hagg, 2009). Our findings of various neurotransmitter responses during differentiation of postnatal brain-derived neurosphere cultures are in line with our other studies of neurosphere cultures with embryonic origin and importantly, therefore indicate that the differentiation of neurospheres *in vitro* is to a major extent a comparable process (Castrén et al., 2005, II, III, Louhivuori et al., 2013; 2015; 2018; 2020; and unpublished observation). Moreover, the process probably reflects with some limitations the properties of the actual neurogenesis of cortical progenitors as earlier suggested (Qian, 2000; Caldwell et al., 2001; Shen et al., 2006; Gaspard et al., 2008). The data combined, our results suggest differentiation time-dependent changes in the neurotransmitter responsiveness patterns and indicate that receptor activation patterns are of significance in the fate determination of NPCs towards different lineages, likely contributing to the generation of vast neuronal diversity in the mature mammalian brain (Muotri and Gage, 2006). The finding that an individual NPC responds to various neurotransmitters (ATP, NE, mGluR5, Ach and OxoM) during early days of differentiation may reflect an early immature stage of the cell and consequently, it may not be unreasonable to suggest that particularly at this stage, the fate and migration of the cells could be sensitively influenced by changes in the levels of neurotransmitters. However, to assess cell subtype specification, further investigations with pharmacological agonists and antagonists together with new imaging methods are needed to address this matter more closely.

The finding that differentiating NPCs respond to Ach and muscarinic receptor agonist, OxoM agree with prior literature of purinergic and muscarinic Ach receptor signaling enhancing progenitor proliferation (Ma et al., 2000; Lin et al., 2007) and suggest that self-renewal property of progenitors remains at early differentiation. To this note, our time-lapse video Cell-IQ imaging studies further supports this suggestion. Additionally, muscarinic stimulation has been shown to promote neurogenesis and molecular programs that instruct progenitor specification to neuronal lineages (Zhao et al., 2003). The positive correlation of OxoM/Ach responding progenitors with high K^+ responding NPCs is in line with this and further, with the result that some progenitors with neural characteristics (iGlu responses, high K^+ and Tuj-1, Map-2 or NeuN immunopositivity) still responded to Ach at later phases of differentiation.

Only SP of the examined neuropeptides (others including Ang II, NT and Ox-A) gave calcium responses in a significant proportion of differentiated NPCs. Indeed, consistent with our finding, previously a role for in the regulation of neuronal maturation has been indicated (Jacquin et al., 1992). SP responding cells showed a trend of decline as the differentiation proceeds, similarly to NE responding cells and further, the magnitudes of their responses were also positively correlated, suggesting that NPCs responding to SP, respond as well to NE. These cells also responded to mGlu stimulation that displayed a similar declining trend as SP and NE when differentiation process ensued. Whereas Ach/OxoM-responding cells were negatively correlated to SP/NE responding cells, suggesting that these groups might respresent two different subpopulations of cells already at very early stages of differentiation. ATP responses remained constant at first 4 days of differentiation and positively correlated to SP, NE and mGluR5 responses, in line with our data from E14 differentiated neurosphere cultures (**Article III**; Castrén et al., 2005; unpublished

observations, Louhivuori et al.,). Importantly, these gathered data indicate that GPCRs such as Ach and NE play a role in determining the neuronal differentiation of progenitors.

6.2. Identification of glutamate responding subpopulations and their significance as maturation markers in neurosphere differentiation (I)

We revealed with pharmacological receptor antagonist studies that specifically mGluR5 activation is seen with glutamate stimulation in Ca^{2+}-free conditions. The data is in line with our previous studies on differentiating neurosphere cultures, which revealed cells showing Ca^{2+} mobilization through mGluR5 (type I cells) and cells, demonstrating only ionotropic glutamate responses (type II cells) (Castrén et al., 2005). The findings of robust mGluR5 activation already at early stages of neurosphere differentiation are in an agreement with the prior literature of mGluR5s role in neuronal development, where the mGluR5 is expressed in the regions of active neurogenesis in the embryonic and postnatal brain, and a role for the activation of mGluR5 receptors in the regulation of early fate determination suggested (Di Giorgi Gerevini et al., 2004; 2005; Baskys et al., 2005; Cappuccio et al., 2005; 2005; Gandhi et al., 2008). Specifically, mGluR5 seems to have a central role in the control of proliferation and survival of NPCs *in vivo* (Di Giorgi Gerevini et al., 2004; Gandhi et al., 2008), in line with our indication of more immature phenotype for mGlu responding cells. Adult mice lacking mGluR5 show a striking reduction in the number of dividing progenitors in the SVZ (Di Giorgi Gerevini et al., 2005). Interestingly, the expression levels of mGluR5, have been shown to change dramatically in relation to changes in the culture conditions and cell phenotype (Di Giorgi Gerevini et al., 2004). Hence, fitting to a bit surprising finding, that also some cells in the periphery of the differentiated neurospheres showed immunoreactivity to mGluR5, even if not any or very small mGlu responses were seen in calcium-measurements. This finding may indeed indicate developmental changes in the activity of mGluR5 signaling pathway during neuronal maturation in specific subpopulations of cells.

A significant portion of differentiated progenitors responded to mGluR5, and it appears that cells expressing mGluR5 subtype (and other metabotropic receptors) represent an early developmental stage of a cell population, which subsequently progresses towards a more neuronal phenotype**.** Thus, these results intriguingly suggest that, at this early phase, NPCs subsequent fate could be influenced or determined by a variety of transmitter substances. We further revealed that the differentiation is a dynamic process, where glutamate responsiveness of differentiating progenitors seems to reflect different maturational stages of cell differentiation and specifically iGluR responses seem to dictate later stages of differentiation towards neural lineage, whereas mGluR5 responses (with also other metabotropic receptor responses) indicate more immature cell phenotype. Interestingly, very similar results have been also obtained with 5 to 7 days differentiated neurosphere cultures derived from E14 mice brains (unpublished data, Louhivuori et al.,), indicating a universal role for Ca^{2+} dependent GluR activation in the differentiation dynamics of neurosphere derived NPCs. The findings of depolarization with high potassium and iGlu responses, along some with responses to NMDA are in line with our functional calcium measurements detecting VGCC profile changes for LVA and HVA calcium channels in differentiating neurospheres, the HVA channel activation dominating the Ca^{2+} responses when the differentiation ensues, and more mature neuronal characteristics appear (Louhivuori et al., 2013).

Our findings indicate the following sequence of events: at the initiation of differentiation by the removal of mitogens, cell migration ensues and the individual progenitors mostly located near the differentiated neurospheres with some immunoreactivity for RG markers (BLPB/GLAST) show mostly metabotropic responses to a variety of neurotransmitters, specifically mGluR5 (type I cells), as these cells subsequently lose their contact with the microenvironment offered by the neurosphere

(RG ICC), initiation of the differentiation programs is enabled and their ability to react through metabotropic receptors is gradually lost together with progenitor characteristics. In the most migrated cells, the gain of ionotropic responses to glutamate and robust responses to cell depolarization with potassium chloride (type II cells) leads to a more neural phenotype as also shown with neuronal markers (Tuj-1+/NeuN+). This inverse correlation between type I and type II cells with a differentiation time-dependent increase of type II cells and reduction of type I cells clearly indicate that type I cells are converted to type II cells, which are further converted to neurons. In line with previously reported endogenous activation of mGlu5 receptors supporting specifically the survival of neuronal-restricted precursors (Castiglione et al., 2008).

Therefore, the loss of cells showing metabotropic responses and gain of ionotropic responses appears to be a key event in the differentiation process. The data presented here should be useful for future studies of the effects of receptor agonists and antagonists along with growth factors on the dynamics of differentiation. As suggested in the article, the relative proportion of metabotropic responses compared to ionotropic ones could provide a 'vital' indicator for the maturational stage of NPC differentiation.

6.3. The regulatory role of glutamate via mGluR5/TRPC3 and neuregulin/ErbB4 to radial glial process growth in differentiating neurospheres (II)

Due to the essential role of mGluR5 in neurogenesis (Di Giorgi Gerevini et al., 2004; 2005; Baskys et al., 2005; Cappuccio et al., 2005) and its strong expression in RG like cells (**Article I**) we wanted to study the molecular mechanisms that are involved in mediating its actions. In **Article II**, our results show that in embryonic cortical NPCs, neuregulin/ErbB4 signaling is able to rescue the disrupted RG growth induced by mGluR5/TRPC3 inhibition (i.e., by MPEP). This together with the finding that blocking neuregulin/ErbB4 signaling impedes RG growth in a similar manner as blocking mGluR5/TRPC3, suggests that neuregulin/ErbB4 signaling works downstream of mGluR5/TRPC3 to maintain the RG scaffold. Additionally, we show that ErbB4 is the prevailing transducer of neuregulin signaling in cortical NPCs.

