Differentiation of neural stem cells in fragile X syndrome

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ACADEMIC DISSERTATION
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LIST OF ORIGINAL PUBLICATIONS

I Tervonen T, Åkerman K, Oostra BA, Castrén M (2005) Rgs4 mRNA expression is decreased in the brain of the Fmr1 knockout mouse. Mol Brain Res. 133: 162-165. a


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<td>82-FIP</td>
<td>82-kDa FMRP interacting protein</td>
</tr>
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<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>actNotch1</td>
<td>activated domain of Notch1</td>
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<tr>
<td>ADAM</td>
<td>a desintigrin and metalloproteases</td>
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<tr>
<td>AGO1</td>
<td>mammalian ortholog of Argonite 1</td>
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<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
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<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
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<td>BCL2</td>
<td>B-cell lymphoma 2</td>
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<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<td>BLBP</td>
<td>brain lipid binding protein</td>
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<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>Brca1</td>
<td>breast-ovarian cancer susceptibility gene 1</td>
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<td>Brgl1</td>
<td>Brahma-related gene 1</td>
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<td>C/EBP</td>
<td>CAAT/enhancer-binding protein</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
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<tr>
<td>CP</td>
<td>cortical plate</td>
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<td>CRE</td>
<td>E-box-cAMP response element</td>
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<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>maintenance DNA methyltransferase 1</td>
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<td>Fmr1-knockout</td>
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<td>FXR1</td>
<td>fragile X related 1</td>
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<td>FXS</td>
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<td>FMRP</td>
<td>fragile X mental retardation protein</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GLAST</td>
<td>astrocyte-specific glutamate transporter</td>
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<td>GS</td>
<td>glutamine synthase</td>
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<td>GSC</td>
<td>germ line stem cells</td>
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<td>GUSB</td>
<td>(\beta)-glucuronidase</td>
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<td>HAT</td>
<td>histone acetyltransferases</td>
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<td>histone deacetylases</td>
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<td>HGF</td>
<td>hepatocyte growth factor</td>
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<tr>
<td>I304N</td>
<td>substitution of isoleucine to asparagine at amino acid 304</td>
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<td>inhibitor of differentiation</td>
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<td>insulin-like growth factor II mRNA-binding protein 1</td>
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<td>inositol 1,4,5-trisphosphate</td>
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<td>IPC</td>
<td>intermediate precursor cell</td>
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<td>ISWI</td>
<td>imitation switch</td>
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<td>intermediate zone</td>
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<td>KH</td>
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<td>LSD</td>
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<td>long-term potentiation</td>
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<td>mGluR</td>
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<td>N-CoR</td>
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<td>neurotrophic growth factor</td>
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<td>Ngn</td>
<td>neurogenin</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>NPC</td>
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<td>nuclear respiratory factor 1</td>
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<td>NUFIP</td>
<td>nuclear FMRP interacting protein</td>
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<td>PKC</td>
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<td>PLC</td>
<td>phospholipase C</td>
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<td>PNS</td>
<td>peripheral nervous system</td>
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<td>PSA-NCAM</td>
<td>polysialylated neural cell adhesion molecule</td>
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<td>PUR</td>
<td>purine-rich single stranded DNA-binding</td>
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<td>REST/NRSF</td>
<td>RE1-silencing transcription factor</td>
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<td>RGG</td>
<td>Arginine-Glycine-Glycine</td>
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<td>RGS</td>
<td>regulator for G-protein signaling</td>
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<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<td>scl</td>
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<td>SCF</td>
<td>stem cell factor</td>
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<td>SCNT</td>
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<td>stromal cell-derived factor 1 alpha</td>
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<td>SGZ</td>
<td>subgranular zone</td>
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<td>SH2</td>
<td>Src-homology-2</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
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<td>SMAD</td>
<td>mothers against decapentaplegic homologue</td>
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<td>SOX2</td>
<td>SRY-related high-mobility group (HMG)-box protein-2</td>
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<td>SSC</td>
<td>standard saline citrate</td>
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<td>T-box transcription factor</td>
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<td>transforming growth factor</td>
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<td>tenascin-C</td>
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<td>Trk</td>
<td>tyrosine kinase receptor</td>
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<td>TrkB.T1</td>
<td>truncated T1 isoform of TrkB receptor</td>
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<td>TuJ1</td>
<td>βIII-tubulin</td>
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<td>upstream stimulatory factor 1</td>
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<td>UTR</td>
<td>untranslated region</td>
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<td>VEGF</td>
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<td>VZ</td>
<td>ventricular zone</td>
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<td>WNT</td>
<td>Wingless type</td>
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ABSTRACT

Multipotent stem cells can self-renew and give rise to multiple cell types. One type of mammalian multipotent stem cells are neural stem cells (NSC)s, which can generate neurons, astrocytes and oligodendrocytes. NSCs are likely involved in learning and memory, but their exact role in cognitive function in the developing and adult brain is unclear.

We have studied properties of NSCs in fragile X syndrome (FXS), which is the most common form of inherited mental retardation. FXS is caused by the lack of functional fragile X mental retardation protein (FMRP). FMRP is involved in the regulation of postsynaptic protein synthesis in a group I metabotropic glutamate receptor 5 (mGluR5)-dependent manner. In the absence of functional FMRP, the formation of functional synapses is impaired in the forebrain which results in alterations in synaptic plasticity. In our studies, we found that FMRP-deficient NSCs generated more neurons and less glia than control NSCs. The newborn neurons derived from FMRP-deficient NSCs showed an abnormally immature morphology. Furthermore, FMRP-deficient NSCs exhibited aberrant oscillatory Ca$^{2+}$ responses to glutamate, which were specifically abolished by an antagonist of the mGluR5 receptor. The data suggested alterations in glutamatergic differentiation of FMRP-deficient NSCs and were further supported by an accumulation of cells committed to glutamatergic lineage in the subventricular zone of the embryonic Fmr1-knockout (Fmr1-KO) neocortex. Postnatally, the aberrant cells likely contributed to abnormal formation of the neocortex. The findings suggested a deficit in the differentiation of distinct glutamatergic mGluR5 responsive cells in the absence of functional FMRP. Furthermore, we found that in the early postnatal Fmr1-KO mouse brain, the expression of mRNA for regulator of G-protein signalling-4 (RGS4) was decreased which was in line with disturbed G-protein signalling in NSCs lacking FMRP.

Brain derived neurotrophic factor (BDNF) promotes neuronal differentiation of NSCs as the absence of FMRP was shown to do. This led us to study the effect of impaired BDNF/TrkB receptor signaling on NSCs by overexpression of TrkB.T1 receptor isoform. We showed that changes in the relative expression levels of the full-length and truncated TrkB isoforms influenced the replication capacity of NSCs. After the differentiation, the overexpression of TrkB.T1 increased neuronal turnover.

To summarize, FMRP and TrkB signaling are involved in normal differentiation of NSCs in the developing brain. Since NSCs might have potential for therapeutic interventions in a variety of neurological disorders, our findings may be useful in the design of pharmacological interventions in neurological disorders of learning and memory.
REVIEW OF THE LITERATURE

1. Definition of stem cells

Characteristics of stem cells are their ability to self-renew and give rise to one or usually many different cell types (reviewed in Lovell-Badge, 2001). In adult animal, stem cells participate in the maintenance of tissue homeostasis and the repair of damaged tissue. The potential of stem cells can be defined based on the amount of cell types they can produce. Totipotent stem cells, which only include in mammals the fertilized egg, are the most potent and they can give rise to every cell type in the animal. Pluripotent stem cells differ from totipotent stem cells only by their inability to give rise to embryonic trophectoderm cells. Multipotent stem cells are tissue specific stem cells, which can give rise to more than one cell type. Finally, unipotent stem cells, which include spermatogonial stem cells in the testis, can give rise to only one cell type.

1.1. Adult stem cells in their niches

The term stem cell “niche” was already introduced long time ago (Schofield, 1978) but only after studies in invertebrate Drosophila, microenvironment supporting adult stem cells was accepted as stem cell niche (Xie and Spradling, 2000; Moore et al., 2004; Guasch and Fuchs, 2005; Li and Xie, 2005). The niche is comprised of an environment of surrounding cells that support adult stem cells and mediate signals of self-renewal, survival and differentiation. To maintain the adult stem cell pool, the niche must prevent excessive apoptosis or proliferation which could lead to the depletion of stem cells or the development of cancer, respectively. The main function of multipotent adult stem cells is to preserve tissue homeostasis in case of naturally occurring apoptosis of terminally differentiated cells, injury and disease.

Well-characterized niches for multipotent adult stem cells include hematopoietic stem cells in the bone marrow (Spangrude et al., 1988), intestinal stem cells in the villus of the small intestine (Booth and Potten, 2000), hair follicle stem cells in the skin (Cotsarelis et al., 1990), germ line stem cells in the testis (Brinster, 2002), and neural stem cells (NSC)s in the subventricular zone (SVZ) and hippocampus (Gage, 2000). In addition to these established niches, other niches for multipotent adult stem cells have also been proposed (Seale et al., 2001; Beltrami et al., 2003; Laugwitz et al., 2005; Buchstaller et al., 2004; Kim et al., 2005).

2. NSCs and their niches

Cells that are undifferentiated, self-renewing multipotent progenitors for neurons and glia in the developing and adult brain are termed as NSCs (Gage, 2000; Temple, 2001; Merkle and Alvarez-Buylla, 2006). NSCs were first isolated from the embryonic mammalian
central nervous system (CNS) (Temple, 1989; Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993) and the peripheral nervous system (PNS) (Stemple and Anderson, 1992). Adult NSCs were found subsequently (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993) and are located in two established niches, which are the SVZ and subgranular zone (SGZ) (Lois and Alvarez-Buylla, 1993; Palmer et al., 1997; Doetsch et al., 1999). In addition, adult NSCs have been isolated and cultured from brain regions including caudal portions of the SVZ, cortex, striatum, septum, corpus callosum, hypothalamus, spinal cord, optic nerve, retina, and olfactory bulbs (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). However, in the adult human neocortex, neorogenesis does not likely occur (Spalding et al., 2005; Bhardwaj et al., 2006). NSCs in the early embryonic brain are neuroepithelial cells (Haubensak et al., 2004), which are suggested to turn into radial glia at the onset of neurogenesis (Malatesta et al., 2003; Anthony et al., 2004; Götz and Huttner, 2005). Adult NSCs in the prominent brain niches are radial glial derived astrocyte-like cells (Doetsch et al., 1999; Imura et al., 2003; Garcia et al., 2004) thus suggesting the same lineage for embryonic radial glia and NSCs in the adult brain (Tramontin et al., 2003; Merkle et al., 2004).

How to discriminate between NSCs, progenitor cells and precursor cells? Progenitor cells and precursor cells (both termed as NPCs from here on) are multipotent daughter cells of NSCs with limited capacity to self-renew. These cells are dividing symmetrically at the SVZ to enlarge their population (Gage et al., 1995; Weiss et al., 1996; McKay, 1997; Haubensak et al., 2004; Noctor et al., 2004; Martinez-Cerdeno et al., 2006). This review will mainly focus on the properties of NSCs, since they are largely overlapping with properties of NPCs and there is only very thin and partially undefined line between these multipotent cell types. This issue will be discussed more closely when reviewing the development of neocortex.

NSCs, as other stem cells with a potential to create different types of cells, need to be controlled during brain development to make correct structures at a certain time. This requires patterning of signals from the environment and intrinsic expression of genes. In embryonic mammalian forebrain development, this means that neurogenesis and gliogenesis follow neural tube closure. Initially during brain development, NSCs are required to generate proper cell numbers in the brain and subsequently to produce the right types of differentiated cells in the correct place. NSC self-renewal and proliferation must be constantly controlled to prevent the depletion of stem cell pool or cancer. This is especially important in the adult brain. The relevance of NSCs in the adult brain is perhaps not as obvious as, for example, the relevance of hematopoietic stem cells in the bone marrow. Nevertheless, in the adult brain NSCs are associated with the creation of new olfactory neurons, hippocampal granule neurons and oligodendrocytes (Gage, 2000; Kempermann et al., 2004; Merkle and Alvarez-Buylla, 2006). The renewal of granule neurons may be important for learning, memory and emotion (Emsley et al., 2005; Paizanis et al., 2007). To some extent, NSCs can also be recruited to repair damaged brain tissue (Singec et al., 2007). As is true for other types of stem cells, NSCs can be manipulated in vitro to exhibit features that are absent in vivo.
2.1. Self-renewal/proliferation of NSCs

Self-renewal and maintenance of NSCs in the SVZ and SGZ niches are mediated by attachment and various signals emanating from endothelial cells of blood vessels and the specialized basal lamina (Doetsch, 2003; Shen et al., 2004). β-catenin and cadherins form adherens junctions, which play a role in the maintenance of NSCs, and Wingless type (WNT) signaling overexpression of β-catenin leads to exaggerated proliferation of NSCs (Chenn and Walsh, 2002, 2003). By contrast, deletion of β-catenin leads to a loss of cortical NPCs (Zechner et al., 2003; Backman et al., 2005). Cortical WNT signaling mediates NPC proliferation through Emx2 (Muzio et al., 2005). At later stages of cortical development the role of WNT signaling is shifted to promote differentiation and inhibitors including Axin and Dkk1 can act to retain the undifferentiated state of NPCs at this point (Hirabayashi et al., 2004). Primarily genes that interfere with differentiation programs regulate NSC maintenance in the undifferentiated state. The polycomb family transcriptional repressor Bmi-1 is important for NSC self-renewal but not for their survival or differentiation (Molofsky et al, 2003). Hes proteins, which are downstream of Notch, promote the undifferentiated state as repressors of proneural transcription and inhibitors of neurogenesis (Kageyama and Ohtsuka, 1999). An RE1-silencing transcription factor (REST/NRSF) is a transcriptional repressor of neuronal gene expression in NSCs and neurons. Activation of neural differentiation from NSCs requires repression and degradation of RE1-silencing transcription factor (REST/NRSF) (Ballas et al., 2005). A binding site for REST/NRSF in promoter region of proneural Mash1, further suggests a role for REST/NRSF in retaining multipotency (Ballas et al., 2005).