Interestingly, activating other G_q-coupled GPCRs (i.e., muscarinic Ach receptors, OxoM) was also able to rescue the RG disruption by MPEP, and these rescues were prevented by inhibiting neuregulin/ErbB4 signaling, implying that both of G_q-coupled receptors are employing the same downstream mechanisms. Common to both these G_q-coupled receptors is the activation of TRPC3, as seen with the blocking effect of pyr3. Inhibiting TRPC3 prevented the rescue of the RG processes by G_q activation, suggesting that common to both pathways is the influx of Ca^{2+} via the non-selective ion channel TRPC3. Ectodomain shedding of ErbB ligands has been shown to be regulated by Ca^{2+}-signaling, for example proteolytic activation of NRG by disintegrin and metalloprotease (ADAM) and/or MMP (Mei and Xiong, 2008). Our results with Ilomastat, a pan matrix metalloprotease inhibitor, displayed also a reduced length in RG processes, and this effect was reversed by soluble neuregulin. These combined results clearly suggests that NRG signaling plays an important part in mediating the growth of RG cells and it appears likely that the activation of proteolytic cleavage of ErbB receptor ligands is a downstream effector mechanism of mGluR5/TRPC3 activation. Our suggested RG process growth mechanism is illustrated in **Figure 11** on page 60.

Importantly, we revealed that the same signal pathways studied, and the pathways studied in **article I** as well, function "*in vivo*" in brain slices. It therefore might not seem unreasonable to suggest that neurotransmitters released from nearby neurons (and similarly, vascular mediators like

endothelins, Louhivuori et al., 2020) through activation of GPCRs actuate the NRG3/ErbB4 pathway which would lead to growth of the radial glial scaffold and via attraction guiding of neurons. The presented work demonstrates how mGluR5/TRPC3 signaling complex activates neuregulin/ErbB4 to mediate neuronal and RG interaction and highlights the importance of glutamatergic signaling in normal brain development. Glutamate signaling especially via mGluR5 is altered in the FXS (Richter et al., 2015). In the light of our findings of altered glutamatergic neurogenesis in the absence of FMRP (**Article IV**), the signaling mechanisms described here in this thesis may thus shed light on the pathophysiology of FXS and it would be important to address further in future studies.

6.4. The involvement of BDNF/TrkB-signaling to FXS and its significance (III)

Number of studies have documented various defects in synaptic plasticity in *Fmr1*-KO mice (for review, see Pfeiffer and Huber, 2009; Contractor et al., 2015; Huber et al., 2015; Richter et al., 2015; Banerjee et al., 2018). In the mature brain, depending on neuronal activity, BDNF regulates synapse functions with wide range of structural and functional effects that result from its complex downstream signaling cascades (Nagappan and Lu, 2014). In rodents, BDNF positively modulates the formation of LTP (Korte et al., 1995; Patterson et al., 1996; Kang et al., 1997) and BDNF exerts positive feedback to synapse function by a process that requires local protein synthesis in the postsynaptic dendrites (Kang and Schuman, 1996). The BDNF-TrkB signaling upregulates translation activity at the synapse (for review, see Leal et al., 2014). We showed that dysregulation of TrkB/BDNF signaling in the *Fmr1*-KO mice leads to alterations in brain development and likely contributes to previously seen impaired synaptic plasticity defects in the absence of functional FMRP (Pfeiffer and Huber, 2009), indicating a role for BDNF-signaling in the pathophysiology of FXS. Altered BDNF/TrkB-signaling already in FMRP-deficient NPCs indicates that perturbations of brain development in FXS occur at early stages of development, further highlighting an important role for FMRP throughout cortical development. Our results further demonstrate that alterations of BDNF and TrkB bring about cell type-specific changes in the differentiation and migration of neuronal cells lacking FMRP and provide a molecular mechanism that may explain specific changes in the cellular and synaptic plasticity resulting in cognitive defects seen in FXS.

The dendritic targeting of *Bdnf* mRNA was increased under basal conditions in the hippocampal CA1 and cortical layer V neurons of *Fmr1*-KO mice and further enhanced by pilocarpine-treatment, which also caused an accumulation of *Bdnf* mRNA transcripts in the most proximal segments of dendrites in cortex but not in HC of *Fmr1*-KO mice, suggesting regional differences in the regulation of subcellular *Bdnf* mRNA expression. This combined with differential expression pattern of BDNF protein in the cortex and hippocampus suggest that BDNF/TrkB-mediated feedback mechanisms for strengthening the synapses are compromised in the absence of FMRP. In agreement with our results, it has been suggested by Kim and Cho, that activity-dependent alterations in the sensitivity to BDNF-TrkB signaling may promote excessive dendritic arborization and spinogenesis in FXS in order to compensate compromised postsynaptic activity (Kim and Cho, 2014).

Many transcripts bound by FMRP encode plasticity-related and other pre- or postsynaptic proteins, and FMRP represses their translation by stalling ribosomal translocation (for review, see Banerjee et al., 2018; Bagni and Zukin, 2019). However, not all FMRP targets are yet revealed and some other FMRP functions remain to be determined (Dockendorff and Labrador, 2019). Acute and genetic loss of FMRP relieves this stalling process and increases protein synthesis, showing that the loss of a translational brake on the synthesis of a subset of synaptic proteins contributes to FXS (for review, see Bassell and Warren, 2008; Huber et al., 2015; Richter et al., 2015). Therefore, it

has led to a suggestion that slowing ribosomal translocation might help restore the brake on translation that is lost in FXS. Importantly, the role of FMRP in regulating synaptic plasticity does not limit only through the control of local translation in synapses, but to the bulk of the protein in the cell body and FMRP is present in the neuronal cell body, proximal dendrites and axons (Christie et al., 2009), suggesting that FMRP plays a part in homeostatic synaptic plasticity (Turrigiano, 2008; Zhang et al., 2018). Consequently, FMRP could act to repress the neuronal activity stimulated translation to generate a feedback loop restricting neuronal responses to activity at the level of the neuron rather than a certain synapse (Darnell et al., 2011). Our findings of differential expression of BDNF protein in the cortex and hippocampus along with regional differences of activity-dependent dendritic targeting of *Bdnf* mRNA in the absence of FMRP suggests regional alterations in BDNF/TrkB feedback signaling for synaptic strengthening. The findings are in line with reported defects in protein synthesis dependent plasticity seen as an impairment of LTP in the Cx and HC (Li et al., 2002; Zhao et al., 2005; Desai et al., 2006; Meredith et al., 2007, Bureau et al., 2008) and as an augmentation of LTD in the HC and cerebellum of *Fmr1*-KO mice (Huber et al., 2002; Koekkoek et al., 2005; Volk et al., 2006). FMRP might inhibit translation in response to activation of BDNF or muscarinic Ach receptors, since synaptic plasticity defects specifically related to these pathways in *Fmr1*-KO mice have been reported (Lauterborn et al., 2007; Volk et al., 2007).

Neuronal plasticity requires not only local protein translation but also actin cytoskeleton remodeling in response to extracellular signals. Previous studies imply that FMRP may regulate neuronal function by actin dynamics-dependent mechanism (Schenck et al., 2003; Reeve et al., 2005; Santini et al., 2017). A role for FMRP in the control of actin cytoskeleton remodeling, also relevant to deficits in LTP, have been shown and perturbations to their activities could explain the abnormal spine morphologies associated with FXS (Castets et al., 2005; Lauterborn et al., 2007). Intriguingly, also fitting to our finding of altered differentiation of Tbr2+/Tbr1+ lineage cells in the developing neocortex of *Fmr1*-KO mice that leads to abbreviations in postnatal cortical structures (**Article IV**), it has been reported that FMRP suppresses the transition from RGCs to IPCs during neocortical development by regulating the actin cytoskeleton and detachment of the apical processes from the ventricular surface (Saffary and Xie, 2011). These findings rise an intriguing scenario, where FMRP function-regulated actin dynamics-dependent cytoskeleton remodeling, that plays a major role in variable neuronal processes, from the control of RGCs transition to IPCs to dendritic spine morphology and synaptic plasticity, could explain variable shortcomings seen in FXS. Therefore, in the future more studies of the cellular and molecular mechanisms affecting different brain structures and synaptic plasticity in the absence of FMRP and its versatile role in various processes from early development to adulthood are needed.

BDNF acts via Rho GTPases to regulate the assembly of the actin cytoskeleton in developing neurons (Ozdinler and Erzurumlu, 2001; Gehler et al., 2004; Miyamoto et al., 2006), and aspects of these signaling pathways are preserved into adulthood in hippocampus that is important for learning (Rex et al., 2007), making BDNF a plausible candidate for a treatment to counteract the problems in spine reorganization postulated to arise when FMRP is lacking. Alternatively, the neuronal actin cytoskeleton modulation is sensitive to calcium (Rosenmund and Westbrook, 1993; Furukawa et al., 1995) and there is evidence that unreliable calcium signaling causes increased threshold for spike-timing-dependent plasticity in the cortex of *Fmr1*-KO mice (Meredith et al., 2007). Furthermore, compromised calcium signaling and dysregulation of PI3-kinase-AKT-mTOR signaling in the brain of *Fmr1*-KO mice (Meredith et al., 2007; Selby et al., 2007; Hu et al., 2008; Gross et al., 2010; Sharma et al., 2010; Hoeffer et al., 2012; Gross et al., 2015) are consistent

with abnormalities in TrkB-signaling during corticogenesis, that via PI3-kinase activation, is involved in the tangential migration of interneurons (Polleux et al., 2002), and the delayed maturation of fast-spiking interneurons in the sensory cortex of *Fmr1*-KO mice that is rescued by chronic delivery of a TrkB agonist (Nomura et al., 2017). Also, other molecules that are important in the actin cytoskeleton dynamics have been previously identified as translational targets of FMRP, mRNAs such as MAP1B and EF1a (Zhang et al., 2001; Sung et al., 2003; Lu et al., 2004). However, more studies are needed to exactly determine these biochemical mechanisms and their contribution to FXS.

Moreover, we revealed alterations in the TrkB expression pattern in the developing *Fmr1*-KO mouse. TrkB+ cell fraction was significantly increased in the IZ of the embryonic neocortex in *Fmr1*-KO mice, wheras in the CP significantly reduced, when compared to their WT littermates. TrkB expressing cells in the IZ of developing brain likely represent tangentially migrating interneurons, which initially travel within marginal and IZ before entering to the CP (Polleux et al., 2002). These data are consistent with abnormalities seen in the differentiation and migration of newborn cells with impaired FMRP function (**Article IV**) and suggest that changes of local TrkB expression might contribute to the aberrances of neocortex formation in *Fmr1*-KO mice. Intriguingly, because there is evidence that Tbr2 expressing INPs control the subpallial interneuron numbers through a non-cell-autonomous mechanism (Sessa et al., 2010), the alterations in the TrkB expression pattern in the *Fmr1*-KO mouse brain could indeed result from the shown aberrancies of the INP differentiation (**Article IV**). Furthermore, these findings agree with reported increase in the TrkB immunoreactivity in a subpopulation of cells with parvalbumin expression in the brain of *Fmr1*-KO mice (Selby et al., 2007). However, further studies are needed to define in detail the spatial and temporal changes of interneuron maturation in the developing neocortex of *Fmr1*-KO mice. The altered cortical lamination seen in **articles III and IV** is indicative of the effect of BDNF overexpression (Ringstedt et al., 1998; Polleux et al., 2002; Ohmiya et al., 2002; Alcantara et al., 2006). The mechanisms involved in altered layering is most likely due to BDNF-mediated induction of Reelin expression (Dicou et al., 2009; Wasser and Herz, 2017). However, future studies are needed to examine this. Specifically, an important question is, what is the effect of BDNF overexpression to glutamatergic cells when FMRP is lacking at different stages of development?

The involvement of BDNF signaling in the pathophysiology of FXS was further investigated in our later study, where we examined the effects of reduced BDNF expression on the learning and behavioral phenotypes, in the *Fmr1*-KO mouse crossed with a mouse carrying a deletion of one copy of the *Bdnf* gene (Uutela et al., 2012). On this article, we reported age-dependent alterations in the expression of BDNF in the hippocampus, mild deficits in water maze learning and significantly impaired contextual fear learning in double transgenics. Reduced BDNF expression did not alter central hypersensitivity or basal nociceptive responses in *Fmr1*-KO mice. The locomotor hyperactivity and deficits in sensorimotor learning, and startle responses, typical to *Fmr1*-KO mice, were improved by reducing BNDF. Collectively, these findings combined to findings of cell region and subtype specific alterations of TrkB-BDNF signaling presented on **article III** indicate that BDNF modulation is involved in synaptic dysfunction that underlies behavioral phenotype in FXS and suggest in line with numerous other molecular and genetic studies that BDNF/TrkB signaling plays a role in the pathogenesis of developmental disorders (Perry et al. 2001; Nelson et al. 2001; Miyazaki et al. 2004; Belmonte & Bourgeron 2006; Shim et al., 2008; Correia et al. 2010; Nomura et al., 2017).
Further support of the participation of BDNF signaling in the pathophysiology of FXS comes from our prior *BDNF* polymorphism study. A single-nucleotide polymorphism (SNP) in the human

BDNF gene, that leads to a methionine substitution for valine at amino acid 66 (Val66Met), interferes with the intercellular trafficking and the activity-dependent secretion of BDNF in cortical neurons (Egan et al., 2003, Chen et al., 2004). In a small sample report of Finnish FXS male patients, we found similar mental age between Val/Val and Val/Met subgroups, but the men bearing the Met66 allele showed poorer adaptive behavior scores in the Vineland Adaptive Behavioral Scale (Sparrow, 1984) than the men with the Val66Val genotype (Louhivuori et al., 2009). We also revealed that the Val66Met *BDNF* polymorphism associates with epilepsy (Louhivuori et al., 2009). Yet, this was not confirmed in a group study of 77 patients with FXS (Tondo et al., 2011). However, there were differences between age-groups and gender heterogeneity in the studies and thus, can account for the variable results. Interestingly, in our study sample, an intronic SNP in the *BDNF* gene (rs6484320) was also found to be associated with epilepsy in FXS individuals, so one could argue that it might also play a part. Nevertheless, a recent meta/analysis study performed by Xu et al., showed a significant association of *BDNF* rs6265 G>A (Val66Met) polymorphism in epilepsy susceptibility, especially in the Asian population (Xu et al., 2018).

Additional confirmation of an association of *BDNF* polymorphisms in the clinical manifestations of FXS came from recent drug efficiency study (AlOlaby et al., 2017). A significant association between the *BDNF* polymorphism and improvements of several clinical measures, including the Clinical Global Impression scale and the cognitive T score, was observed in a double-blind randomized placebo-controlled clinical trial of selective serotonin reuptake inhibitor, sertraline, in young children with FXS (AlOlaby et al., 2017). In addition, an open-label study in youth with FXS showed a significant increase in BDNF level after treatment with the $GABA_A$ agonist, acamprosate, indicating that BDNF might be a useful pharmacodynamic marker in future acamprosate studies (Erickson et al., 2013).

As combined, these studies identify BDNF signaling and *BDNF* genotype as potential molecular biomarkers in FXS. However, further studies are urgently needed to clarify the exact functional role of BDNF signaling during different developmental timepoints and specific brain regions, and *BDNF* polymorphisms in the pathophysiology of FXS.

6.5. Diffentiating progenitors, neocortical layer formation and significance to FXS pathophysiology (IV)

In general, our findings point out that abnormalities in NPC properties and in their differentiation contribute to the pathophysiology of FXS and further confirm that in the absence of FMRP, many developmental problems can be seen already before synaptogenesis and therefore have challenged the classical views of FXS being caused only by aberrations in synaptic connections. Intriguingly, several research articles suggest that FMRP's function in the CNS may be tightly linked to developmental timing (for review see, Li and Zhao, 2014). Our research groups pioneer work on the role of FMRP in NPC function during development, has previously reported that the loss of FMRP results in an altered early maturation of FMRP-deficient cortical progenitors derived from human FXS fetus or *Fmr1*-KO mice brains (Castrén et al., 2005). Another study of human NPCs found that loss of *FMR1* leads to notable changes in overall progenitor cell gene expression and further that neural differentiation resulted in the abnormal expression of several neurodevelopmental genes (Bhattacharyya et al., 2008). Very recent paper also implicates FMRP in controlling aNSC differentiation through regulatory discrimination of mRNAs (Liu et al., 2018). In line with our findings of altered neuronal differentiation and less immature neural phenotype (**Articles III and IV**), FMRP has been shown to play an important role in the proliferation of NSCs in the *Drosophila* larval brain by controlling the precise timing of re-entry to into the cell cycle

(Callan et al., 2010) and in the proliferation and differentiation of NSCs in the adult mouse brain (Luo et al., 2010). Furthermore, it has been shown that FMRP ablation in adult NSC causes disturbances in hippocampus-dependent learning (Guo et al., 2011).

The canonical Wnt/β-catenin signaling pathway have been implicated in the regulation of neuron-glia proliferation and the decision to differentiate into neurons or astrocytes in the adult mouse brain when FMRP is lacking (Luo et al., 2010; Guo et al., 2012). Also, at early stages of development, a link between Wnt-signaling and FMRP in FX-hESCs has been suggested (Telias et al., 2013). In aNSCs isolated from the DG of the HC, the loss of FMRP results in fewer neurons and excess glial cells when compared to WT, and this alteration in the ratio between neurons and glial cells is connected to decreased levels of *Neurogenin1* due to dysregulated Wnt signaling (Luo et al., 2010). By using a cyclin-dependent kinase 4 (CDK4) chemical inhibitor the overproliferation defects of *Fmr1*-KO aNPCs were partially rescued, indicating that CDK4 mediates at least in part FMRP's function in regulating aNPC cell cycle (Luo et al., 2010). Interestingly, CDK4 and cyclin D1, a regulatory subunit of cyclin-dependent kinases CDK4 and CDK6 that is required for progression through the G1 phase of the cell cycle, have been also identified as candidate mRNA targets associated with FMRP, and altered expression of CDK4 in FMRP deficient synapses has been reported (Miyashiro et al., 2003). Furthermore, inhibition of GSK3β, that is upregulated in *Fmr1*-KO aNPCs (Portis et al., 2012) improves hippocampus-dependent learning and reportedly rescues neurogenesis in a mouse model for FXS (Guo et al., 2012). The identification of GSK3β as an mRNA target indicates a possible role for FMRP in regulating the output of the Wnt pathway in NPCs.