Another approach to maintain NSC in an undifferentiated state is to prevent proneural proteins from acting normally. The inhibitor of differentiation (Id) proteins sequester E proteins, which are their dimerizing partners and this leads to inhibition of neurogenesis (Yokota, 2001). The cell cycle regulator and cell fate determinant Geminin inhibits the interaction of proneural basic helix-loop-helix (bHLH) proteins with Brahma-related gene 1 (Brg1) thus blocking their action and leading to inhibition of neurogenesis (Seo et al., 2005; Spella et al., 2007). The SRY-related high-mobility group (HMG)-box protein-1 (SOX1), SOX2, and SOX3 of B1 group proteins of the SOX family participate in maintaining the NSC pool (Bylund et al., 2003; Graham et al., 2003). These SOX proteins block proneural bHLH protein activity without affecting proneural gene expression and inhibit neurogenesis (Bylund et al., 2003). Furthermore, it seems that proneural genes inhibit SOXs expression (Bylund et al., 2003). In addition, proneural gene regulated expression of SOX21 is blocking the action of SOX1-3 possibly by interfering with their targets (Sandberg et al., 2005).

Extracellular signals mediated by basic fibroblast growth factor (FGF2) and Notch1 are also important for maintaining the NSC pool and promoting self-renewal within the SVZ niche (Hitoshi et al., 2004; Zheng et al., 2004). In fact, Notch pathways are well established in promoting an undifferentiated state in NSCs during developmental neurogenesis (Artavanis-Tsakonas et al., 1999; Mizutani and Saito, 2005). For example, endothelial cells secrete factors that stimulate NSC proliferation promoting Notch activation in neurogenic niches involving consolidation of cell-cell contacts required for Notch signaling (Shen et al., 2004). In vitro self-renewal and proliferation can be mediated by high concentration of mitogens, epidermal growth factor (EGF) and FGF, in the culture medium (Reynolds and Weiss, 1992; Palmer et al., 1995; Craig et al., 1996; Tropepe et al., 1999). In addition, ciliary neurotrophic factor (CNTF) augments self-renewal of neural precursors by promoting the expression of Notch1 in vitro (Chojnacki et al., 2003). Early
embryonic NSCs require FGF to proliferate, and later NSCs require FGF or EGF for proliferation (Kalyani et al., 1997; Gritti et al., 1999; Tropepe et al., 1999; Vaccarino et al., 1999; Raballo et al., 2000). More committed NPCs are responsive to sonic hedgehog (Shh), FGF, and neurotrophin-3 (NT-3). Shh, the soluble form of amyloid precursor protein (APP) and EGF signalling, act in concert to promote proliferation of NSCs/NPCs (Machold et al., 2003; Caille et al., 2004; Palma and Ruiz i Altaba, 2004). The transforming growth factor (TGF)α, another ligand for EGF receptor, may also have a role in regulating proliferation of NSCs/NPCs (Enwere et al., 2004).

Extracellular signaling molecules such as FGF2, TGFα, and CNTF are secreted by astrocytes and the relative amount of astrocytes in neurogenic SVZ and SGZ is high suggesting a possible explanation for the maintenance of multipotent NSCs in those regions (Emsley and Hagg, 2003). There are other regions in the CNS in which astrocytes are abundant but no neurogenic proliferation is occurring, which suggest that the amount of astrocytes is not the rate limiting factor. Actually, the important thing would be to distinguish neurogenic proliferating adult NSCs, which have been shown to be of glia lineage from differentiated glia (Götz and Huttner, 2005). The actual mechanisms of maintaining proliferative regions in the adult brain may involve desintegrin and metalloproteases (ADAM)s which can activate TGFα, Notch1, and APP by cleaving their extracellular domains (Huovila et al., 2005; Yang et al., 2005). Since astrocytes and ADAMs co-localize in non-neurogenic regions, there must be another way of defining the neurogenic niche.

Neurotransmitters as well as their receptors and transporters are already expressed in the embryonic cortex (Dammerman and Kriegstein, 2000; Haydar et al., 2000; Olivier et al., 2000). GABA and glutamate directly regulate NSC/NPC proliferation by changes in DNA synthesis (LoTurco et al., 1995; Haydar et al., 2000), and alterations in intracellular calcium concentrations (LoTurco et al., 1995; Owens et al., 2000; Weissman et al., 2004). These effects may be area specific since the NPC response to glutamate and GABA is opposite in the VZ compared to the SVZ (Haydar et al., 2000). Interaction between neurotransmitters and growth factors may underlie this difference (Antonopoulos et al., 1997). D2/3-dopamine receptor activation plays a role in regulation of proliferation of NSCs in the SVZ presumably in a diverse cell type specific manner (Baker et al., 2004; Höglinger et al., 2004; Baker et al., 2005; Kippin et al., 2005). Dopamine can have a direct effect on proliferation, since its receptor, D2, is expressed in both NSCs and NPCs (Höglinger et al., 2004; Kippin et al., 2005). Another neurotransmitter, 5-hydroxytryptamine (serotonin or 5-HT), can also promote NPC proliferation (Banasr et al., 2004). Interestingly, dopaminergic and serotonin releasing projections converge in the SVZ suggesting some kind of coordinated action of these neurotransmitters (Simpson et al., 1998). Supporting this idea, a similar overlap between serotonergic and noradrenergic projections occurs in the SGZ (Goldman-Rakic et al., 1990).

Hormones regulate NSC/NPC proliferation, since thyroid hormone increases NSC proliferation through its α-receptor and by regulating c-MYC expression (Lenkine et al., 2005). Prolactin together with TGFα seems to promote proliferation in the SVZ (Shingo et al., 2003). Estrogen receptor activity promotes the proliferation of NSCs in the embryonic brain (Brännvall et al., 2002). Furthermore, evidence indicates that IGF-1 increases proliferation presumably in an estrogen dependent manner (Perez-Martin et al., 2003). On the other hand, testosterone analogue, 19-nortestosterone, was shown to reduce the proliferation of NSCs in the adult rat brain (Brännvall et al., 2005), and glucocorticoid hormones seem to reduce the proliferation of NSCs by inhibiting the cell cycle (Sundberg
et al., 2006). Again, SVZ and SGZ NSCs/NPCs respond differentially to proliferative signals, which this time are mediated by hormones (Shingo et al., 2003; Abrous et al., 2005).

Cell cycle regulation is of course essential for maintaining a proliferative state. Activation of RAS/RAF/MEK/ERK protein kinase pathway shortens cell cycle length, and thus alters proliferation rate (Edgar, 1995). This pathway operates through p53, retinoblastoma tumor suppressor (Rb), and E2F families of proteins (Yoshikawa, 2000). Of these, the E2F family of proteins may be essential for promoting self-renewal in NSCs. Rb and p53 have another role in promoting proliferation by mediating telomerase signaling in a cell type specific manner (van Steensel and de Lange, 1997).

FGF2 has a major role in cerebral cortex development by promoting proliferation of NPCs (Ghosh and Greenberg, 1995; Raballo et al., 2000). EGF and its receptor are also expressed in the neocortex during development and act to increase NPC proliferation (Burrows et al., 1997; Morrow et al., 2001). Interestingly, the EGF receptor is asymmetrically inherited by its progeny when the cortical NPC divides, which probably contributes to a cell fate determination by EGF receptor distribution in NPC populations (Sun et al., 2005). Heparan sulfate proteoglycans, components of the extracellular matrix, can participate in growth factor actions (Dutton and Bartlett, 2000). Furthermore, FGF and EGF are differently regulated by these components of extracellular matrix (Ford et al., 1994; Ferri et al., 1996) and, on the other hand, growth factors modulate the production of extracellular matrix components by NPCs (Drago et al., 1991). The extracellular matrix seems to be important in regulating NSC/NPC proliferation by directly affecting cell numbers and indirectly regulating the actions of growth factors. Integrons expressed by NPCs are activated by binding to the extracellular matrix proteins or cell surface integrins in other cells, which results in the activation of intracellular pathways. In this manner, α5β1 integrins promote proliferation by activating intracellular phosphatidylinositol-3-kinase (PI3K) and AKT (Jacques et al., 1998).

2.2. Survival of NSCs

During neurogenesis and gliogenesis in the developing mammalian brain, NSCs proliferate and differentiate into neurons and glia to give rise to a stratified cell diversity of the mature brain. This proliferation is exaggerated since only 15-40% of post-migratory cells survive implicating some kind of selection system to decide which cells are required (Finlay and Slattery, 1983; Oppenheim, 1991; Ferrer et al., 1992). A substantial amount of programmed cell death or apoptosis occurs in mitotically active NSCs and progenitor cells in various regions during brain development (Kuan et al., 2000). The principle of NSC apoptosis is to regulate the number of proliferating cells, which will eventually affect brain size. This has been demonstrated by abnormal brain size and morphology caused by disrupting the function of genes in the apoptotic pathway (Frade and Barde, 1999; Haydar et al., 1999; Kuan et al., 2000; Depaepe et al., 2005; Putz et al., 2005).

Apoptosis is regulated by caspases (Raff, 1998) of which the telencephalic transcripts are especially linked with NSCs survival (Kuan et al., 2000; Ceccatelli et al., 2004). Staurosporine activation or caspase inhibitor-mediated blocking of caspase-3 leads to apoptosis or excess survival of NSCs, respectively, thus suggesting a caspase dependent death pathway in NSCs (D’Sa-Eipper and Roth, 2000). Inactivation of brain caspases results in hyperplasias in a similar manner as inactivation of another proapoptotic gene,
Apaf-1 (Cecconi et al., 1998; Yoshida et al., 1998). Caspase-3 and caspase-9 as promoters of apoptosis seem to be essential for early forebrain development in a region or cell population specific manner (Kuida et al., 1996; Kuida et al., 1998; Levison et al., 2000). Apoptosis in NSCs via caspase activation can be blocked by activation of anti-apoptotic Bcl-2 (Cheng et al., 2001; Esdar et al., 2001). Another pathway that activates caspase-3 via Fas does not seem to cause apoptosis in NSCs (Tamm et al., 2004). Antiapoptotic gene Bcl-X which regulates neuron survival is not involved with NSC apoptosis revealed by normal VZ after genetic manipulation of this gene (Motoyama et al., 1995; Roth et al., 2000). However, proapoptotic gene Bax which is important for developmentally occurring neuronal death seems to be also important for naturally occurring apoptosis of adult NSCs through caspase and inositol 1,4,5-trisphosphate (IP3) activation (White et al., 1998; Shi et al., 2005).

Extrinsic factors are important in preventing apoptosis since neuroepithelial NSCs survival is mediated by FGF, EGF, insulin/IGF, and antidepressant mediated activation of antiapoptotic Bcl-2 in vitro (Kalyani et al., 1997; Diaz et al., 1999; de la Rosa and de Pablo, 2000; Chen et al., 2007; Huang et al., 2007). Moreover, the importance of insulin for neuroretinal NSCs survival has been demonstrated in vivo with a blocking antibody, which increases apoptosis in intrinsic areas (Diaz et al., 2000). Some factors like c-Jun N-terminal kinases (Jnk)s seem to affect NSC survival in a regional and temporal manner, since Jnks decrease apoptosis in neuroepithelial NSCs and promote it in the forebrain VZ (Kuan et al., 1999). Neurotrophic factors play a role in cortical NSC/NPC survival as shown by increases in apoptosis when endogenous TrkB and TrkC mediated signaling are blocked by antibodies (Barnabe-Heider and Miller, 2003). This kind of action involves downstream signaling target of Trks, PI3K and its adapter protein ShcA, suggesting an autocrine/paracrine action of neurotrophins in NSC/NPC survival (Barnabe-Heider and Miller, 2003; McFarland et al., 2006). Platelet-derived growth factor (PDGF) also acts through the PI3K pathway to promote survival of NSCs (Guillemot, 2007). The well-characterized tumor suppressor p53 is expressed in NSCs/NPCs and it decreases the survival of these cells (Akhtar et al., 2006; Meletis et al., 2006). Ephrin signaling appears to be one factor controlling brain size by negatively regulating the survival of NPCs (Depaepe et al., 2005). Amyloid-beta peptide induces Fas-independent apoptosis in NPCs (Millet et al., 2005) and prostate apoptosis response 4 (PAR-4) prevents apoptosis in NSCs (Wang et al., 2006). The neurotransmitter glutamate promotes the survival of SVZ-derived NPCs (Brazel et al., 2005), and on the contrary, hydrogen peroxide and rotenone induce apoptosis in NSCs/NPCs (Lin et al., 2004; Li et al., 2005).

2.3. Epigenetic control of NSC fate

Epigenetic modifications occur without affecting DNA sequence and expose certain genes to be available for expression. Histone acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation, DNA methylation, noncoding RNAs, and chromatin remodeling are known mechanisms of epigenetic control of gene expression (Strahl and Allis, 2000; Hsieh and Gage, 2004; Lund and van Lohuizen, 2004; Kondo, 2006). Cellular diversity in the CNS has especially provoked interest in epigenetic mechanisms as controllers of cell fate (Branchi et al., 2004).

Chromatin remodeling through covalent changes in histones has emerged as one of the epigenetic mechanisms regulating gene expression (Strahl and Allis, 2000). Chromatin
structure can be modified through histone acetylation, and particularly by lysine acetylation, which is a well characterized type of histone modification (Strahl and Allis, 2000). Histone acetyltransferases (HAT)s mediate the acetylation, and histone deacetylases (HDAC)s counteract this by removing acetylation. Non-acetylated DNA is packed into tight nucleosomes, which blocks the attachment of transcription activators to the promoter sites of certain genes leading to gene silencing. Action by HATs facilitates nucleosomal relaxation and access to gene promoter elements. In NSCs, class II HDAC, which are one human transcript of HDACs expressed also in brain, seem to be increased upon differentiation (Ajamian et al., 2003) and particularly affect oligodendrocyte differentiation (Marin-Husstege et al., 2002; Shen et al., 2005). To control cell fate HDACs are pivotal for repressing neuronal genes in non-neuronal cell types (Chong et al., 1995; Schoenherr and Anderson, 1995). These genes share a common NRSE element, which is a binding site for REST/NRSF. This factor interacts with mSin3A/B complex (Naruse et al., 1999), the nuclear receptor co-repressor (N-CoR) (Jepsen et al., 2000), CoREST/HDAC2 (Ballas et al., 2001), and the H3K9 histone methyltransferases G9a and SUV39 (Lunyak et al., 2004; Ballas et al., 2005). CoREST recruits methyl DNA binding protein MeCP2, heterochromatin protein 1 and the histone lysine methyltransferase, suppressor of variegation 39H1 to silence REST/NRSF target genes (Lunyak et al., 2002). Valproic acid inhibition of HDACs promotes neuronal differentiation and suppresses glial differentiation from adult NPCs (Hsieh et al., 2004). Inhibitors of HDACs are generally promoting specifically neuronal differentiation from NPCs (Hao et al., 2004; Hsieh et al., 2004; Acharya et al., 2005).

Histone methylation of lysine residues in histones H3 and H4 are thought to be involved with molecular imprinting of gene expression in eukaryotes (Sims et al., 2003). In embryonic neuroepithelial NSCs, acetylation and methylation of histone H3 in the promoter of SOX2 regulates the NSC proliferation and maintenance (Kondo and Raff, 2004). Histone methylation has emerged as a transcriptional regulator in the CNS, since dimethylated and trimethylated histone H3 show brain region and developmental stage dependent changes in human glutamate receptor promoters (Stadler et al., 2005).

DNA cytosine methylation is another epigenetic modification implicated in genomic imprinting and X-chromosome inactivation (Jaenisch and Bird, 2003). The mechanism of DNA methylation includes repressing transcription factor binding by methylation of CpG sites or reversing methylation by methyl-CpGs binding proteins (MBD)s and HDAC repressor complexes. The absence of an enzyme that establishes and maintains DNA-methylation, maintenance DNA methyltransferase 1 (Dnmt1), results in impaired neuronal function and premature death in mice (Fan et al., 2001). On the other hand, mice deficient in methyl-CpGs binding protein 1 (MBD1) exhibit impaired neurogenesis in the dentate gyrus, decreased neuronal differentiation of NSCs, increased aneuploidy in NSCs, and resemblance with the genomic instability found in cancer cells (Zhao et al., 2003). Inappropriate and early expression of glial specific genes can be blocked in NSCs and non-glial cells by, for example, by CpG methylation of the binding site of signal transducer and activator of transcription-3 (STAT3) (Takizawa et al., 2001; Namihira et al., 2004).

Noncoding RNAs, such as repressor microRNAs, are beginning to reveal their importance as epigenetic regulators of gene expression in the CNS (Grewal and Moazed, 2003; Bartel, 2004; Smirnova et al., 2005). Remarkably, a novel noncoding dsRNA was shown to be able to interact with REST/NRSF and convert it to activator of neuronal gene expression in a manner opposite to its proposed function (Kuwabara et al., 2004). Chromatin remodeling protein complex family, switching (SWI/SNF), which uses ATP
hydrolysis to disrupt the connection between histones and DNA, interact with HATs or HDACs and/or specific transcription factors to upregulate or downregulate target genes (Knoepfler and Eisenman, 1999; Cho et al., 2004). Loss of function in members of this family of proteins Brahma, Breast-ovarian cancer susceptibility gene 1 (Brca1), Brg1, and Bmi-1 leads to promotion of NSC self-renewal and maintenance (Molofsky et al., 2003; Kondo and Raff, 2004; Seo et al., 2005). Another chromatin remodeling system, imitation switch (ISWI), which is expressed in the embryonic and postnatal brain, is sure to play a role in NSCs/NPCs, since it induces bone morphogenetic protein 4 (BMP4) expression and inhibits Shh expression (Dirscherl et al., 2005).

2.4. Regulation of NSC differentiation

NSCs normally differentiate in multiple steps to produce the final cellular diversity found in the mammalian CNS. Beginning from NSCs, through NPCs to maturing terminally differentiated cell types including a vast variety of neurons, as well as glial cells including astrocytes and oligodendrocytes. During brain development, the generation of neurons and glia is temporally organized. Neocortical layer formation especially occurs in a highly orchestrated manner. The secrets behind the generation of neuron subtypes are only now beginning to be elucidated (Muotri and Gage, 2006). Finally, the onset of shaping of neuronal networks is occurring during postnatal development and lasting to some extent throughout mammalian life.

2.4.1. Neuronal differentiation

WNT signaling pathways play an important role in the NSC differentiation process after the switch to support neuronal differentiation of NSCs/NPCs instead of proliferation. Consequently, WNT7A or stabilized β-catenin promote cell cycle arrest and neuronal differentiation of cortical NPCs both in vivo and in vitro (Hirabayashi et al., 2004; Hirabayashi and Gotoh, 2005). WNT signaling is suggested to promote neurogenesis by directly activating the expression of proneural genes, Neurogenin1 and Neurogenin2 (Ngn1 and Ngn2) in clonal NPC culture (Machon et al., 2003; Hirabayashi et al., 2004; Israsena et al., 2004). Furthermore, WNT signaling is also required for adult hippocampal neurogenesis (Zhou et al., 2004; Lie et al., 2005). WNT signaling in NPCs may be regulated by the presence of FGF2 signaling to switch to support neuronal differentiation instead of proliferation (Hirabayashi et al., 2004; Israsena et al., 2004).

PDGF is suggested to promote neuronal fate in NPC cultures by binding to a tyrosine kinase receptor which, in turn, activates the intracellular SHP2-mitogen-activated-protein-kinase kinase (MEK)-ERK pathway that mediates neurogenic signals of a variety of growth factors (Johe et al., 1996; Williams et al., 1997; Menard et al., 2002; Barnabe-Heider and Miller, 2003; Gauthier et al., 2007). SHP2-MEK-ERK further phosphorylates transcription factors of the CAAT/enhancer-binding protein (C/EBP) family to activate neuronal genes including Tα1 α-tubulin (Menard et al., 2002) and Math2 (Uittenbogaard et al., 2007). Perturbations in C/EBP activity direct NPCs towards glial fate, which suggests that growth factor mediated SHP2-MEK-ERK-C/EBP signaling pathway is contributing to neurogenesis by promoting neuronal fate determination (Paquin
et al., 2005). In addition, SHP2 directly represses the nonreceptor tyrosine kinase Janus (JAK)-STAT signaling to promote neuronal fate over glial fate (Gauthier et al., 2007).

Adult neurogenesis is in part promoted by neuronal activity received by hippocampal NPCs, which increases intracellular calcium dependent expression of differentiation factors such as NeuroD and repressing inhibitors of neurogenesis including Hes1 and Id2 (Deisseroth et al., 2004; Overstreet Wadiche et al., 2005; Tozuka et al., 2005). GABAergic signaling promotes neuronal network integration related properties of newly born neurons in the CNS, which include neurite outgrowth and synaptogenesis (Represa and Ben-Ari, 2005). Noggin inhibits BMP4 signaling to favor neuronal differentiation of NPCs in the adult SVZ and SGZ (Chmielnicki et al., 2004).

The transcription factor Pax6, which has been implicated in neurogenesis in the neocortex and the adult SVZ, is a direct activator of Ngn2 in neocortical NPCs suggesting a mechanism for regulating neurogenesis through proneural genes (Heins et al., 2002; Scardigli et al., 2003; Hack et al., 2005). Perturbations in Pax6 action result in loss of cortical neurons (Heins et al., 2002; Hack et al., 2005). However, Pax6 also acts through distinct pathways independent of proneural proteins, because Pax6 promotes neurogenesis in postnatal astrocytes, and loss of Pax6 does not result in gliogenesis (Heins et al., 2002). Neuronal differentiation of NSCs/NPCs seems to be regulated by dual action of Pax6 with or without proneural proteins.

Proneural genes encode bHLH transcription factors, which have fundamental role in neurogenesis (Bertrand et al., 2002; Ross et al., 2003). These genes expressed in the mammalian telencephalon include Mash1, Ngn1, and Ngn2. Mash1 is expressed in basal ganglia NPCs as well as in neocortical NPCs, whereas Ngn1 and Ngn2 are expressed only in neocortical NPCs (Britz et al., 2006). The most important function for proneural genes is to direct NSCs/NPCs towards neuronal fate instead of astroglial fate (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001). Other functions of these genes include converting NSCs into mature neurons (Bertrand et al., 2002; Helms and Johnson, 2003; Schuurmans et al., 2004; Hand et al., 2005). When proneural gene expression is absent in vivo, loss of neurons and NPCs occurs and astroglial fate determination is promoted (Tomita et al., 2000; Nieto et al., 2001). In vitro, proneural genes have been shown to promote neuronal lineage of NPCs by direct transcriptional activation of downstream genes such as NeuroD (Nieto et al., 2001; Sun et al., 2001; Parras et al., 2004). In contrast, glial fate inhibition by proneural genes occurs through inhibiting the signaling pathways JAK-STAT and BMP-mothers against decapentaplegic homologue (SMAD) (Nieto et al., 2001; Sun et al., 2001; Parras et al., 2004; He et al., 2005). Proneural bHLH genes require other transcription factors to act in concert to regulate NSCs/NPCs differentiation. The evidence suggests that Ngn proteins interact with histone acetylase CBP/P300 to activate target genes (Sun et al., 2001; Ge et al., 2006) and with a component of chromatin remodeling complex, Brg1, to promote neurogenesis in mammalian embryonic carcinoma cells (Seo et al., 2005). However, absence of Brg1 expression in embryonic neocortex results in neuronal differentiation and the inhibition of astroglial fate (Matsumoto et al., 2006). Surprisingly, one of the proastrocytic bHLH genes, stem cell leukemia (scl), appears to promote neuronal differentiation and maturation in addition to promoting astrogenesis (Bradley et al., 2006).

Ngn1 and Ngn2 are able to initiate neural differentiation (Farah et al., 2000; Mizuguchi et al., 2001; Nakada et al., 2004) and their sequential downstream targets include bHLH transcription factors NeuroD1, NeuroD2, Math2, Math3, Nse11 and T-box proteins Tbr1 and Tbr2 (Schuurmans et al., 2004; Englund et al., 2005; Hevner et al., 2006). Tbr1 is essential to neural differentiation of some cortical NPCs and Tbr2 is useful
as a marker for intermediate cortical NPCs committed to glutamatergic fate (Englund et al., 2005; Hevner et al., 2006). The actual function of Tbr2 is yet unknown. Ngn1 and Ngn2-mediated expression of Tbr1 and Tbr2 is restricted to cortical NPCs and neurons, and it is absent in subcortical regions or basal ganglia (Schuurmans et al., 2004). NeuroD and its related partner Math2/Nex may be involved in the differentiation of neurons located in the dentate gyrus (Miyata et al., 1999; Liu et al., 2000; Schwab et al., 2000). These two genes also have a role in promoting adult hippocampal neurogenesis (Deisseroth et al., 2004; Tozuka et al., 2005). Ngn1 and Ngn2 have been specifically implicated in inducing neurogenesis and neural differentiation in the NSCs/NPCs of the developing dorsal telencephalon in contrast to Mash1, which is implicated in the basal ganglia development (Fode et al., 2000; Schuurmans et al., 2004). This was elucidated in knockout studies, which showed that Ngn1 and Ngn2 were controlling differentiation of cortical NPCs into a glutamatergic phenotype through activation of their target transcription factors, while on the other hand; Mash1 seems to promote neural differentiation into a GABAergic phenotype through activation of Dlx homedomain genes (Schuurmans et al., 2004). Furthermore, Ngn1 and Ngn2 appear to repress Mash1 activation and action in cortical NPCs and Ngn2 appears to be able to initiate a neocortical glutamatergic program independent of Mash1 repression.

Other factors that specify certain neuronal subtypes are Gsh2, which induces differentiation of striatal projection neurons through production of retinoic acid (Waclaw et al., 2004); GDNF, which is involved in neocortical interneuron maturation (Pozas and Ibanez, 2005); and Dlx1, which participates in the morphological development of a certain population of neocortical interneurons (Cobos et al., 2005).

2.4.2. Astroglial differentiation

Notch signaling has been associated with converting neuroepithelial NPCs into radial glia and radial glia into mature astrocytes. Neuroepithelial NPCs and radial glia share the common apical to basal polarity and interkinetic nuclear motility (Götz and Huttner, 2005). The change from neuroepithelial NPCs to radial glia requires increased expression of several astroglial-specific genes including astrocyte-specific glutamate transporter (GLAST), S100β, glutamine synthase (GS), vimentin and tenascin-C (TN-C) and this is where Notch signaling plays a key regulative role (Gaiano et al., 2000; Anthony et al., 2005; Götz and Huttner, 2005). Direct targets for Notch in radial glia are brain lipid binding protein (BLBP) through activation of CSL/CBF-1 and the Neuregulin receptor ErbB2 through activation of Deltex (Schmid et al., 2003; Anthony et al., 2005; Patten et al., 2006). Astrocytes are differentiated from radial glia when Neuregulin-ErbB2 signaling is suppressed (Schmid et al., 2003). Overexpression of Notch receptor promotes astrogensis in the adult brain and the differentiation of astrocytes from NPCs in culture (Gaiano et al., 2000; Tanigaki et al., 2001). The Notch pathway has been shown to be active in radial glia and immature astrocytes and to promote astrogenesis directly through activation of GFAP expression (Ge et al., 2002; Tokunaga et al., 2004; Kohyama et al., 2005). Notch signaling plays a role in maintaining NPCs in undifferentiated state during neurogenesis through the members of the Hes family of transcription repressors, Hes1 and Hes5 (Nakamura et al., 2000; Ohtsuka et al., 2001; Hatakeyama et al., 2004). Moreover, Hes proteins repress target proneural genes Ngn1, Ngn2 and Mash1 to promote astrogenesis. In addition, Hes proteins activate JAK-STAT signaling to induce astrocyte
differentiation (Kamakura et al., 2004). In summary, Notch promotes astrogenesis both directly and indirectly by inducing GFAP expression and through Hes protein activation, respectively. The absence of Numb and Numb-like, which are intracellular inhibitors of Notch signaling, lead to perturbations in neocortex development in mice (Li et al., 2003; Petersen et al., 2004). The effect of Numb cultured cortical NSCs/NPCs appears to be stage dependent and results in premature neuronal differentiation or increased proliferation. Furthermore, around midneurogenesis Numb unequally segregates with the daughter cell to promote neuronal fate (Shen et al., 2002).