Indeed, *Fmr1*-KO aNPCs exhibit higher levels of GSK3, which results in an increased degradation of beta-catenin and this in turn represses the expression of Wnt pathway genes such as *Neurogenin1*, which controls the choice between neuronal and astrocyte differentiation (Luo et al., 2010). Hence providing a mechanistic explanation for the increase in astrocytes and decrease in neurons observed in FMRP deficient aNPCs. Interestingly, during neocortical development upregulation of Wnt/β-catenin signaling induces early differentiation of IPs into neurons and lead to accumulation of these newly born neurons at the SVZ/IZ border and promotes RG self-renewal (Munji et al., 2011). The relationship between FMRP and GSK3 seems to be, however both, developmental stage specific and species specific since no evidence for a pathological increase in GSK3β protein levels upon cellular loss of FMRP in FX-hESCs, derived from FX human blastocysts, were found (Luo et al., 2010 vs. Telias et al., 2015a). Instead, they suggested a novel role for the *SOX* superfamily of transcription factors in delayed neurogenesis observed in FX cells (Telias et al., 2015a). Furthermore, recent paper showed that a major toxicity of GSK3 inhibition, stabilization of β-catenin, could be avoided by selective inhibition of GSK3α, but not GSK3β, and it was sufficient to correct a range of disease phenotypes in a mouse model of FXS (McCamphill et al., 2020). Therefore, implying GSK3α selective inhibitors as new potential therapeutic reagents for treating FXS.

Intriguingly, very recent study also suggests the involvement of FMRP in the epigenetic regulation of Wnt/β-catenin, Ras/MAPK, and mTOR signaling pathways during neocortical development (Casingal et al., 2020). In fact, both Ras/MAPK and PI3K-AKT-mTOR signaling cascades have been implicated in FXS (for review, see Borrie et al., 2017). Overactivation of mTOR signaling during neurogenesis can increase protein synthesis and induce neuronal differentiation (for review, see Licausi and Hartman, 2018) and further, mTOR signaling is exaggerated in the absence of FMRP (Gross et al., 2010; Sharma et al., 2010). Fitting in line with our findings of aberrant glutamatergic neurogenesis that were linked to altered lamination of postnatal neocortex of *Fmr1*-

KO mice and likewise, related to the finding of altered migration of TrkB+ cells in the developing neocortex of *Fmr1*-KO mice (**Article III**).

No matter the various pathways implicated, and mechanical differences found in different developmental stages and culturing systems, notably, all these stem cell reports are consistent with the presence of cell fate abnormalities and signaling pathway aberrations that could lead to circuitry defects in *Fmr1*-KO brains. However, further research is required to reveal the complete sequence of molecular events that mediate FMRP function during development and adult neurogenesis.

FMRP function is required for normal brain development and formation of functional neuronal connections (Contractor et al., 2015). We showed during neocortical development that when the function of FMRP is compromised with a missense point mutation I304N in the KH2 domain, increased production of progenitors and defects in the migration of newborn cells are seen. These results agree previously shown increased expression density of recently divided BrdU-positive cells in the SVZ of *Fmr1*-KO mice (Castrén et al., 2005) and with the finding of increased cell density in the CP of *Fmr1*-KO at E16 mice brain sections and further with increased number of Ki67-positive dividing cells and higher cell density in the CP in the 18-week-old FXS brain when compared to the age-matched WT controls, suggesting aberrations in the neuronal lamination of deeper neocortical layers in the absence of FMRP.

During earlier cortical development or adult neurogenesis in the absence of FMRP alterations of RG cells have been seen (Luo et al., 2010; Saffary and Xie, 2011). However, we detected no differences in RG markers (BLBP and GLAST) in our experiments. Nonetheless, these results do not rule out possible alterations in the RG NSCs at other stages of cortical development. Fitting in line with our findings of aberrant differentiation of Tbr-2+ cells in the neocortex of mice lacking FMRP, with shRNA electroporation approaches, it has been reported that the loss of FMRP causes a shift in the relative ratio of stem cell-like RGCs to the IPCs in the neocortex of embryonic mice (Saffary and Xie, 2011). More specifically they show, that a specific knock-down of FMRP during early neocortical neurogenesis leads to severe depletion of RGCs or in *Fmr1*-KO mouse embryos, a mild but significant reduction in the size of the RGC pool at E14.5 is seen. These results were based on the expression of Pax6, a nuclear marker for RGCs, which as a nuclear marker would allow more accurate cell counting than cytoplasmic markers (BLBP and GLAST), because of the high density of RGCs in neocortical sections. Consistent with our findings of normal expression of cytoplasmic RGC markers at E16.5 in *Fmr1*-KO mice, they also observed normal number of Pax6+ cells at E16.5 in the brain of *Fmr1*-KO mouse embryos.

Excitingly, a mechanistic link that fits to our findings of aberrant Tbr2-positive cortical progenitors (Article IV) and altered migration of TrkB-positive interneurons in the absence of FMRP (Article III) has been reported. It has been shown that Tbr2-positive cortical INPs dictate the migratory route and control the amount of subpallial GABAergic interneurons in the SVZ through a non-cell-autonomous mechanism, with Cxcl12 chemokine signaling, since its forced expression in the Tbr2 mutants can rescue, to some extent, SVZ cell migration (Sessa et al., 2010). It has led to an assumption that INPs are able to control simultaneously the increase of glutamatergic and GABAergic neuronal pools and by interneuron attractive activity to intrinsically balance their relative accumulation (Sessa et al., 2010). Future studies shall shed light on these intriguing findings and a possible link with FMRP function.

In line with our finding of altered differentiation of glutamatergic lineage Tbr2+ and Tbr1+ cells that leads to abbreviations in postnatal cortical structures in *Fmr1*-KO, one more study showed that FMRP regulates the positioning of neurons in the cortical plate and multipolar-to-bipolar transition

affecting neuronal migration and the establishment of neuronal networks (La Fata et al., 2014). Additionally, in agreement with our findings of altered migration and differentiation of glutamatergic neurons that disrupt normal formation of cortical layers when FMRP is lacking, Gonçalves et al., reported state-dependent network defects in the developing neocortex of *Fmr1*-KO mouse brain that affect developmental processes involved in neuronal circuit formation and function (Gonçalves et al., 2013). Taken together, these results imply abnormalities in the neurogenesis of nIPCs and specifically in the differentiation of cells committed to glutamatergic lineage in the absence of FMRP. These results are consistent with various studies showing NPC alterations in FMRP-lacking cells (Castrén et al., 2005; Bhattacharyya et al., 2008; Callan et al., 2010; Luo et al., 2010; Saffary and Xie, 2011; Gonçalves et al., 2013; La Fata et al., 2014; Liu et al., 2018).

ER81 is a known downstream target of Pax6 which has been identified to be sequentially expressed before glutamatergic cell lineage markers; Ngn2, Tbr2, and Tbr1 in the developing cortical progenitors (Englund et al., 2005; Tuoc and Stoykova, 2008). Therefore, offering a direct link between glutamatergic progenitors and ER81 expressing neurons. Abnormally long and tortuous spines in higher densities have been seen particularly in large corticospinal projection neurons of the postnatal and adult somatosensory neocortex of *Fmr1*-KO mouse (Nimchinsky et al., 2001; Galvez and Greenough, 2005). The observed increase in the ER81+ cells of early postnatal neocortical layer V in *Fmr1*-KO suggests alterations in the development of cortico-cortical and sub-cerebral projection neurons in the absence of FMRP, since distinctively these neuron types are known to express ER81 (Yoneshima et al., 2006). Consequently, the abnormalities seen in the density of ER81 expressing cells in the layer V in the brain of *Fmr1*-KO mouse at P5 may be linked with previously reported dendritic spine abnormalities present in the human and mouse FXS brains (Rudelli et al., 1985; Hinton et al., 2001; Irwin et al., 2001; reviewed in, He and Portera-Cailliau, 2013). Therefore, our findings suggesting that in the absence of FMRP, cell type-specific changes caused by defects in the early differentiation of NPCs may be associated with aberrances in sprouting and result in abnormalities seen in the development and maintenance of synapses in the brains of *Fmr1*-KO mice.

Furthermore, in neurons differentiated from patient derived ESCs or iPSCs aberrant gene expression profiles during neurogenesis as well as morphological and functional abnormalities have been shown when the function of FMRP is altered (Urbach et al., 2010; Sheridan et al., 2011; Telias et al., 2013, Doers et al., 2014, Telias et al., 2015a, Telias et al., 2015b; Halevy et al., 2015; Boland et al., 2017; Sunamura et al., 2018; Achuta et al., 2017; Danesi et al., 2018). In agreement with our findings, very recent transcriptome analysis study of the NPCs derived from *FMR1*-KO human iPSCs showed altered expression of neural differentiation markers, particularly a marked induction of the astrocyte marker GFAP, implying that iNPC neither solely obtained nor lost the tendency to differentiate into neuron, but rather dysregulation of differentiation to neural lineages (Sunamura et al., 2018). No matter some variations between results, which may be due to intrinsic differences between the respective cell lines, importantly, all the studies constantly report defects in neurogenesis and that neurons differentiated from patient derived ESCs or iPSCs show an immature phenotype with impaired excitability and synaptic connectivity (Telias et al., 2015a, Telias et al., 2015b, Halevy et al., 2015, Boland et al., 2017, Sunamura et al., 2018). These studies further demonstrate the importance of patient derived ESCs and iPSCs as a model system to study molecular mechanisms of FXS. All the findings combined, it is clear, that FMRP is required for proper development of neuronal activity during differentiation from progenitors and imply that incorrect differentiation of FMRP lacking NPCs may be a common pathological mechanism underlying FXS.