JAK-STAT signaling is the major promoter for astroglial fate and differentiation in the neocortex. CNTF, LIF and cardiotrophin-1 are cytokines expressed by newborn neurons, which mediate activation of JAK-STAT signaling upon binding with the glycoprotein-130-LIFR receptor complex on NSCs/NPCs (Johe et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998; Barnabe-Heider et al., 2005). Activated STATs bind to the promoter of GFAP causing transcriptional activation of the target (Bonni et al., 1997; Nakashima et al., 1999a). In addition, cytokines such as CNTF can phosphorylate and inactivate co-repressor N-CoR on the GFAP promoter to induce astrocyte differentiation (Hermanson et al., 2002). In early NPCs, BMP cytokines promote neurogenesis but later they will switch to promote astrocyte differentiation and inhibit other cell fates (Gross et al., 1996; Li et al., 1998a; Nakashima et al., 2001). BMPs recruit downstream transcription factor SMADs to bind the promoter region of GFAP and induce astrogenesis (Nakashima et al., 1999b). However, astrogenesis promoting BMP signaling involves interaction with the JAK-STAT pathway and complex interaction with Notch and its downstream partner Hes5 (Takizawa et al., 2003). BMP2 can inhibit neurogenesis and oligodendrogenesis through activation of repressing Id proteins and Hes5 (Nakashima et al., 2001; Samanta and Kessler, 2004; Vinals et al., 2004). BMP signaling is present in the adult SVZ, where it acts coordinately with the inhibitor Noggin to produce astrocytes or neurons (Lim et al., 2000).

Growth factors, such as FGF2 interacting with other extrinsic signals, induce astrocyte differentiation (Qian et al., 1997). Furthermore, NPCs expressing high quantities of EGF receptor can have increased expression and activation of STAT3 by cytokine action and promote astrocyte fate (Burrows et al., 1997; Viti et al., 2003; Lillien and Gulacsi, 2006).

The most important proastrocytic transcription factors seem to be nuclear factor-1 (NFI) family of proteins, which are expressed ubiquitously but with partially differential patterns (Gronostajski, 2000). In the absence of certain NFIs, corpus callosum development is perturbed along with a reduction of GFAP mRNA. Most importantly, the timing and extent of astrogenesis are affected (Cebolla and Vallejo, 2006; Gopalan et al., 2006). Proastrocytic bHLH proteins, scl and Ngn3, are essential for the differentiation of astrocytes and oligodendrocytes in specific regions of the embryonic spinal cord (Lee et al., 2003; Muroyama et al., 2005).

2.4.3. Oligodendroglial differentiation

Oligodendrocyte precursors (OLPs) are generated from embryonic ventro-medial telencephalon, late embryonic ventro-lateral telencephalon and postnatal dorsal telencephalon (Kessaris et al., 2006). There are a few extrinsic signaling pathways promoting oligodendroglial fate. For example, generation of embryonic OLPs from
NSCs/NPCs can be induced by Shh signaling through activation of two important oligodendrocyte fate determinants, bHLH genes Olig1 and Olig2 (Lu et al., 2000; Yung et al., 2002). In addition, FGF2 has been shown to promote OLP generation from NPCs independently in vitro (Chandran et al., 2003; Kessaris et al., 2004). PDGF signaling appears to play a role in the oligodendrocyte differentiation in the adult SVZ by favoring oligodendrocyte fate over neuronal fate (Jackson et al., 2006). Furthermore, in the adult SVZ, GFAP-positive NSCs can generate OLPs and mature oligodendrocytes expressing Olig2, PDGF receptor α, and polysialylated neural cell adhesion molecule (PSA-NCAM) (Menn et al., 2006).

The function of Olig genes appears to be promoting both oligodendrogenesis and neurogenesis and inhibiting astrogensis (Zhou and Anderson, 2002). Olig1 and Olig 2 are expressed in OLPs and mature oligodendrocytes but ectopic expression of these genes can induce oligodenrocyte fate in the NSCs/NPCs of embryonic and postnatal brain (Lu et al., 2000; Zhou and Anderson, 2002; Marshall et al., 2005). Simultaneous or separate deletion of Olig1 and Olig2 leads to depletion of OLPs and oligodendrocytes in the brain (Zhou and Anderson, 2002). Olig2 seems to act during earlier developmental stages generating OLPs and oligodendrocytes and Olig1 is required by more mature oligodendrocytes and myelination (Arnett et al., 2004). One specific adult OLP population expressing chondroitin sulfate proteoglycan NG2 appears to be Olig2 dependent (Ligon et al., 2006). Interestingly, Olig2 is expressed in common NPCs for oligodendrocytes and neurons in the spinal cord and also in the embryonic telencephalon and is required for fate determination of both lineages (Tekki-Kessaris et al., 2001; Yung et al., 2002; Zhou and Anderson, 2002; Furusho et al., 2006). Olig2 represses neurogenesis by competing with Ngn proteins for same promoter place and Olig2 expression must be downregulated before neuronal differentiation can occur (Lee et al., 2005; Furusho et al., 2006). Repression of astrogensis by Olig2 occurs through inhibition of STAT3 and co-activator P300 activity (Fukuda et al., 2004). In NPCs, BMP signaling inhibits Olig protein function by causing dimer formation with downstream Id proteins (Yung et al., 2002; Samanta and Kessler, 2004). Inhibition of Oligs can also occur through nuclear export initiated by AKT, which is downstream of cytokine signaling (Setoguchi and Kondo, 2004). In mature astrocytes, Olig2 expression is low or absent but some astrocytes can begin to express it again upon brain injury (Buffo et al., 2005). In the early postnatal SVZ, Olig2 is expressed in astrocytes and oligodendrocytes but not in neurons (Marshall et al., 2005). Olig2 may have a temporally regulated role in astrocyte differentiation during embryonic and postnatal brain development. The homeobox gene Nkx2.2 interacts with Olig2 to produce OLPs and differentiated oligodendrocytes in the developing CNS but the role of this interaction is not entirely clear (Fu et al., 2002). One of the proneural bHLH genes, Mash1, is co-expressed in the closely-related and overlapping regions with Olig2 and is involved with specification of certain populations of oligodendrocytes differentiated from NPCs in the developing and postnatal brain (Kondo and Raff, 2000; Parras et al., 2004). Recently, it was shown that Mash1 cooperates with Olig2 during the early embryonic neurogenic period to produce a distinct population of oligodendrocytes from OLPs by controlling PDGF receptor α expression in the dorsal telencephalon (Parras et al., 2007).

SOX E transcription factors including SOX8, SOX9 and SOX10, play a role in glial development in the CNS (Kordes et al., 2005). They have largely overlapping expression patterns in developing oligodendrocytes, which are temporally sequential. SOX9 is expressed in OLPs and in immature myelinating oligodendrocytes in the embryonic VZ. SOX8 expression appears later and it is present only in the ventral VZ. SOX10 is expressed in specified OLPs emerging after the onset of SOX8 and SOX9 expression.
Furthermore, SOX8 and SOX10 expression persists in mature oligodendrocytes after SOX9 expression has been switched off. SOX9 may participate in NSC fate determination, since in the absence of SOX9 expression, neural lineage is promoted at the expense of glia (Stolt et al., 2003; Stolt et al., 2005).

Unexpectedly, proastrocytic genes such as NFIs have been shown to be required for normal oligodendrocytic lineage choice and parallel inhibition of neurogenesis (Deneen et al., 2006). Oligodendrocytes have been thought to be of ventral forebrain origin but recent evidence suggests that a distinct population of oligodendrocytes is generated from neocortical NPCs instead (Kessaris et al., 2006).

3. Neocortex development

The CNS is made from cells that divide to form neuroepithelium, which folds into the fluid-filled neural tube. During the onset of neurogenesis, neuroepithelial cells divide asymmetrically in the VZ and SVZ of the anterior and dorsal neural tube to give rise to radial glia, which produces radially migrating newly born neurons of the neocortex. These neurons find their place in the six neocortical layers in an inside out fashion and mature to exhibit various neuronal phenotypes. GABAergic interneurons are generated mainly in subcortical regions and they migrate tangentially to the neocortex. The last steps of neocortical development are synaptogenesis and neuronal network formation, which include making new connections and removing unnecessary ones in activity dependent manner (McConnell, 1988; Marin and Rubenstein, 2003; Guillemot et al., 2006) (Fig. 1).
3.1. Corticogenesis

Dividing NSCs in the mammalian neocortical VZ and SVZ, also known as apical and basal NSCs, first give rise to the subplate and cortical layer I. After this, cortical layers are formed in order VI, V, IV, and last II/III. Layer I is the most superficial and the layer VI is the deepest. The mouse is widely used as a model for mammalian neocortex development so only the events during mouse neocortical development will be discussed (Fig. 1 and 2).
Figure 2 The neocortex contains apical and basal progenitor cells, which generate neurons and glia. a In neuroepithelial cells, nucleus migrates from the apical end to basal end of the cell and these cells divide at the apical surface. b In radial glial cells, basal side of the interkinetic nuclear migration is limited to a boundary between ventricular and subventricular zones. These cells divide at the apical surface. c In basal progenitors, nucleus migrates to basal boundary and the cells divide in that position at the basal end of the ventricular zone or at the subventricular zone. G1, S, G2 and M are phases of the cell cycle. Adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, Götz and Huttner, copyright 2005.

At the onset of cortical neurogenesis, NSCs located at the VZ divide symmetrically first and then change to produce more restricted NPCs by asymmetric divisions and give rise to mainly projection neurons and also astrocytes (Davis and Temple, 1994; Williams and Price, 1995; Nieto et al., 2001). A rather complex inhibition of astrocyte differentiation plays a major role in the sequential generation of neurons before the generation of astrocytes in the neocortex (Qian et al., 2000; Morrow et al., 2001; Sun et al., 2001; Fan et al., 2005; He et al., 2005). As aforementioned, proneural genes, which include Ngn1, Ngn2 and Mash1 are crucial for the neuronal differentiation program and mediating neuronal commitment during neocortical neurogenesis (see 2.4.1. Neuronal differentiation) (Fode et al., 2000; Nieto et al., 2001).

The regulation of cell cycle progression, cell cycle length and cell cycle exit affects the number of neurons produced during neurogenesis and the neocortical lamination (Polleux et al., 1997; Caviness et al., 2003; Lukaszewicz et al., 2005). Proneural bHLH genes promote cell cycle exit in some areas of the CNS but this has not been shown in the neocortex (Mizuguchi et al., 2001; Lo et al., 2002). Differentiated neocortical neurons are originally generated from either radial glial apical VZ NPCs or basal SVZ NPCs (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Basal NPCs originate from apical NPCs and this process may be mediated by Ngn2 action on the properties of mitotic cells (Miyata et al., 2004). Neocortical neuronal differentiation may involve sequential expression of certain transcription factors including Pax6, Tbr2, NeuroD and Tbr1 in a temporal order although this has not been shown conclusively (Hevner et al., 2001; Englund et al., 2005; Hevner et al., 2006). Neural subtype identity is regulated by proneural bHLH genes, Ngn1 and Ngn2, and homeodomain genes, such as Pax6, in the developing neocortex (Bertrand et al., 2002; Shirasaki and Pfaff, 2002; Lee and Pfaff, 2005).
Pax6 directly regulates the transcription of Ngn2 in cortical NPCs (Scardigli et al., 2003). Pax6 and Emx2 are required to promote corticogenesis (Muzio et al., 2002). On the other hand, interactions between Pax6 and homeobox gene Gsh2 contribute to the formation of the pallial-subpallial border (cortical-subcortical) (Toresson et al., 2000). Pax6 is also required to generate appropriate functional phenotype in the neocortical neurons (Schuurmans et al., 2004; Kroll and O'Leary, 2005). The highest expression levels of Pax6 are found in the NPCs of the ventral neocortex accompanied by region specific expression of sFrp2 and Dbx1 genes (Kim et al., 2001; Yun et al., 2001; Assimacopoulos et al., 2003). Furthermore, Pax6 or nuclear orphan receptor Tlx are essential for inducing expression of sFrp2 in NPCs of ventral neocortex implicating a function for these genes in the patterning of the lateral telencephalon (Stenman et al., 2003). Pax6 expression in the NPCs of the ventral neocortex is required for the development of the claustrum, endopiriform nucleus, piriform cortex, amygdalar lateral nucleus, amygdalar basolateral nucleus, amygdalar basomedial nucleus and the nucleus of the lateral olfactory tract (Stoykova et al., 2000; Tole et al., 2005). The radial glia functions contributing to neurogenesis are supported by Pax6 (Heins et al., 2002; Haubst et al., 2004). Cortical layer formation involves a series of complex events (Mallamaci and Stoykova, 2006) and Pax6 participates in these events by mainly specifying later born neurons of upper neocortical identity (Götz et al., 1998; Fukuda et al., 2000). Supporting studies report abnormalities affecting upper but not lower layers of the neocortex in the absence of Pax6 expression (Tarabykin et al., 2001; Noctor et al., 2004; Schuurmans et al., 2004; Zimmer et al., 2004). SVZ is possibly the origin of upper layer neurons in the primate neocortex (Łukaszewicz et al., 2005) and in mice (Tarabykin et al., 2001). Subventricular tag (Svet1), a marker for both SVZ NPCs and upper cortical layers at perinatal stages, seems to be abolished from these areas in the absence of Pax6, which further supports a role for Pax6 in upper neocortical layer specification (Tarabykin et al., 2001). Other genes expressed in the VZ and in proliferating SVZ cells that migrate to form upper neocortical layers are Cux1 and Cux2, which also seem to specify upper layers in a Pax6 dependent manner (Nieto et al., 2004; Zimmer et al., 2004). It is not known whether Svet1 and Cux genes are expressed intrinsically or extrinsically in these defined populations of neocortex cells. Tlx is also involved in the generation of upper cortical layers and may cooperate with Pax6 (Stenman et al., 2003; Schuurmans et al., 2004).

So far a number of layer specific genes in the neocortex have been identified (Gray et al., 2004; Guillemot et al., 2006). Some of them such as SOX5, Klf6, Zfp312 and NR4A3 are clearly markers for deeper layers and others such as FoxO1, NR4A2 (Nurr1), bHLHb5 and Lmo4 are markers for upper layers. Some of them also exhibit anteroposteriorly and mediolaterally specific or dynamic expression patterns in addition to dorsoventral layer specific patterns. Such genes include Cadherin-6 and ephrin-45, which are expressed in the parietal cortex. On the other hand, Lmo4 is expressed in the frontal cortex and Clim1a is expressed in the occipital cortex. Decreasing mediolateral gradient expression of NR4A3 has been reported in the deeper layers of neocortex. Some layer-specific genes are also essential for the formation of a given layer or layers. In experiments, where layer-specific genes were rendered inactive or functionally blocked, genes such as Brn1, Brn2, were shown to perturb layer II-IV formation, whereas homeobox transcription factor Otx1 affected layer V and Tbr1 caused abnormalities in layers I and VI and the subplate.
3.2. Radial glia

At the onset of neocortical neurogenesis, radial glia forms from neuroepithelial cells upon downregulation of tight junction genes (Aaku-Saraste et al., 1996) and apical to basal separation of certain proteins (Aaku-Saraste et al., 1997). The radial glial cells exhibit a partially neuroepithelial as well as an astroglial phenotype (Campbell and Götz, 2002; Kriegstein and Götz, 2003). The radial glia as direct followers for neuroepithelial cells represents a more restricted form of neocortical NPCs and most of the neocortical neurons and astrocytes are originated from radial glia (Malatesta et al., 2003; Anthony et al., 2004). Radial glia emerges around embryonic day 10 (E10) in the mouse neocortex and is marked by expression of RC1/RC2 and little later by BLBP (Götz and Barde, 2005). Radial glia retain features from neuroepithelial cells including expression of intermediate-filament protein nestin (Hartfuss et al., 2001), an apical localization of centrosomes and prominin-1 (Weigmann et al., 1997; Chenn et al., 1998), adherence junctions and associated proteins at the apical end of the lateral plasma membrane (Aaku-Saraste et al., 1997; Wodarz and Huttner, 2003), and basal lamina contact (Halfter et al., 2002). In addition, radial glial cells exhibit apical-basal interkinetic nuclear migration, although this is more restricted compared to neuroepithelial cells (Fig. 2). Several astrocytic markers are expressed in radial glia after the onset of neurogenesis including GLAST, BLBP, S100β, GS, TN-C and vimentin (Götz and Barde, 2005; Götz and Huttner, 2005). The radial glia astrocytic structural phenotype includes the emergence of the glycogen granules that are storage sites for glycogen in these cells (Gadisseux and Evrard, 1985). Notch signaling through Hes proteins seems to be essential for maintaining radial glia during neurogenesis (Hatakeyama et al., 2004). Radial glia have limited ability to give rise to only a single type of progeny either astrocytes or oligodendrocytes, or neurons (Malatesta et al., 2003; Anthony et al., 2004). The traditional role for radial glial fibers, reaching from ventricle lumen to pial surface, is to provide a surface on which the newly born neurons can migrate to their destined neocortical layers (Rakic, 1978; Kriegstein et al., 2006). The radial glia seem to be the progeny of neuroepithelial cells with a more restricted potential to generate different kinds of cell types in the neocortex (Götz and Huttner, 2005).

3.3. Gliogenesis

The onset of gliogenesis occurs at time when neocortical NPCs become responsive to BMP, EGF or the cytokines CNTF, LIF and cardiotrophin-1, and glial specific genes such as GFAP and S100β begin to be expressed (Guillemot, 2007). The competence of neocortical NPCs to respond to cytokine signals is achieved by the action of FGF2 and EGF (Lillien and Gulacsi, 2006). Newly born cortical neurons secrete cytokines to promote gliogenesis, which act through the JAK-STAT pathway (Barnabe-Heider et al., 2005). An essential cytokine may be cardiotrophin-1, since other cytokines of the IL-6 family, CNTF and LIF, are only expressed postnatally (Barnabe-Heider et al., 2005; Miller and Gauthier, 2007). In culture, cytokine TGFβ1 signaling through the activation of SMADs appears to be one of the factors that promote gliogenesis by inducing radial glia to produce astrocytes instead of neurons (Stipursky and Gomes, 2007). BMP and Notch signaling favor gliogenesis primarily by blocking the action of proneural genes (Nakashima et al., 2001; Louvi and Artavanis-Tsakonas, 2006). As Notch signaling is required to maintain radial glia, it seems to be equally important in gliogenesis (Taylor et
Notch signaling through DNA binding protein RBP/J seems to be a key element in glial differentiation and it involves the regulation of glial specification gene Sox9 expression (Taylor et al., 2007).

A proportion of the neocortical oligodendrocytes are generated in subcortical regions from which they migrate tangentially into the neocortex in the same manner as GABAergic interneurons and other proportion are generated from cortical NSCs/NPCs (Thomas et al., 2000; He et al., 2001; Tekki-Kessaris et al., 2001; Ross et al., 2003) (Fig. 3).

**Figure 3** Induction of neurogenesis and gliogenesis in the embryonic neocortex. Multiple extracellular signals and transcription factors determine whether progenitors adopt neuronal or astroglial fate. Thick black arrows show transcriptional activation and thin grey arrows show non-transcriptional positive interactions. Thick grey lines represent transcriptional repression and thin grey lines represent non-transcriptional negative interactions. BMP, bone morphogenetic protein; C/EBP, CAAT/enhancer-binding protein; CNTF, ciliary neurotrophic factor; CSL, DNA-bound transcription factor; CT-1, cardiotoxin-1; EGF, epidermal growth factor; E10, embryonic day 10; FGF2, fibroblast growth factor 2; Hes1/5, bHLH transcription factors; ICD, intracellular domain; Id1/3, inhibitors of differentiation 1/3; LIF, leukemia inhibitor factor; N-COR, nuclear receptor co-repressor; PDGF, platelet-derived growth factor; SMAD, mothers against decapentaplegic homologue; STAT, signal transducer and activator of transcription; Wnt, wingless. Reprinted from Progress in Neurobiology, Vol 83, Guillemot, Cell fate specification in the mammalian telencephalon, Pages 37-52, Copyright 2007, with permission from Elsevier.
3.4. Modes of neural migration in the developing telencephalon

In the developing brain, the migration of newborn cells is directed away from the proliferative zones the VZ and the SVZ. Radial migration is the main mechanism that creates the layered structure of excitatory neurons in the neocortex. The migration of interneurons occurs in a largely tangential manner from subpallium to pallium. In the olfactory bulb, new neurons migrate from the SVZ along the rostral migratory stream (RMS).

The migration of pallial neocortical neurons, which occurs in a ventral to dorsal direction from the embryonic VZ towards the pial surface, is termed radial migration (Rakic, 1971; Marin and Rubenstein, 2003). An important scaffold for migrating neurons is provided by radial glial cells, which are also the progenitors for a subset of neocortical neurons (Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2002). Radial migration of a newborn neocortical neuron involves four stages including initiation of movement, attachment to radial glial fiber, and locomotion with nucleokinesis, detachment, and laminar positioning (Marin and Rubenstein, 2003). During their radial migration newborn neocortical neurons also make retrograde and tangential moves and acquire a transient multipolar phenotype (Tabata and Nakajima, 2003; Noctor et al., 2004).

Tangential migration is a mode of neuronal migration that is not associated with radial glial guidance or direction in the CNS (O’Rourke et al., 1992; Walsh and Cepko, 1992), but cell motility includes the same steps as radial migration (Kriegstein and Noctor, 2004; Marin et al., 2006). Tangentially migrating cells can acquire diverse morphological appearance with short compact processes, long elongated processes, or branched processes (Marin et al., 2006). Tangential migration includes cell motility along the neuronal scaffold, along axons, or dispersed migration from the subpallium to the pallium. First two modes are involved in the migration of olfactory bulb interneurons along the rostral migratory stream and the migration of Gonadotrophin-releasing hormone neurons (Lois and Alvarez-Buylla, 1994; Wray, 2001). The third mode involves migration of interneurons and oligodendrocytes to the neocortical and hippocampal regions during forebrain development (Wichterle et al., 1999; Corbin et al., 2001; Letinic et al., 2002; Marin and Rubenstein, 2003).

After creating the neuronal variety in the layered neocortex, synaptic connections are established and strengthened mainly in an activity-dependent fashion. This operation is very complex and new information is altering the overall picture frequently (Muotri and Gage, 2006; Price et al., 2006; Merkle et al., 2007; Toni et al., 2007) and so will not be discussed further.

4. NSCs in disease

Altered adult hippocampal neurogenesis has been suggested to occur in several neurological disorders. Epileptic seizures are known to induce proliferation of NSCs and increase neurogenesis, which may contribute to the dentate granule cell abnormalities found in many cases of temporal lobe epilepsy (Bengzon et al., 1997; Parent et al., 1997; Scharfman et al., 2000). Increased neurogenesis occurs in Alzheimer’s disease and Huntington’s disease (Curtis et al., 2003; Jin et al., 2004a). On the other hand, in a Parkinson’s disease model, NSC proliferation and neurogenesis are reduced in the SVZ and in the dentate gyrus (Höglinger et al., 2004), although, it seems unlikely that
neurogenesis of dopaminergic neurons of the adult substantia nigra would be affected (Zhao et al, 2003; Frielingsdorf et al, 2004). Interestingly, major depression has been proposed to be accompanied by a defect in hippocampal neurogenesis, although this kind of depression is not primarily a hippocampal disorder (Jacobs et al., 2000; Kempermann and Kronenberg, 2003).

Some of the brain tumors are lethal with resistance to conventional treatments. Only a few cells in the brain tumor may be responsible for generating cancer cell mass as well as for the recurrence of cancer. This cell type has the properties of a somatic stem cell with the capability to initiate malignant cell proliferation and has been termed a brain tumor stem cell (Vescovi et al., 2006). The most typical types of brain tumors are glioblastomas, medulloblastomas, and ependymomas in which the brain tumor stem cell type has been already identified. Brain tumor stem cells exhibit the ability to self-renew and they have the potential to differentiate into neurons, astrocytes, and oligodendrocytes in vitro (Ignatova et al., 2002; Hemmati et al., 2003; Singh et al., 2003; Galli et al., 2004; Tunici et al., 2004; Taylor et al., 2005; Vescovi et al., 2006; Nicolis, 2007).

NSCs may be exploited to establish well-standardized cell assays for drug screening and toxicology to replace assays which do not properly represent the actual human system (Singec et al., 2007). For disease modeling, NSCs carrying known pathological defects derived from different developmental stages of the CNS may be useful when seeking an understanding of the mechanisms causing these defects (Martinat et al., 2004; Singec et al., 2007).

Based on current knowledge, pathological neuron loss in the adult CNS may in some extent recruit quiescent endogenous NSCs in neuronal repair and rescue, which can be beneficial in NSC-based therapeutic interventions.

5. NSC-based cell therapy

The potential of NSCs or NPCs to populate the brain and restore function has been shown during the last decade (Snyder et al., 1992; Flax et al., 1998; Ourednik et al., 2002). In vitro, NSCs have been propagated successfully in the presence of EGF and/or FGF2 in cell clusters termed neurospheres for relatively long time, but still a standardized method for NSC culture is lacking (Snyder et al., 1992; Singec et al., 2006). Fundamental to NSC therapy is the fact that new neurons are generated in the olfactory bulb and hippocampal dentate gyrus in the adult brain (Lledo et al., 2006). Another important observation has been that radial glial cells and a yet undefined subtype of astrocytes are actually NSCs in the developing and in the adult brain, respectively (Noctor et al., 2001; Anthony et al., 2004; Ihrie and Alvarez-Buylla, 2007). Therapeutic strategies for using NSCs in neurological diseases involve different types of approaches and possibilities including cell replacement, chaperon effects, NSC-based gene therapy, standardized cell assays, disease modeling, and somatic cell nuclear transfer (SCNT) (Singec et al., 2007). The potential of NSCs in cell replacement was shown in the adult mammalian neocortex by an experimental model of induced pyramidal neuron death, in which NSCs were able to replace lost neurons with the same type of neurons (Snyder et al., 1997). The potential of NSCs has to be carefully validated according to the individual circumstances since transplants can die during cell preparations, after the implantation, or due to a cell craft size (Bakshi et al., 2005; Karlsson et al., 2005). Improved transplantation techniques, anti-
apoptotic drugs, and immunosuppression after transplantation are required for utilizing NSCs in clinical cell replacement therapy (Takagi et al., 2005; Singec et al., 2007).

The ability to secrete neurotrophic factors brain derived neurotrophic factor (BDNF), GDNF, nerve growth factor (NGF), and NT-3, and cytoprotective molecules to rescue diseased neurons manifest the inherited chaperone effect of crafted NSCs (Ourednik et al., 2002). These crafted cells also deliver other factors that decrease scarring, promote angiogenesis, and prevent inflammatory action. This type of NSC action can be considered to promote the homeostatic recovery of the diseased tissue (Singec et al., 2007).

Endogenous and crafted NSCs can be used as vehicles for transgene delivery into the diseased brain, since these cells exhibit the ability to target pathology with incredible integration capabilities and stably express a foreign gene. Furthermore, the migration tendency of NSCs is guided by chemoattractants released from inflammation and during acute or chronic tissue damage. One of the molecules secreted during the inflammatory response, stromal cell-derived factor 1 alpha (SDF1-α), is a strong attractant for human NSCs migrating even from long distances (Imitola et al., 2004). Thus, transgenic NSCs may present a powerful tool in long-range delivery of drugs and therapeutic molecules into an injured CNS.