6.6. Calcium-mediated receptor responses in progenitor differentiaton and role in FXS

Ca^{2+}-mediated signals regulate many processes throughout neuronal development, including progenitor cell proliferation, migration and differentiation, dendrite growth and axon guidance (Zheng and Poo, 2007; Rosenberg and Spitzer, 2011). Immature neuronal morphology is one of the main characteristics in the FXS (Rudelli et al., 1985; Hinton et al., 2001; Irwin et al., 2001) and *Fmr1*-KO mouse brain (reviewed in, He and Portera-Cailliau, 2013). FMRP-deficient neocortical and hippocampal pyramidal neurons display an immature dendritic spine phenotype, alterations in neurite length and the morphology of GABAergic interneurons in the neocortex of *Fmr1*-KO mice have been reported (reviewed in, He and Portera-Cailliau, 2013). Differentiating FMRP-deficient NPCs in culture displayed both fewer and shorter neuronal processes and aberrant intracellular calcium oscillations to Ach and mGluR5 activation (Castrén et al., 2005). In line with our findings of aberrancies of ATP responses in progenitors lacking FMRP. These findings of immature neuronal morphology further imply a delayed maturation of FMRP-deficient neurons and potential defects in neuronal function. Indeed, the $[Ca^{2+}]_i$ signaling is involved in early morphological maturation and the neurotransmitter phenotype acquisition of neurons (Ciccolini et al., 2003) and oscillating $[Ca^{2+}]_i$ release can enhance gene expression (Dolmetsch et al., 1998; West et al., 2001). Therefore, aberrant $[Ca^{2+}]_i$ release may be contributory for the immature phenotype of FMRP-deficient neural progenitors. Furthermore, the increased number of neural progenitors differentiated from FMRP-deficient cells during cortical development may be explained by an augmented fate determination into neurons as a result of intense oscillatory $[Ca^{2+}]_i$ transients (Gu and Spitzer, 1995).

The aberrant $[Ca^{2+}]_i$ transients are not only present in FMRP-deficient differentiating progenitors, as well spines and dendrites of neurons in the mature prefrontal cortex have been shown to exhibit similar alterations, thus implying that alterations in Ca^{2+}-signaling may be a general feature of FMRP-deficient neural cells (Meredith et al., 2007). Since intracellular Ca^{2+} oscillations are most affected in subsets of neurons sensitive to mGluR5 antagonist MPEP, the altered Ca^{2-} signaling in the FMRP-deficient neural cells might substantially intensify overall gene expression together with excess mGluR5 dependent protein synthesis (Bear et al., 2004; Castrén et al., 2005). These results indicate not only morphological abnormalities in cultured NPCs, but also suggest that FMRP is required for proper function of glutamatergic neurons and are in line with our findings of altered cortical neurogenesis of glutamatergic lineage Tbr2+/Tbr1+ cells in *Fmr1*-KO mice brains (**Article IV**). Our finding of the importance of an inter-receptor crosstalk, through the release of neurotransmitters such as glutamate and acetylcholine acting via TRPC3 channel proteins and MMPs that regulates the activity of NRG1, whereby the transactivation of ErbB4 by G_q-coupled receptor proteins combines the broad diversity of GPCRs with the potent ErbB signaling pathway in RG-neuron interactions and RG scaffold maintenance in normal conditions (**Article II**), highlights the regulatory control of these signaling pathways during cortical development. Ultimately, potential deficiencies caused by the lack of functional FMRP in these signaling pathways, and therefore neural migration and fate choice determination, could in part explain the aberrations seen in cortical development of FXS. However, future studies shall shed light on this intriguing suggestion.

Our studies further revealed enhanced intracellular calcium responses to ATP along with temporal changes of ATP responding cell population in differentiating neurospheres lacking FMRP (**Article III**) suggesting delayed alteration in the maturation of ATP responding cells. Indeed, extracellular ATP is essential for the initiation and propagation of calcium waves in the cells of the proliferative VZ (Kriegstein and Noctor, 2004; Weissman et al., 2004) and for the migration of INPs (Liu et al.

2008). At later stages of differentiation, the amount of ATP responding cells was increased in a FMRP-lacking cell population which spatial location near the mother neurosphere in a layer of cells with positivity to RG markers and responsiveness to other neurotransmitters, such as mGluR5 activation which corresponds well to more immature RG cell phenotype. In line with our findings of glutamate, ATP and NMDA responding cells and their correspondence to different maturational stages of cells, we have recently shown that mGluR5 responses do indeed dictate the differentiation of progenitors to NMDA-responsive neural cells in FXS (Achuta et al., 2017). NMDA responses in combination with the immunostaining results indicate a more mature neural phenotype for these NMDA responding cells and agree with our previous findings in **Article I** and later publication, where we showed that differentiating neurospheres with BDNF increases the migration of a particular NMDA responsive cell population (Jansson et al., 2012).

Furthermore, our findings showed temporal changes in the differentiation and migration of Tbr2 expressing cells during neocortical development of *Fmr1*-KO mice. Interestingly, prior study, which provides a mechanistic link between ATP signaling and Tbr2-positive cell population, reported that ATP signaling regulates the migration of Tbr2-positive cells (Liu et al., 2008). Therefore, these findings suggest that in the absence of FMRP, the aberrant ATP signaling could contribute to the alterations seen in the Tbr2 cell population and consequently might play an important role in the pathogenesis of differentiating NPCs in FXS. Moreover, BDNF exposure restored the enhanced amplitude of ATP responses to WT levels in a subpopulation of differentiating NPCs lacking FMRP, suggesting that perturbations of BDNF/TrkB signaling are involved in the enhanced ATP responses and thereby in the impaired migration of INPs in the absence of FMRP. However, further studies are required to reveal exactly, how the lack of FMRP function affects ATP signaling and thereby the migration and fate determination of INPs.

Collectively, these findings suggest FMRP function dependent changes in progenitor cell fate determination, maturation and differentiation dynamics that are developmental time specific and fit the current scenario of imbalance of inhibitory and excitatory neuron development, which affects circuit network formation and ultimately synaptic plasticity changes seen in FXS (Li and Zhao, 2014; Berry-Kravis et al., 2018). Altogether, these findings indicate that in the absence of FMRP, the aberrances in the early differentiation of glutamatergic neuronal cells are associated with alterations in the lamination and positioning of cortical neurons in a mouse model for FXS. It will be of high importance to study these findings in the near future with human derived cells. In addition, our findings of calcium-dependent neurotransmitter mediated signaling mechanisms during cortical development as mediators of neural-RG interactions and cell determination, it will be crucial to reveal the potential role of FMRP function in these processes in different models of FXS.

6.7. Future possibilities and challenges with neural stem cell research and therapy in general -methodological view

The (re-)discovery of the presence of neural stem cells and progenitors in the adult mammalian brain in the 1990s (Altman, 1962 vs. Reynolds and Weiss, 1992, Lois and Alvarez-Buylla, 1994), has opened novel possibilities to study the maturation and function of neuronal cells, in addition to developing therapies for brain injury and different neurodevelopmental and neurodegenerative diseases (reviewed in Lois and Kelsch, 2014). Stem cells and their progenitors can be utilized in culture systems in addition to disease modeling to establish highly standardized cell assays for toxicology and drug screening to replace assays which do not accurately represent the actual human system (reviewed in, Singec et al., 2007). Our data presented here and in previous studies indicate

that that the same metabotropic and ionotropic signaling pathways operate in mouse embryonic and postnatal NPC cultures, mouse embryonic slices, and cultured human NPC cells (Castrén et al., 2005). This indicates that the neurosphere model used in this study is valid for functional studies on mechanisms involved in neuronal development and NPC migration. In general, the utilization of NSCs and NPCs in different applications can thus be considered as a powerful research tool to unravel the mechanisms of neuronal cell fate determination and henceforth the culturing and differentiating of progenitors provides an exceptional *in vitro* model for cellular and developmental studies. As shown in this study, the neurosphere model is particularly suited for quantitative single cell imaging methods. The model also allows quantification of different motility patterns and cell-cell contacts. Like previous studies, our research thus substantiates the usefulness of neurosphere cultures as a well-established tool to study the differentiation and migration of neuronal cells.

One challenge in NSC research has been that it has proven rather difficult to separate specific cell types or subpopulations of cells from mixed cell populations, since most propagation and differentiation protocols produce mainly heterogeneous cell populations. Likewise, despite the availability of various markers, the lack of highly specific cell lineage or cell type identifying markers is still evident, therefore slowing cell type identification and limiting the possible clinical applications to use these cells (for review on astrocytic markers, see Chandrasekaran et al., 2016 and for neurogenesis markers, see Zhang and Jiao, 2015). Naturally, functional studies are needed in combination with molecular studies to truly identify the vast variety of different types of neuronal cells. Our functional calcium measurements of neurotransmitter responsiveness provide valuable new information of progenitor cell receptor dynamics and reveal distinct subpopulations of migrating and maturing cells that can be distinguished based on their responsiveness. Immunostainings of the differentiated cells combined with cell localization based on their transmitter responsiveness were not solely specific for certain cell types, further highlighting the need for more specific immunomarkers to distinguish different subpopulations of progenitors. The existing lack of specific cell lineage markers for subpopulations of RG and NPCs should be overcome with recent developments in the field of next generation sequencing techniques combined to new imaging techniques (reviewed in, Poulin et al., 2016; Zhang et al., 2018; Ben Jehuda et al., 2018; Mayer et al., 2019).