Endogenous NSCs may be induced to produce new neurons upon injury. Some regions of the adult brain are suggested to contain NSCs but they are quiescent under normal homeostatic inhibitory conditions in contrast to the hippocampal dentate gyrus and olfactory bulb, which constantly generate new neurons (Bernier et al., 2002; Lie et al., 2004). Non-neurogenic NSCs can be induced to proliferate and generate neurons in vitro or when transplanted to neurogenic regions in vivo or upon CNS injury (Snyder et al., 1997; Shihabuddin et al., 2000). However, it is unclear whether these endogenous NSCs can be recruited without causing exhaustion or tissue malformations and whether NSC-derived new neurons can establish correct synaptic contacts. Furthermore, activating endogenous NSCs may be useless when considering therapeutic treatment of diseases with inherited genetic mutations.

SCNT or therapeutic cloning may be one possibility to generate NSCs specifically for individual patients by deriving the nucleus from a fully differentiated cell and transferring it into an egg which will be allowed to develop into a blastocyst. In theory, cells from the blastocyst could be manipulated to differentiate into a neuronal lineage and transplanted into the CNS, although this method has not been successfully employed in the CNS or in humans in general (Rideout et al., 2002; Singec et al., 2007).

Various brain disorders can potentially benefit from NSC therapy, including Parkinson’s disease, Huntington’s disease, lysosomal storage diseases, and brain cancer. Parkinson’s disease is caused by the degenerative death of nigro-striatal dopaminergic neurons that results in the clinical symptoms including tremor, rigidity, and bradykinesia (Clarke, 2007). Transplantation of fetal mesencephalic tissue into diseased striatum which is lacking dopamine, and is innervated by axons from the substantia nigra, has proven to be a successful method in improving Parkinson’s disease (Lindvall and Björklund, 2004). Due to difficulties of getting human fetal tissue for transplantation, immortalized animal NSC lines and human embryonic stem (ES) cell lines producing unlimited numbers of specific type of midbrain dopaminergic neurons have been established (Kim et al., 2002; Singec et al., 2007). In animal models of Parkinson’s disease, transplantation of these stem cell-derived differentiated cells have improved the functional phenotype (Björklund et al., 2002), but this needs to be shown in human CNS in vivo.

Inherited trinucleotide CAG-repeat expansion mutation on chromosome 4, which results in nuclear and cytoplasmic accumulation of huntingtin fragments and the death of
medium spiny striatal GABAergic neurons, causes Huntington’s disease (Ramaswamy et al., 2007; Singec et al., 2007). This devastating disease, which exhibits motor, cognitive, and psychiatric symptoms, leads to the eventual death of patients 15 to 20 years after disease onset. Transplantation of NSCs may provide therapy for improving the odds of Huntington’s disease patients, since animal models have shown that crafted fetal neural tissue can survive, grow, and establish functional contact with the host brain (Peschanski et al., 2004). Clinical trials on neural transplantation in Huntington’s disease patients have provided positive evidence on improving the condition of these patients and shown that fetal striatal tissue crafted into patient’s brain can survive, produce synaptic contacts, and resist the process of the disease (Bachoud-Levi et al., 2006; Singec et al., 2007).

Lysosomal storage diseases (LSDs) such as human MPS VII has been studied via mouse models in experiments were the potential of crafted migratory NSCs to deliver important molecules has been demonstrated. MPS VII is caused by a deficiency in a β-glucuronidase (GUSB) enzyme, which is involved in the degradation of glycosaminoglycans. The symptoms of this disease include neurodegeneration throughout the brain, and mental retardation in humans (Singec et al., 2007). NSCs crafted into the ventricles of the newborn mouse disseminated throughout the neural axis, corrected lysosomal function and increased the levels of GUSB (Snyder et al., 1995). Similar progress has been observed in transplantation experiments in other types of LSDs, but despite promising results in animal models clinical trials have not yet been initiated (Lacorazza et al., 1996; Shihabuddin et al., 2004).

Other neuropathological conditions presenting NSC trophism in which NSC-based therapy may be useful include amyotrophic lateral sclerosis (Muller et al., 2006), stroke (Kelly et al., 2004), traumatic brain injury (Wennersten et al., 2004), multiple sclerosis (Totoiu et al., 2004), Alzheimer’s disease (Tuszynski et al., 2005), acute spinal cord injury (Cummings et al., 2005), and epilepsy (Chu et al., 2004).

NSCs may be used as novel therapies to treat malignant brain cancers, since NSCs and brain tumor stem cells have very similar properties (Yip et al., 2006). In fact, NSCs can migrate long distances and home tumor cells (Aboody et al., 2000). Transgenic NSCs can deliver drugs into tumors and significantly reduce tumor cell mass (Aboody et al., 2000). Cells related to brain tumors may express various factors such as chemokine receptor CXCR4, stem cell factor (SCF), monocyte chemoattractant protein-1 (MCP1), and vascular endothelial growth factor (VEGF) etc. that are involved in chemoattractions of NSCs (Yip et al., 2006). Efforts are currently under way to exploit NSCs as vehicles in brain cancer treatment (Yip et al., 2006).

In conclusion, NSC-therapy may prove valuable in treating CNS disorders involving neoplastic, chronic/degenerative, metabolic, acute/traumatic, and inflammatory lesions (Muller et al., 2006). However, the problems of NSC-therapy such as survival, differentiation and correct network integration of transplanted cells remain as challenges for the future stem cell research (Singec et al., 2007).

6. Neurotrophins and their receptors in the CNS

Neurotrophins are a family of structurally and functionally similar proteins that were first characterised as survival factors for sensory and sympathetic neurons, and have since been demonstrated to regulate many aspects of survival, development and function of neurons in both the PNS and the CNS. The neurotrophin family in mammals contains four
structurally and functionally related members: NGF, BDNF, NT-3, and NT-4. Shared structural similarities between neurotrophins such as tertiary fold and cystein knot are also features of other growth factors including TGFβ and PDGF. The action of neurotrophins is mediated through the Trk family of receptor tyrosine kinases (TrkA, TrkB, and TrkC) and p75 neurotrophin receptor (p75NTR), all of which are transmembrane receptors (Huang and Reichardt, 2001; Lu et al., 2005; Reichardt, 2006; Lei and Parada, 2007) (Fig. 4).

**Figure 4** Neurotrophins and their receptors. Trk receptors and p75 receptor are membrane spanning receptors that have extracellular domain which neurotrophins bind. Upon ligand binding, Trk receptors can activate such intracellular pathways as MAPK-pathway, PI3K-pathway and PLC-γ-pathway. Upon ligand binding, p75 activates such intracellular pathways as NF-κB and JNK. See text for abbreviations. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Chao, copyright 2003.
6.1. BDNF and TrkB receptors

Of the neurotrophins, BDNF is most prominently involved in activity-dependent neuronal organization during nervous system development (McAllister et al., 1999; Bibel and Barde, 2000; Thoenen, 2000; Poo, 2001; Aid et al., 2007). In the adult brain, BDNF modulates synaptic plasticity, and thus, is involved in processes including learning, memory, and mood (Nestler et al., 2002; Lu, 003; Castrén, 2004; Castrén et al., 2007). BDNF has been implicated in the modulation of pain, and in Alzheimer’s disease as well as in other neurodegenerative disorders (Allen and Dawbarn, 2006; Chao et al., 2006). A Val66Met polymorphism in the gene encoding BDNF disrupts hippocampal function, episodic memory, and has been connected to schizophrenia and depression (Egan et al., 2003; Hariri et al., 2003; Frey et al., 2007). In synaptic plasticity-related events, BDNF promotes hippocampal LTP through TrkB activation, while proBDNF has been suggested to enhance hippocampal LTD through p75NTR activation (Patterson et al., 1996; Minichiello et al., 1999; Minichiello et al., 2002; Zakharenko et al., 2003; Pang et al., 2004; Woo et al., 2005).

The effects of BDNF are mediated through binding to TrkB and p75NTR receptors (Klein et al., 1989; Rodriguez-Tebar et al., 1990). The p75NTR has high affinity for proBDNF whereas TrkB binds mature BDNF (Lu et al., 2005). In addition to the full-length isoform bearing the catalytic domain, TrkB has three truncated isoforms TrkB.T1, TrkB.T2, and TrkB.shc, which lack the tyrosine kinase domain in their intracellular part (Klein et al., 1990; Middlemas et al., 1991; Stoilov et al., 2002). Full-length TrkB receptors are highly expressed in neurons during development, while postnatally, TrkB receptor expression is attenuated (Allendoerfer et al., 1994; Escandon et al., 1994). Like BDNF expression, TrkB expression is upregulated in the hippocampus and neocortex in an activity-dependent manner and lack of TrkB leads to the loss of specific neuron populations in those areas (Xu et al., 2000; West et al., 2002; Kingsbury et al., 2003). Depolarized cultured cortical neurons have increased expression of TrkB receptor (Kingsbury et al., 2003). TrkB receptor expression is altered by stress, seizure, exercise, antidepressant treatments, and antipsychotic agents in vivo (Lei and Parada, 2007). Furthermore, TrkB receptors are activated upon antidepressant treatment (Saarelainen et al., 2003). TrkB is required for the formation of hippocampal Schaffer collateral synapses (Luikart et al., 2005) and cerebellar GABAergic synapses (Rico et al., 2002).

Truncated TrkB.T1 receptors are expressed in neurons as well as in non-neuronal cells and their expression increases postnatally and predominates in the whole telencephalon (Klein et al., 1990; Allendoerfer et al., 1994; Escandon et al., 1994; Fryer et al., 1996). It was previously thought that these truncated receptors did not transmit any intracellular signals but their function was to repress BDNF signaling through ligand sequestering or forming dominant negative hetero dimers with full-length TrkB receptors (Biffo et al., 1995; Eide et al., 1996; Ninkina et al., 1996; Li et al., 1998b; Haapasalo et al., 2001). However, this has been challenged by the finding that TrkB.T1 receptors can signal through distinct pathways of their own (Baxter et al., 1997; Rose et al., 2003; Ohira et al., 2005; Cheng et al., 2007). TrkB.T1 may mediate BDNF-evoked G-protein and IP3-dependent intracellular calcium release in astrocytes as well as control glial Rho GTPase activity and thereby regulate glial cell morphology (Rose et al., 2003; Ohira et al., 2005). Both full-length and truncated TrkB receptor expression is increased as a result of CREB activation in cultured neurons (Deogracias et al., 2004). TrkB can be recruited into cholesterol-rich rafts upon ligand binding and internalized through activity and Ca²⁺ influx dependent potentiation of the tyrosine kinase domain (Du et al., 2003). TrkB expression is
altered in several pathological and physiological conditions including brain trauma, motoneuron axotomy, Parkinson’s disease, Alzheimer’s disease, schizophrenia, and aging (Lei and Parada, 2007).

6.2. The role of BDNF action in NSCs and in the developing brain

BDNF signaling regulates the in vitro survival and differentiation of NSCs and NPCs derived from the developing embryonic and postnatal brain (Ahmed et al., 1995; Lachyankar et al., 1997; Shetty and Turner, 1998; Takahashi et al., 1999; Benoit et al., 2001; Caldwell et al., 2001; Barnabe-Heider and Miller, 2003). BDNF and nitric oxide generate a paracrine positive feedback loop to inhibit NPC proliferation and promote differentiation in the mammalian brain (Cheng et al., 2003). BDNF increases the expression of bHLH proneural genes Mash1 and Math1 to facilitate differentiation of NSCs (Ito et al., 2003). In the developing and adult brain, p75NTR expression defines BDNF-responsive neurogenic NPCs in the SVZ (Young et al., 2007). BDNF signaling through p75NTR is required for neuronal differentiation of fetal forebrain NPCs in vivo and lack of p75NTR increases proliferation and nestin expression in these cells (Hosomi et al., 2003). Furthermore, a sequential activation of p75NTR and TrkB is involved in dendritic development of SVZ derived differentiating NPCs (Gascon et al., 2005).

The expression of truncated TrkB.T1 directs neocortical NSCs towards a glial fate through a new signaling mechanism (Cheng et al., 2007) and neuron-specific overexpression of TrkB.T1 can promote proliferation of adult SGZ progenitors which is accompanied by reduced survival of newborn granule neurons in vivo (Sairanen et al., 2005).

During neocortex development, administering BDNF at E13 but not at E14 results in the differentiation of deeper layer neurons instead of superficial neurons (Ohmiya et al., 2002). Furthermore, BDNF alters the position, gene-expression properties and projection of neurons of superficial layers to match those of the deeper layers, and in the absence of BDNF migration of layer II/III neurons is affected (Fukumitsu et al., 2006). During development, TrkB receptor expression regulates the temporal migration of neocortical neurons and the differentiation of neurons and oligodendrocytes (Medina et al., 2004). TrkB expression in thalamic axons is important for normal development of the barrel cortex (Lush et al., 2005).

In the developing cerebellum, BDNF directly and acutely stimulates the migration of newborn granule neurons in the cerebellum (Borghesani et al., 2002). These cells migrate along a BDNF gradient, which involves vesicle trafficking as an important component for responding to BDNF-mediated chemotactic stimulus (Zhou et al., 2007). Furthermore, NPC migration from the SVZ along the rostral migratory stream is promoted by local expression of both BNDF and TrkB receptors (Chiarameillo et al., 2007). In migrating cells, this neurotrophic effect causes the activation of CREB through PI3K and MAPK pathways (Chiarameillo et al., 2007).

TrkB signaling is important for the growth and stabilization of excitatory synapses during development in a cell-autonomous manner both in pre- and postsynaptic cells (Luikart and Parada, 2006). Overexpression of the full-length TrkB receptor leads to increased expression of plasticity related genes in several brain regions (Koponen et al., 2004). The expression of both full-length TrkB and TrkB.T1 receptors are suppressed by thyroid hormone in the developing rat brain (Pombo et al., 2000).
The expression of TrkB receptors has been associated with malignant neuroblastomas (Brodeur et al., 1997; Schramm et al., 2005). TrkB receptor expression can suppress anoikis, which is a form of apoptosis caused by the loss of cell-matrix interactions, and induce metastasis in non-malignant epithelial cells (Douma et al., 2004). Transiently overexpressed TrkB.T1 receptor competes with the full-length TrkB receptor, and is able to increase process and filopodia formation in neuroblastoma cells thus directing them toward differentiated morphology (Haapasalo et al., 1999). TrkB activation can also promote the survival of human ES cells by counteracting apoptotic signals (Pyle et al., 2006).

To summarize, BNDF signaling through TrkB and p75NTR signaling seems to play a role in NSC/NPC survival, differentiation, and proper neocortical development.

6.3. TrkB.T1 overexpressing mouse

Overexpression of a truncated form of the TrkB receptor, TrkB.T1, may suppress the normal function of BDNF (Eide et al., 1996). To test this hypothesis a transgenic mouse model was generated, which harboured N-terminally flag-tagged TrkB.T1 cDNA under the control of the Thy1 promoter (Caroni, 1997; Saarelainen et al., 2000a). Transgene expression in these mice is neuron specific in mature brain, and the main regions for expression are the hippocampus and neocortex. Initial characterization revealed that these mice exhibit impaired long-term spatial memory and normal hippocampal LTP (Saarelainen et al., 2000b). These mice have increased susceptibility for ischemia induced neocortical injury, implicating impaired neurotrophin mediated rescue of neocortical neurons upon ischemic insult (Saarelainen et al., 2000a). Adult hippocampal neurogenesis is altered in these mice overexpressing TrkB.T1 receptor, since the survival of newborn neurons in the dentate gyrus was reduced, and was accompanied by precursor proliferation which was likely induced by a homeostatic feedback mechanism (Sairanen et al., 2005). Based on these observations, the mouse model overexpressing the TrkB.T1 receptor can be considered as a model for mildly impaired learning and memory.

7. Fragile X syndrome

Fragile X syndrome (FXS) is the most common form of inherited mental retardation with a prevalence of 1 in 4000 males and 1 in 8000 females (Turner et al, 1996; O'Donnell and Warren, 2002). The mental retardation linked to fragile X chromosome was first termed as Martin-Bell syndrome in the early 80s (Richards et al., 1981), and it is now known as FXS. FXS belongs to a large group of human X-linked mental retardation syndromes (Chiurazzi et al., 2004). The fragile X site lies in locus Xq27.3 of the X-chromosome between factor IX gene and marker st14 (Harrison et al., 1983; Oberle et al., 1986). FXS is caused by a mutation in a single gene, fragile X mental retardation 1 (FMR1), which was cloned in the early 90s (Heitz et al, 1991; Verkerk et al., 1991). The mutation leads to a lack of fragile X mental retardation protein (FMRP) (Pieretti et al., 1991). Cloning of the FMR1 gene accelerated research on the syndrome and the gene proved to be well conserved between species. This allowed the generation of animal models to study the syndrome.
7.1. Phenotype of FXS

7.1.1. Behavioral phenotype in FXS

Characteristic of FXS is mental retardation, with IQ normally ranging from 20 to 70 (Fisch et al., 2002). The cognitive skills that are the most affected include speech, short-term memory, and visuospatial abilities. Developmental delay in speech is the symptom that is usually first noticed in FXS. A wide variety of non-cognitive symptoms of FXS include hyperactivity, attention deficit, hypersensitivity to sensory stimuli, and autistic like behavior (O’Donnell and Warren, 2002). Gaze avoidance, tactile defensiveness, and repetitive behaviors, which are characteristic for autistic individuals, are also observed in 15% to 50% of FXS patients. In addition, FXS patients show a delayed return of glucocorticoids to baseline levels following stress, which may be considered to be part of autistic like features (Hessl et al., 2004).

7.1.2. Anatomical and neuroanatomical phenotype in FXS

In FXS, facial morphology is usually altered in a manner that prominent jaws, forehead and large ears accompany a long and narrow face (Chudley and Hagerman, 1987) (Fig. 5). This is typical for macrocephalic facial morphology. A common and characteristic of FXS is enlarged testicles or in other words macroorchidism (Turner et al., 1980). Other physical features include a high arched palate, flat feet, hypotonia, mitral valve prolapse, and increased joint laxity that are thought be a result from a connective tissue abnormality in FXS (Hagerman et al., 1983; Loehr et al., 1986; Hjalgrim et al., 2000). Increased birth weight, increased stature, and macrocephaly are part of the unusual growth patterns associated with FXS. This is believed to be caused by neuroendocrine dysfunction, and in particular, by hypothalamus dysfunction (Hessl et al., 2004).

FXS brains exhibit no major gross abnormalities (Rudelli et al., 1985; Hinton et al., 1991; Wisniewski et al., 1991; Irwin et al., 2001). There are, however, observations about ventricular enlargement and mild cortical atrophy (Wisniewski et al., 1991). MRI images of FXS brains have revealed other abnormalities such as decreased size of posterior vermis of cerebellum and increased size of caudate nucleus. The abnormalities seen in the caudate nucleus, posterior vermis and ventricles correlate with the expression of the FMR1 gene and may result from the lack of FMRP (Reiss et al., 1991a; Reiss et al., 1991b; Reiss et al., 1995; Mostofsky et al., 1998; Eliez et al., 2001). Interestingly, hippocampal volume was observed to be enlarged in children and young adults with FXS (Reiss et al., 1994; Kates et al., 1997) but not in older adults (Jäkälä et al., 1997) suggesting a developmental defect in hippocampus of FXS patients. Recently, a tensor-based morphometry analysis revealed minor neuroanatomical alterations in FXS brains including an increase in parietal and temporal white matter (Lee et al., 2007). This analysis also confirmed the earlier findings in FXS brains showing increases in lateral ventricle and caudate nucleus volumes. Periventricular heterotopia has been found in two FXS patients, which suggests that neuronal migration is impaired in FXS (Moro et al., 2006). Structural abnormalities in the lateral geniculate nucleus of the visual pathway have been reported in FXS patients (Kogan et al., 2004b). These abnormalities are shown to affect only magnocellular neuron
layers of the lateral geniculate nucleus in which FMRP is normally expressed thus suggesting a cell type specific function for FMRP in certain parts of the visual pathway.

Figure 5 A boy with fragile X syndrome. Reprinted, with permission, from Annual Reviews of Genomics and Human Genetics, Volume 8 © 2007 by Annual Reviews, www.annualreviews.org.

7.1.3. Neuronal ultrastructure: Dendritic spine phenotype in FXS

FMRP is expressed widely in the human CNS and highly in neurons (Abitbol et al., 1993; Devys et al., 1993). Certain excitatory neurons show protrusions in their dendrites that are important for synapse formation. These are called dendritic spines and they are dysmorphed in FXS in such a manner that they generally exhibit an immature phenotype with an increased density of longer, thinner and tortuous spines compared to healthy controls (Rudelli et al., 1985; Hinton et al., 1991; Wisniewski et al., 1991; Irwin et al., 2001) (Fig. 6). During normal development, dendritic spine maturation and pruning are
important steps in the formation of functional synaptic contacts and the elimination of unnecessary ones. Dendritic spine abnormalities have also been observed in other syndromes with mental retardation (Dierssen and Ramakers, 2006). Increased dendritic spine density and the resulting increased excitatory activity are postulated to play a role in epileptic seizure susceptibility in FXS patients (Incorpora et al., 2002). Spine morphogenesis involves signaling pathways that eventually lead to rearrangement of actin cytoskeleton. At the postsynaptic site, FMRP synthesis may affect related proteins and signaling pathways involved in dendritic spine morphology including Rho GTPase Rac1, microtubule-associated protein-1B (MAP1B), calcium-calmodulin-dependent kinase II (CaMKII), calbindin, alpha-glucocorticoid receptor, p21-activated kinase (PAK) and cadherins (Grossman et al., 2006a; Hayashi et al., 2007).

![Figure 6](image)

**Figure 6** Dendritic spines of fragile X neurons have an immature appearance. Adapted from Irwin et al, 2001 and reprinted with permission from Wiley-Liss, Inc.

### 7.2. Causes of FXS

The 5’ untranslated region (UTR) of the *FMR1* gene contains a CGG trinucleotide repeat region, which is unusually expanded to over 200 repeats in FXS compared with 6 to 44 repeats in healthy controls and 55 to 200 repeats in premutation carriers (Verkerk et al., 1991). The expansion to the full-mutation usually occurs when the premutation is transmitted from a female, since male spermatogenesis is unable to preserve the long repeat extension (Malter et al., 1997). The repeat extension, and the upstream CpG-island are hypermethylated and the premutation-length extension increases in size when transmitted to a pedigree (Heitz et al., 1991; Oberle et al., 1991; Eichler et al., 1993). Microsatellite sequences, similar to the one causing FXS, are common in the human genome and are usually relatively stable when transmitted to a pedigree whereas
trinucleotide repeat sequences are much more unstable and can be lengthened upon transmission (Richards and Sutherland, 1997). This type of dynamic mutation usually results in a genetic disorder. FXS is one of the 16 disorders known so far to be caused by this type of mutation mechanism (Cummings and Zoghbi, 2000). The trinucleotide repeat extension mechanism is thought to involve slipped-strand mispairing during DNA replication that occurs especially in Okazaki fragments (Kunkel, 1993; Richards and Sutherland, 1997). Long trinucleotide repeat regions containing Okazaki fragments are unstable and can suffer a slippage which results in the addition of new complementary nucleotides to template strand by DNA repairing enzymes and thus generates an expansion. This suggests defect in the mismatch repair system although an evidence of this is lacking. Within the FMR1 gene, the full-mutation trinucleotide repeat and CpG-island are hypermethylated together with the surrounding regions, which causes repression or silencing of the FMR1 gene that leads to a lack of FMRP in FXS (Pieretti et al., 1991; Sutcliffe et al., 1992; Hornstra et al., 1993). Methylation occurs very early in embryonic development and the short time period when the full-mutation FMRP is expressed does not seem to affect the severity of the FXS phenotype (Willemsen et al., 1996; Losekoot et al., 1997). In FXS, methylation of the FMR1 gene prevents direct transcription factor-binding and induces chromatin condensation which impairs the binding of transcriptional machinery (Penagarikano et al., 2007). Four upstream footprints for transcription factor-binding sites, which are inactivated after hypermethylation of the FMR1 gene in FXS, include a GC rich palindrome, two GC boxes, and E-box-cAMP response element (CRE) site (Drouin et al., 1997; Schwemmle et al., 1997).

Although over 95% of FXS cases are caused by the CGG trinucleotide repeat mutation (O’Donnell and Warren, 2002), it is not the only cause for FXS since other mutations can also lead to a loss of functional FMRP. Several deletions (Hammond et al., 1997) and some point mutations (De Boulle et al., 1993; Lugenbeel et al., 1995) affecting the FMR1 gene have been found to cause FXS. A point mutation may lead to the expression of non-functional protein and may offer an opportunity to characterize the functional domains of any given protein. In fact, in one case of FXS with a severe phenotype, a missense point mutation in the nuclear ribonucleoprotein K Homology 2 (KH2) domain of FMRP at amino acid site 304 caused by substitution of isoleucine (I) to asparagine (N) (I304N) was reported (De Boulle et al., 1993; Siomi et al., 1994). Since then, this mutation has been studied and has provided valuable information of the molecular binding partners of FMRP. In some patients, the FXS phenotype, which does not involve a trinucleotide repeat expansion or a mutation in the gene, has been found (Clarke et al., 2004). Overall, the bottom line is that the absence or loss-of-function mutation of FMRP causes the phenotype in FXS. Recent research has shown that the premutation size of the CGG trinucleotide repeat expansion results in a distinct phenotype of its own referred to as fragile X-associated tremor/ataxia syndrome (Hagerman and Hagerman, 2004) but will not be discussed further in this thesis.

7.3. The structure and expression of the FMR1 gene

The FMR1 gene spans 38 kb and encodes a 4.4 kb transcript consisting of 17 exons (Fig. 7). The gene encodes a protein, which has several splice variants and a maximum length of 632 amino acids resulting in a molecular weight of 80 kDa (Ashley et al., 1993; Eichler et al., 1993). The CGG trinucleotide repeat region is located within the first exon of the
The fragile X mental retardation 1 gene (FMR1) and the translation initiation sequence is located 69 bp downstream (Ashley et al., 1993).

Transcription factors that actively regulate FMR1 promoter activity include upstream stimulatory factor 1 (USF1) and USF2, nuclear respiratory factor 1 (NRF1) and NRF2, specificity protein 1 (Sp1) and Sp3, alpha-Pal/Nrf-1, and cAMP response element binding protein (CREB) (Kumari and Usdin, 2001; Kumari et al., 2005; Smith et al., 2006). Of these transcription factors, Sp1 is the strongest in regulating FMR1 transcription with apparent resistance to DNA methylation, although the presence of MeCP2 and the chromatin-remodeling protein Brahma may disrupt its association with the FMR1 promoter in FXS cells (Smith et al., 2004; Harikrishnan et al., 2005). Transcriptional regulation of the Fmr1 gene in the embryonic but not adult mouse brain seems to involve a transcription factor AP2 in a gene dosage dependent manner (Lim et al., 2005a).

Normally, the FMR1 gene is associated with acetylated histone proteins H3 and H4 at the 5’ region with acetylation of H3 being the most important for transcription, but in FXS cells the acetylation of these proteins is reduced (Coffee et al., 1999; Coffee et al., 2002). In addition, normal methylation patterns of the FMR1 gene show that histone 3 has a methylated lysine 4 and unmethylated lysine 9 (Coffee et al., 2002). In FXS, opposing methylation patterns occur (Coffee et al., 2002). The FMR1 CGG trinucleotide repeat sequence is normally interrupted by an AGG trinucleotide sequence approximately every 9 to 10 repeat units, and if these interruption sequences are missing, as in some premutation cases, the risk of full-mutation is greatly increased. Thus, the loss of these interrupting trinucleotide sequences increases the instability of the trinucleotide repeat region (Eichler et al., 1993; Kunst and Warren, 1994). Premutations, with at least 90 trinucleotide repeats, are converted to full-mutation when transmitted to a pedigree and contain perhaps 2 AGG interruptions in all. This suggests that for full-mutation, 70 pure trinucleotide repeats are required. This amount of repeats fits to a theory of Okazaki fragment slipped-strand mispairing as a mechanism for trinucleotide repeat expansion.