For therapeutical purposes or specific cell type studies, it is important to take in consideration that there are also variations in the potency to produce certain kind of differentiated cells from SCs, since only few cells in a heterogeneous colony could possess the right cell fate (Schroeder, 2013). Consequently, one partially unachieved future challenge in NSC research, is to differentiate specific lineages and/or fates for clinical applications and cell transplantation therapies (reviewed in, Lederer and Santama, 2008; Cohen and Melton, 2011). Another problem is possible misidentification of model cells that needs to be addressed in the scientific community in order to develop cells that mirror those found in the human brain (Lu et al., 2021). However, recent advancements in the field of single-cell transcriptomics which can be used to profile gene expression in individual cells and uniquely classify neural cell types based on combinatorial gene expression deliver means to deepen our understanding of the genetic programming underlying cortical development (reviewed in, Poulin et al., 2016). Intriguingly, very recently single-cell transcriptomic analysis of mouse neocortical development that enables the direct molecular comparison of all cell types of the developing cortex under the same experimental scheme was achieved (Loo et al., 2019). This provides an essential resource for future studies directed at understanding how genetic and environmental factors affect cell composition, cell states and fates and in uncovering correct cell identity. Combination of such approaches with cellular imaging methods would provide further mechanistic information.

It is intriguing to consider, since NSCs are a subpopulation of glial astrocyte-like RG cells (for reviews, see Ihrie and Alvarez-Buylla, 2008; Rowitch and Kriegstein, 2010 and Bayraktar et al., 2014), whether different forms of possible future CNS stem cell therapies could utilize these subpopulations of glial cells in the adult brain? Could it be that they may retain the capacity to be latent NSCs, and thus be induced to become NSCs and even different types of highly specialized neural cells by turn over? (For introduction of this idea, see Doetsch, 2003). This possibility could prove to be especially important tool for treatment in neurodevelopmental or neurodegenerative diseases (for review, see Barker et al., 2018). Thrillingly, very recent report has claimed to do this in a mouse model for Parkinson's disease (Qian et al., 2020). Indeed, it has been shown that astrocytes in parenchyma might have an increased potential to produce neurons in the setting of injury through sonic hedgehog (SHH) dependent signaling mechanism (Sirko et al. 2013) and suggesting that NSC properties might be retained under certain circumstances. In addition, cultured astrocytes have been induced to function as neuronal progenitors with modifying transcription factors (Heins et al., 2002; Berninger et al., 2007), opening further possibilities to exploit these cells in future cell therapy applications. However, to date it is still unclear which molecular characteristics exactly distinguish classical astroglial cells that do not function as NSCs from astroglial cells with progenitor properties.

Adult NSCs are likely confined within the developmentally transforming NE-RG-astrocyte lineage (Alvarez-Buylla et al., 2001), named slowly dividing type-B-cells (reviewed in, Gonzalez-Perez and Quinones-Hinojosa, 2012). Remarkably, type-B stem-cells are not a homogeneous population; instead, it seems they comprise restricted subpopulations with diverse neurogenic potential (Merkle et al., 2007), further highlighting the need for specific markers to identify differences between arising populations. Intriguingly, current data also indicates that astrocytes display an astounding diversity in terms of positional identity, partly inherited from their RG ancestors (Bayraktar et al., 2014; John Lin et al., 2017) or instructed by surrounding neurons leading to differences in their morphology and gene expression at different laminar positions (Farmer et al., 2016, Lanjakornsiripan et al., 2018). Our findings of functional neurotransmitter responsiveness, and similarly vascular mediators like endothelins (Louhivuori et al., 2020), combined with morphological changes and migration patterns of differentiating progenitors provide valuable new data for further fate determination and differentiation studies and highlight the importance of G_q-coupled signaling dynamics in the regulation of cell fate specification and RG-neuronal interactions mediated migration and bipolar-to-multipolar transitions.

When considering possible brain repair applications, another promising strategy aside NSC, would be direct neuronal re-programming from local glial cells upon injury, since they maintain some of the original patterning information from their RG ancestors (Grade and Götz, 2017, Barker et al., 2018, Wang and Zhang, 2018, Mattugini et al., 2019). However, the acquisition of adequate neuronal subtype identity and target region innervation is a key for functional repair, since laminar differences of neurons are crucial for correct cortical function. Region-dependent and layer-specific differences in cortical astrocytes might affect the outcome of re-programming in terms of neuronal subtype identity and consequently, inducing neurons that derive from other regions, such as cortical interneurons with subpallial origin might prove problematic. Therefore, more studies of the extrinsic and intrinsic signaling mechanisms affecting these processes are crucially needed.

The important questions whether layer-specific re-programming is driven by cell-intrinsic mechanisms, specific migration and/or environmental cues, remain unanswered. Future investigations with new genetic tools, multiclonal lineage tracing using combinatorial genetic markers together with new imaging methodology and transplantation studies should help determine

exactly how plastic regionally specified astrocytes are and whether their local identity is cell autonomously determined. These further studies shall also shed light on developmental aspects of NSC-RG-neuron and astrocytic cell progeny production and maturation dynamics. Very recent study of the development of cortical astrocytes revealed that local environment likely determines clonal expansion of astrocytes and their final morphotype (Clavreul et al., 2019), suggesting that non-specified astrocyte progenitors produce plastic, intermixed clones, whose daughter cells may adopt different morphotypes through interactions with their environment. Intriguingly, our findings of the interplay between RG-neuronal contacts and their signaling mechanisms would agree with this and importantly, the neurotransmitter release driven G_qPCR-mediated signaling causing transactivation of NRG/ErbB signaling at the onset of cell migration offer one very versatile explanatory mechanism how cells could achieve this control of cell maturation also in astrocytes.
Another exciting possibility when considering future CNS cell therapy, could be the conversion of cells from oligodendroglial origin at specific sites (for review, see García-León and Verfaillie, 2016).

6.7.1. Studies in vitro vs. in vivo and from mice to men

One important thing to keep in mind with SC research in neuroscience is that the progenies generated from NSCs change over the course of development *in vivo* (for reviews see, Merkle and Alvarez-Buylla, 2006; Kriegstein and Alvarez-Buylla, 2009). Even though it is well established that the *in vitro* neurosphere model resembles the precise developmental sequence seen during cortical development and some multipotent progenitors can generate a changing set of different neural cell types along with maturation (Qian et al., 2000; Caldwell et al., 2001; Shen et al., 2006; Gaspard et al., 2008). Still, the actual mechanisms and some affecting factors remain to be elucidated and led us to study these processes *in vitro*. The heterogeneous nature of neurosphere cultures, consisting of cells at various stages of differentiation, is an important matter to note when using a neurosphere culture as a model (reviewed in Jensen and Parmar, 2006). Neurosphere system is not to be considered a model for studying NSCs, but rather a model to study various progenitors arising within the neurospheres. Besides the importance of spatiotemporally controlled intrinsic programs, the contribution of environmental morphogen gradients as ques in the modulation of cell maturation and migration is therefore crucial to grasp. Specifically, the composition of growth media and the concentrations of mitogens present in growing conditions or other extracellular factors during differentiation can naturally modulate clone compositions and maturation (Irwin et al., 2003). Since cells can interact with each other in a cell culture, certain cell progeny could provide fate-modulating signals that might affect clone composition in the differentiating neurospheres and migrating and maturing cells could also affect nearby cells with their signaling (Kriegstein and Alvarez-Byulla, 2009).

In our culture conditions and during differentiation we showed that specifically glutamate signaling is an important factor in mediating migration and maturation, likely indicating that our growing conditions favor glutamatergic cell lineages. However, we have also shown GABAergic responses in a subpopulation of differentiated cells (Jansson et al., 2012). Likewise, we have revealed calcium fluctuations through T-type calcium channels as triggers for early neuronal activity and playing part in the initiation of neural migration and neurite outgrowth (Louhivuori et al., 2013). In addition, we have demonstrated various extracellular factors, like BDNF and NRG to play a part in RG/neural interactions and migration.
Furthermore, it is important to note that since progenitors can be manipulated *in vitro* to display features that are not present *in vivo* (Conti and Cattaneo, 2010), this needs to be carefully regarded and methodologically standardized for different applications, for example, cell passaging method (manual vs. enzymatic) and passaging number may affect the properties of cells. Overall, the

sensitivity of the culture system to variation highlights the importance of standardized cell culture routines. However, to this note it was quite surprising how similar calcium responses and therefore subpopulations of cells, reflected by their neurotransmitter responsiveness, were seen with E14 or P5-7 propagated cells in our culture conditions after differentiation (unpublished observation, Louhivuori et al.,). This indicates universal early differentiation patterns of cortical progenitors in E/P neurosphere cultures and truly validates the usefulness of this culturing system for developmental NSC/NPC migration studies.

Additional important aspects in SC research of the CNS that should be taken in consideration, are the differences between animal species in the rate of neurogenesis and functional purpose of neurogenesis in the different brain areas, that may vary between animals and within animal species alongside with the tight developmental and spatiotemporal regional control, thus further complicating SC research. Despite the tremendous value of rodent models in the investigation of brain development, they cannot provide insight into aspects that are unique or highly derived in humans and consequently, many human psychiatric and neurological conditions, that have developmental origins, cannot be studied adequately in animal models. Furthermore, despite the well-established role for SVZ and hippocampal neurogenesis taking place in the adult mouse brain (for review, see Obernier and Alvarez-Buylla, 2019) to date it is still unclear if significant hippocampal neurogenesis, that holds any functional importance, occurs in the adult human brain. Opposing results have been reported, leading to a big debate in the field (see, Sorrells et a., 2018 vs. Boldrini et al., 2018). However, it is likely that these contradicting findings can be explained with experimental caveats and challenges to study this phenomenon in postmortem human tissues (for review, see Lee and Thuret, 2018). Nevertheless, additional studies are crucially needed to address this matter more closely with different techniques and by increasing sample sizes.

Our findings of aberrant differentiation of Tbr2 positive cell population in the mouse model for FXS could prove rather significant in humans, since neocortical SVZ evolutionarily expands in primates (Zecevic et al., 2005; Noctor et al., 2008; Hansen et al., 2010; Molnar et al., 2011; 2019; Taverna et al., 2014). However, the human cerebral cortex has some unique genetic, molecular, cellular and anatomical features, specifically, known differences in neurogenesis and diversification of RG and progenitor cell subtypes and ultimately the formation of cortical structures which need to be further explored (reviewed in, Molnar et al., 2019). In addition, our finding of altered TrkB positive cell population in the developing cortex in the absence of functional FMRP might be of significance but needs to be carefully inspected in the human FXS brain, specifically since the migration pattern of TrkB-positive interneurons in humans reportedly differs significantly from the one occurring in mouse brain during cortical development (Letinic et al., 2002; Zecevic et al., 2005; 2011).

For future cortical development studies, the characterization of transcription factor expression sequences in progenitors, and analysis of how each transcription factor primes the cell for effective gene regulation by subsequently expressed transcription factors is of great importance (Hevner, 2019). Spatial transcriptomics methods enable *in situ* molecular profiling of cells in their circuit context and recently, with osmFISH technique (a cyclic single molecule fluorescent *in situ* hybridization protocol), the spatial distributions of 31 cell types in adult mouse cortex was revealed (Codeluppi et al., 2018). Importantly, these high-throughput, multi-modal profiling transcriptomics techniques can be applied to human cortex during development to identify the functional consequences of transcriptional profiles, including responsiveness to neurotransmitter signaling in human cortical cells (Mayer et al., 2019).

Some possibilities for cellular transplantation therapies of neural progenitors into the developing and adult CNS have been attempted and the long-term fate of the transplanted cells has been studied intensively (reviewed in, Lindwall and Kokaia 2010; Trounson and McDonald, 2015; Grochowski et al., 2018; Weston and Sun, 2018). Despite the progress, there are still challenges, for example many cells administered show only transient presence for a few days with trophic influences on immune or inflammatory responses. Evidently, currently information gathered about various intrinsic and extrinsic factors affecting proliferation, survival and differentiation mechanisms in the acquisition to a specific type of cell and correct laminar positioning is still lacking and further studies are required to unravel different aspects controlling the self-renewal, fate choice determination and migration. Moreover, targeting of cells to specific site, controlling their fate along with preventing tumor formation and the actual propagation of cells to existing neural networks to form functional synaptic connections are still challenges and further studies are necessary to overcome these issues (reviewed in, Gaspard and Vanderhaeghen, 2010; Zakrzewski et al., 2019).
Consequently, it is obvious that the knowledge gained from mice progenitor studies and brain development cannot be directly translated to apply in humans and therefore a crucial need for advancement toward humanized models is greatly indicated. Indeed, in the last decade of stem cell research, a huge step toward human modeling and cell-based therapy has been taken (reviewed in, Rowe and Daley, 2019).

6.7.2 The use of human stem cells and induced pluripotent stem cells in developmental research and cell therapy

The use of human SCs, which can be turned to certain tissue specific progenitors and afterwards highly specialized cell types, opens possibilities not only for developmental studies and disease modeling, but also for cell therapy. Modeling in humans is highly advantageous, however, no matter the vast developments, there are still issues to overcome. Firstly, there are several ethical issues concerning the use of human SCs and SC therapy in general. There are various laws and restrictions for embryonic SC research and in some countries the use of human embryonic SCs is if not restricted, very limited. In addition, the limited availability of human embryonic SCs and their therapeutical use raises an ethical dilemma (for reviews see, Shufaro and Reubinoff, 2004; Kastenberg and Odorico, 2008; Lo et al., 2010a; 2010b). Postnatal extraction of human SCs is generally more acceptable, even though the expanding of postnatal human SCs is slow and difficult, and for therapeutical purposes highly costly. The recent progress in the field of SC research; the creating of human derived induced pluripotent stem (iPS) cells from somatic cells, with the help of biotechnical tools (Takahashi and Yamanaka), has helped in overcoming these ethical dilemmas, but might arise other ones them self.

Patient-specific iPS cells created with cell re-programming from extracted human cells at any age introduce an overpowering method for *in vitro* disease modeling and drug discovery when compared to animal models, in addition to future cell transplantation or cell replacement therapies. For example, blood or fibroblast cells can be converted to iPS cells upon expression of a set of few transcription factors, following a strategy originally published (Takahashi and Yamanaka, 2006, reviewed in Takahashi et al., 2016). This rather groundbreaking finding, that the commitment of cells can be converted to more immature cell types did challenge the traditional view of developmental cell research, since for a long time it was considered that already specified cells cannot be reversed (for reviews, see Marchetto et al., 2010). Subsequently, these highly specialized types of cells can be transplanted patient-specifically to replace damaged cells or help to restore dysfunctioning tissue homeostasis at targeted site. In addition, with combined biotechnical

engineering, these created cells can function as specific pharmacological drug releasing cells at precise target place.
Latest advancement on the field, the combining of iPS cell methodology with revolutionary technology for genome editing, CRISPR-Cas9 system is newly recognized as a powerful tool for editing DNA at specific loci (Zhang et al., 2018; Ben Jehuda et al., 2018). It offers the possibility to infer cell lineage from gene expression trajectories and this technology can be used to capture a detailed picture of cortical cell fate decisions. However, it is important to note that the combined use of iPS cells and CRISPR/Cas9/technology in human germ lines raises ethical issues (Hockemeyer and Jaenisch, 2016). The importance of ethical transparency and commonly regulated and agreed rules for these experiments should not be overlooked.

Although these recent advancements in the stem cell research field, it is important to keep in mind that modifying cell properties for therapeutical uses with different biotechnical tools may as well raise high resistance and still needs to be carefully investigated more in detail. Specifically, it has been reported that postnatal SCs may have limited proliferation capacity *in vitro* (Rao and Mattson, 2001). Even though postnatal SCs are telomerase competent and thus able to slow down the rate of telomere loss, the lengths of telomeres in somatic SCs and progenitors declines with age and may limit the use of these cells. Additionally, genetic and epigenetic abnormalities associated with reprogramming and *in vitro* culturing of adult cells to iPS cells have been found (Gore et al, 2011; Lister et al., 2011; Hussein et al., 2011). It was shown that the reprogramming process for generating iPS cell is associated with inherent DNA damage; iPS cells had more genetic abnormalities than their originating cells and embryonic stem cells (Hussein et al., 2011). Importantly, these mutations could alter the properties of these cells and thus affect their clinical applications. This discovery has an important implication in the use of these cells for replacement therapies in regenerative medicine and gives researchers new insights into the re-programming process, highlighting the need for further studies to help make future applications of SC creation and subsequent use safer (for reviews see, Gaspard and Vanderhaeghen, 2010; Cohen and Melton, 2011; Zakrzewski et al., 2019). So far, various SC therapies are currently in clinical trials, and some with shown early benefits to patients (reviewed in, Trounson and McDonald, 2015; Alessandrini et al., 2019).
All in all, it can be concluded that despite the rapid progress in SC research and transplantation therapy still many challenges are faced, and more studies are required to conquer these issues. Subsequently, for basic cell differentiation and migration modeling and mechanisms studies, the *in vitro* neurosphere model is even now beneficial and especially in parallel with iPS-culturing and new methodology like transcriptomics, CRISPR/Cas9 technology, and 3D organoids studies (Gordon et al., 2021).

6.8. Future aspects of stem cell research in FXS and general therapeutic implications

FXS is the most common inherited human form of intellectual disability and a known cause of autism spectrum disorder. As a developmental disorder that results from the loss of function of FMRP (Pieretti et al., 1991) with increasingly understood pathophysiology, FXS is a model of developmental disability for targeted drug development efforts, and its mechanistic studies have revealed many molecular signaling cascades that are involved in neuronal development and abnormal synaptic plasticity function in the *Fmr1*-KO mouse (for reviews see, Braat and Kooy, 2015; Contractor et al., 2015; Huber et al., 2015; Richter et al., 2015; Banerjee et al., 2018; Bagni and Zukin, 2019). One most popular one being the mGluR5 theory of FXS, where the absence of FMRP leads to enhanced glutamatergic signaling via Group I mGluRs, which leads to increased protein synthesis and defects in synaptic plasticity, including enhanced long-term depression (Bear

et al., 2004). However, to date little is still known about the actual underlying mechanisms of the disease during early brain development. Since in healthy individuals, FMRP is upregulated during development of the neural lineage (for review, see Bhakar et al., 2012), it is assumably involved in the progression of neural development and at different stages of neurodevelopment FMRP regulates different targets. Indeed, in line with our findings, various developmental functions have been revealed for FMRP, that are linked to maintenance and differentiation of embryonic and adult NSCs (for review, see Li and Zhao, 2014). Consequently, the molecular mechanisms downstream to FMRP are of high interest for the part they could play in neocortical development and in FXS pathology. Our findings of alterations of NPC differentiation and cell-subtype specific properties during corticogenesis, long before synaptogenesis, provide essential information of the pathophysiology of FXS and urge the need for future studies during development.

FMRP is an RNA-binding protein that plays an established role in gene expression and regulates the translation of potentially hundreds of mRNAs, many of which have been implicated in the development and maintenance of neuronal synaptic function and plasticity (for review, see, Zafarullah and Tassone, 2019). In fact, disturbances in neuroplasticity are a key finding in FXS animal models, and an imbalance in inhibitory and excitatory neuronal circuits is considered to underlie many of the variable clinical manifestations of FXS (for review, see Ligsay and Hagerman et al., 2016). As a result, preclinical animal model findings have led to dedicated targeted drug treatment development in FXS on an imbalance between excessive glutamate and deficient GABA neurotransmission (for review, see, Berry-Kravis et al., 2018; Zafarullah and Tassone, 2019). However, despite the efforts and recent advancements in ongoing clinical trials with various drugs and substances, yet no cure has been developed for FXS.

One important newly arisen dilemma that could in part explain variable results gain from different studies and unsuccessful clinical trials is species and timing dependency of FMRP function. First is the fact that the developmentally regulated inactivation of *FMR1* in FXS human embryos starts with full expression of *FMR1* in the pluripotent state, even with an existing full mutation. As a result, the expression of *FMR1* in FXS humans gradually fades until it is completely lost in mature neurons (Willemsen et al., 2002). Importantly, this process is not fully recapitulated by *Fmr1*-KO mice strains, where the absence of functional FMRP is present throughout development (Verkerk et al., 1991; Mientjes et al., 2006). However, by using hESCs, this developmentally regulated inactivation of FMRP can be recapitulated (Telias et al., 2015). Therefore, highlighting the advantage of their use in future mechanistic studies. Secondly, variations in *FMR1*-related measures, like CGG repeat number and the percent of methylation that correlate to *FMR1* mRNA and FMRP expression, in addition to sex differences, affect the clinical outcome of FXS (for review, see Zafarullah and Tassone, 2019). It should be also noted, that the final human FXS phenotype may be modulated in a complex manner by several environmental factors, other epigenetic modulations and gene polymorphisms that may influence the outcome of clinical studies with different experimental approaches. Reportedly, FMRP may broadly affect gene expression during neurogenesis via translational control of epigenetic and transcriptional regulators (Sheridan et al., 2011; Korb et al., 2017). Thus, it is instinctive to assume that FMRP plays a pivotal role during neural development when it is abundantly expressed (Bhakar et al., 2012). Indeed, various approaches have provided evidence to support this suggestion along with present studies (**Articles III and IV)** (for reviews, see Li and Zhao, 2014; Telias, 2019).

7. CONCLUSIONS

The studies presented in this thesis highlight the importance of Ca^{2+} influx pathways in mediating neuronal maturation, migration and development of the nervous system. This research provides new information on progenitor cell properties and differentiation, their neurotransmitter responsiveness and the cellular mechanisms that regulate cell migration, specification and process growth. Particularly, calcium-dependent glutamate-mediated receptor signaling seems to be a key factor in early differentiation of progenitors and in maintaining RG-neuronal interactions and neurite process elongation.

These studies also shed light on neocortical development and reveal disturbances particularly, in the differentiation of glutamatergic cell lineage, expressing sequentially Tbr2 and Tbr1 transcription factors in a mouse model for FXS. Therefore, highlighting the importance of functional FMRP in the regulation of NPC differentiation. In addition, a role for BDNF/TrkB signaling in modulating neocortical development and alterations of dendritic targeting of *Bdnf* mRNA in the absence of functional FMRP was revealed, offering a molecular mechanism by which the regional and cell-type specific changes could be explained in FXS. Both these studies further confirm the importance of functional FMRP already early during development, before synaptogenesis, in parallel to its classical role in synaptic spine modulation.

Based on the findings presented in this thesis, the following conclusions can be drawn:
Collectively in these studies (**I, II, III**) it was revealed that the *in vitro* differentiation of neurospheres is a dynamic process involving spatially and temporally distinct cellular subpopulations, which can be distinguished based on their neurotransmitter responsiveness, morphology and migratory behavior. **Calcium-imaging together with immunostaining and live cell imaging can be used as a combined tool** to indicate these subpopulations of differentiating progenitors.

(I) Two major subpopulations of cells can be found: **1.)** mGluR5 (and SP/NE/ATP) responding cells, with a morphologically more immature phenotype, slower migration, shorter and bulkier processes, likely positioning at the edge of the differentiating neurosphere, and shared immunopositivity to RG markers (i.e.,BLBP and GLAST). **2.)** And cells, that are more likely migrated further away from the neurospheres, have a more neural phenotype with small clear soma, thin and long processes and fast migratory movements, immunopositivity to neural markers (i.e., Tuj-1, Map-2 or NeuN) and robust $[Ca^{2+}]_i$ responses to iGluR activation and 70 mM K^+ induced depolarization, in addition, some respond to NMDA and Ach. Specifically, **changes in gluR responsiveness during differentiation seem to correlate with the maturational stage of the cell, from metabotropic RG to ionotropic neural**.

II) The G_q-coupled receptor mGluR5, acting via TRPC3 channel proteins and MMPs regulates the activity of NRG1, which in turn promotes RG process elongation via receptor tyrosine-protein kinase ErbB4. Since also other G_q-coupled receptors such as muscarinic acetylcholine receptors can replace mGluR5 and cause the transactivation of ErbB receptors, young neurons can promote RG scaffold growth through release of neurotransmitters such as glutamate and acetylcholine. This suggests an inter-receptor crosstalk, whereby the **transactivation of ErbB4 by G_q-coupled receptor proteins combines the broad diversity of GPCRs with the potent ErbB signaling pathway**.

III) **Alterations in the BDNF/TrkB signaling** modulate brain development and affect to the synaptic plasticity impairments seen in the absence of FMRP. Lack of FMRP caused increased TrkB expression in neuronal progenitors, aberrant intracellular calcium responses to BDNF and ATP in subpopulations of differentiating NPCs, changes in TrkB and BDNF expressing cell populations during neocortical development, and spatial differences in the subcellular and regional BDNF expression in the brain of *Fmr1*-KO mice, demonstrating **cell subtype-specific alterations throughout neuronal maturation when FMRP is lacking**. Dendritic targeting of *Bdnf* mRNA was increased under basal conditions and further enhanced by pilocarpine-induced neuronal activity in cortical layer V and hippocampal CA1 neurons of *Fmr1*-KO mice. In line with regional differences of synaptic plasticity in the brain of *Fmr1*-KO mice, BDNF protein levels were increased in the hippocampus but reduced in the cortex of *Fmr1*-KO mice and the pilocarpine-treatment caused an accumulation of *Bdnf* mRNA transcripts in the most proximal segments of dendrites in cortical but, not in hippocampal neurons of *Fmr1*-KO mice. These findings suggest **regional differences in the regulation of BDNF signaling** and that **BDNF/TrkB-mediated feedback mechanisms for strengthening the synapses are compromised in the absence of FMRP,** providing one molecular mechanism that may account for developmental timing and regional specific changes in the cellular and synaptic plasticity resulting in cognitive defects in FXS.

IV) The **differentiation of glutamatergic cell lineage is disturbed** during cortical neurogenesis in the absence of FMRP, as seen with increasements of Tbr2 positive intermediate progenitors in the SVZ and afterward an accumulation of Tbr1 positive postmitotic neurons in the infragranular layers of embryonic neocortex in *Fmr1*-KO mice. These abnormalities in the neocortex formation are associated with **alterations in the structures of deeper neocortical layers and an increased density of ER81 positive projection neurons of layer V** in the early postnatal neocortex of *Fmr1*-KO mice. Altogether, these finding suggest a regulatory role for FMRP in the differentiation of neocortical glutamatergic neurons and further indicate that cell type-specific changes, caused by defects in NPC differentiation in the absence of FMRP, may be associated with aberrances in dendritic sprouting that ultimately result in abnormalities seen in the development and maintenance of synapses in *Fmr1*-KO mice.

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