The FMR1 gene has two autosomal homologs: fragile X related 1 (FXR1) and FXR2 (Siomi et al., 1995; Zhang et al., 1995) and highly conserved orthologs in mammals,
chickens, fruit flies, zebrafishes, and frogs (Ashley et al., 1993; Price et al., 1996; Wan et al., 2000; Tucker et al., 2004; Lim et al., 2005b)

7.4. Fragile X mental retardation protein

The \textit{FRM1} gene product, FMRP, is most highly expressed in the brain and testis in which the major phenotypic symptoms of FXS, mental retardation and macroorchidism, are observed (Devys et al., 1993). In the CNS, the majority of \textit{FMRI} gene and FMRP expression is localized to neurons. Recently, however, a developmentally transient expression of FMRP was also observed in non-neuronal cells in the mouse brain (Abitbol et al., 1993; Devys et al., 1993; Pacey and Doering, 2007). The protein is mainly localized in the cytosol of neurons although it also has a nuclear localization signal (NLS) suggesting a trafficking between the nucleus and the cytosol (Devys et al., 1993; Feng et al., 1997b). Alternative splicing can generate at least 12 different FMRP isoforms (Devys et al., 1993; Siomi et al., 1993), and they show the same expression pattern in different tissues (Verkerk et al., 1993). The most common isoforms lack exon 12, and the isoforms that are the least expressed lack exon 14 (Sittler et al., 1996). The isoforms with low expression also locate to nucleus, since the nuclear export signal (NES) is located within exon 14.

FMRP contains sequence motifs characteristic of RNA-binding proteins. Two heterogeneous KH domains (KH1, KH2) and an Arginine-Glycine-Glycine (RGG) box are found within exons 8, 10, and 15, of FMRP, respectively (Ashley et al., 1993). In addition, the amino-terminal of FMRP contains an RNA-binding domain (Adinolfi et al., 2003; Ramos et al., 2006). \textit{In vitro}, FMRP associates with 4% of fetal human brain mRNAs including its own message (Ashley et al., 1993). FMRP is associated with mRNP complexes and translating polyribosomes and can regulate the localization, stabilization and translation of a subset of mRNAs (Khandjian et al., 1996; Siomi et al., 1996; Feng et al., 1997a,b; Brown et al., 2001; Zalfa et al., 2003; Khandjian et al., 2004; Stefani et al., 2004; Zalfa et al., 2007).

Autosomal homologs for FMRP, FXR1P and FXR2P, also bind to 660 kDa mRNP complex with FMRP and several other proteins (Zhang et al., 1995). These two homologs possess the same structural organization as FMRP including the KH domains, the RGG box, NLS, NES, and a homodimerization domain. They are also mainly localized to cytosol, although they can shuttle between the nucleus and the cytosol. FXR2P shows a different nuclear localization than FMRP and FXR1P (Tamanini et al., 2000).

Nuclear shuttling of FMRP might be facilitated by interaction with Ran-binding protein (RanBPM), which is involved in nuclear trafficking (Nakamura et al., 1998; Menon et al., 2004). Furthermore, FMRP interacts with nuclear export factor NXF2 to promote nuclear export (Lai et al., 2006). In the cytoplasm, MicroSpherule protein 58 (MSP58) interacts with FMRP in polyribosomal mRNPs of neurons and escorts it to the somato-dendritic compartment (Davidovic et al., 2006). FMRPs action as an mRNA transporter may occur through an interaction with neurospecific KIF3C kinesin and dendritic RNA granules (Davidovic et al., 2007). Additional binding partners of FMRP in the mRNP complex include nuclear FMRP interacting protein (NUFIP), cytoplasmic FMRP interacting protein 1 (CYFP1) and CYFP2, an 82-kDa FMRP interacting protein (82-FIP), nucleolin, and Y-Box factor 1/p50 (YB1/p50) (Bardoni et al., 1999; Ceman et al., 1999; Ceman et al., 2000; Schenck et al., 2001; Bardoni et al., 2003). Of these,
YB1/p50 has an interesting function, since it is involved in the transcription, transportation, stabilization and translation of mRNAs (Stickeler et al., 2001; Nekrasov et al., 2003). Although, the proteins present in the FMRP mRNP complex have been identified, the content of the complex may vary spatiotemporally (Fig. 8).

**Figure 8** FMRP transports mRNA cargoes from the nucleus to postsynaptic sites in neurons. Adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Bagni and Greenough, copyright 2005.

Post-translational modifications of FMRP have been shown to include methylation and phosphorylation (Ceman et al., 2003; Dolzhanskaya et al., 2006). Methylation is directed to arginine 544 within the RGG box and is postulated to regulate protein-protein and protein-RNA interactions of FMRP (Dolzhanskaya et al., 2006; Stetler et al., 2006), whereas the phosphorylation directed at the N-terminal end of FMRP, is thought to negatively regulate the association of FMRP with actively translating polyribosomes (Ceman et al., 2003).

### 7. The function of FMRP: Regulating the localization, stability and translation of mRNAs

FMRPs RNA-binding properties are thought to be functionally important and many of the mRNAs binding to FMRP have been identified (Ashley et al., 1993; Adinolfi et al., 1999; Brown et al., 2001; Darnell et al., 2001; Chen et al., 2003; Miyashiro et al., 2003; Zalfa et al., 2003) (Fig. 9). These messages encode proteins associated with the function and maturation of neurons, and synaptic plasticity. FMRP-bound mRNAs are both up- and downregulated suggesting a dual role for FMRP as a translation inducer and repressor (Miyashiro et al., 2003). FMRP binds to its target mRNAs via different mechanisms. The RGG box on FMRP binds to a RNA loop structure called G-quartet, which contains four guanines that are stabilized by hydrogen bonds in a planar conformation (Darnell et al., 2001; Zanotti et al., 2006). FXRP1 can modulate the FMRP affinity for the G-quartet.
RNA structure (Bechara et al., 2007). Another structural class of mRNAs binding to FMRP has U-rich sequences that facilitate the interaction (Chen et al., 2003). The KH2 domain of FMRP binds to a distinct RNA structure named loop-loop pseudoknot or kissing complex (Darnell et al., 2005; Valverde et al., 2007), which appears to compete with binding of polyribosomes. Thus, it seems that the KH2 domain may be important in translation regulation by FMRP. Furthermore, an I304N mutation in the KH2 domain of FMRP has diverse consequences including altered RNA-binding activity (De Boulle et al., 1993; Siomi et al., 1994; Feng et al., 1997a; Brown et al., 1998; Lewis et al., 2000), impaired homodimerization (Feng et al., 1997a; Laggerbauer et al., 2001), inability to inhibit mRNA translation in vitro and assembly of 80S ribosomes on target mRNAs (Laggerbauer et al., 2001; Li et al., 2001). In addition, the mutated form of FMRP is faster in shuttling between nucleus and cytosol (Tamanini et al., 1999). A mutation in KH domains of the fruit fly ortholog of FMRP results in partial loss-of-function phenotypes (Banerjee et al., 2007).

Figure 9 The interaction of FMRP with mRNAs. FMRP interacts with a G-quartet structure, b Poly(U) stretches and e kissing complex in the mRNA. Indirect interaction between FMRP and mRNAs can occur through c the small non-coding RNA brain cytoplasmic RNA 1 (BC1) or d microRNAs (miRNA). eIF2C2, eukaryotic translation initiation factor 2C, 2. Adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Bagni and Greenough, copyright 2005 and, with permission, from Annual Reviews of Genomics and Human Genetics, Volume 8 © 2007 by Annual Reviews, www.annualreviews.org.

Another mechanism by which FMRP may regulate target mRNAs is through its association with the microRNA pathway (Caudy et al., 2002; Jin et al., 2004b). MicroRNAs are small noncoding RNAs that inhibit the translation of partially homologous mRNAs by association with an RNA-induced silencing complex (RISC). Before association with RISC, microRNAs are processed from longer RNAs by Dicer enzyme. FMRP associates with microRNAs, RISC proteins as well as with components of
the Dicer microRNA pathway and the mammalian ortholog of Argonite 1 (AGO1) [also knowns as eukariotic translation-initiation factor 2C, 2 (eIF2C2)] (Jin et al., 2004b). This suggests that FMRP participates in translation repression with microRNA pathway components. FMRPs association with the microRNA pathway is further supported by the reported regulation of translation of FMRP interacting CaMKII mRNA by a microRNA mediated mechanism in neurons (Ashraf et al., 2006; Grossman et al., 2006a). There is evidence that the microRNA pathway participates in inhibition of Lim-domain-containing protein kinase 1 (LimK1), which is important for dendritic spine morphology (Schratt et al., 2006). One function of FMRP and microRNA interaction may involve chromatin remodeling in the nucleus since microRNAs regulate chromosome methylation (Jenuwein, 2002; Bao et al., 2004). The Small nonmessenger cytoplasmic RNA BC1 binds to FMRP (Zalfa et al., 2003; Zalfa et al., 2005). BC1 is also associated with mRNAs that are regulated by FMRP. BC1 participates in the formation of a translational inhibition complex with polyA-binding protein and eIF4A (Wang et al., 2002). Although evidence suggests that BC1 is an adapter between FMRP and target mRNAs, FMRP and BC1 are unlikely to be directly associated since BC1 is present in lighter polysome fractions than FMRP (Krichevsky and Kosik, 2001; Zalfa et al., 2005). A novel mechanism for FMRP mediated translational regulation has emerged, since FMRP and AGO2, which are microRNA pathway components, activate translation in a cell-growth-dependent manner through interacting with AU-rich elements at 3’ UTR of target mRNAs (Vasudevan and Steitz, 2007).

Regulation of translation is not the only role for FMRP, since it has also been shown to participate in the transport and stabilization of neural mRNAs. The activation of metabotropic glutamate receptors (mGluRs) increases transport of mRNA-containing FMRP-granules to synaptic sites (Antar et al., 2004; Antar et al., 2005), and recently, FMRP was shown to stabilize postsynaptic density-95 (PSD-95) mRNA in a mGluR-mediated manner via interaction with the 3’UTR of PSD (Zalfa et al., 2007). In the absence of FMRP, transport granule diassembly is increased in response to mGluR stimulation (Aschrafi et al., 2005). The status for FMRP as a transporter is supported by the fact that some of the confirmed mRNA cargoes of FMRP are not located into the dendritic region when the transporter is absent. Furthermore, ZBP1/insulin-like growth factor II mRNA-binding protein1 (IMP1), which participates in mRNA transport in neurons, can interact with FMRP in mRNA granules in vivo (Rackham and Brown, 2004). Binding partners of FMRP, purine-rich single stranded DNA-binding (PUR) proteins and Staufen, are probably participating in mRNA transport and the regulation of translation by interacting with a kinesin motor (Kohrmann et al., 1999; Duchaine et al., 2002; Ohashi et al., 2002; Kanai et al., 2004; Price et al., 2006).

mGluR-dependent protein synthesis in hippocampal slices of Fmr1-knockout (Fmr1-KO) mice is required for epileptiform discharges and these can be blocked by ERK inhibitors (Chuang et al., 2005). Interestingly, since phosphorylation represses the association of FMRP with polyribosomes and the stimulation of mGluR5 decreases the activity of the mRNA target of FMRP, phosphatase PP2A, this may be a mechanism, which regulates FMRP function (Ceman et al., 2003; Castets et al., 2005; Mao et al., 2005). FMRP shifts between polyribosomes and stress granules after neuronal injury by either arsenite or hippocampal electrode insertion (Kim et al., 2006).

Gene expression analysis has revealed that 90 and 73 genes are abnormally regulated in the absence of functional FMRP in human and mouse, respectively (D’Agata et al., 2002; Bittel et al., 2007). In the brain, the greatest changes in gene expression include decreased expression of MAP2 as well as MAP1B, and increased expression of APP and
Hes1 mRNAs. The absence of FMRP has been associated with brain region specific elevations in protein synthesis (Qin et al., 2005) and decreased Fmr1 expression has been observed upon aging in mice (Singh et al., 2007).

FMRP function at the molecular level includes mRNA transport from the nucleus to somato-dendritic synaptic sites, mRNA stabilization, and translational regulation of a subset of mRNAs via several functional interaction sites. FMRP function may be strictly restricted in a spatiotemporal manner.

7.6. mGluR5 and synaptic plasticity in FXS

FMRP is associated with actively translating polyribosomes in neurons and therefore is involved in the regulation of the translation of neuronal transcripts (Khandjian et al., 2004; Stefani et al., 2004). Protein synthesis is particularly important in the cell bodies and dendritic spines in neurons since it is a part of synaptic plasticity related events. Synaptic plasticity can be mediated by receptors for neurotransmitter glutamate, which include ionotropic N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methylsoxazole-4-propionic acid (AMPA) receptors, and G-protein coupled mGluRs (Hollmann and Heinemann, 1994). Two types of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) are important for learning and memory (Malenka and Bear, 2004). Pre- and/or postsynaptic forms of LTP and LTD contribute to the creation, maintenance and elimination of synapses as well as the regulation of synaptic strength. Postsynaptic LTD is generated by the activation of NMDA receptors or group I mGluRs at the postsynaptic site. These two types of postsynaptic forms of LTD both involve increased internalization and consequently decreased surface expression of AMPA receptors (Huber et al., 2000).

The synthesis of FMRP at the postsynaptic site is increased by activation of mGluR1 and/or mGluR5 (Weiler et al., 1997; Hou et al., 2006). The synthesis of FMRP is important, since in cortical neurons FMRP levels increase after light exposure stimulation of dark-reared/light-exposed rats, whereas mRNA levels remain unchanged (Gabel et al., 2004). FMRP-deficient hippocampal Schaffer collateral synapses of the CA1 area and cerebellar parallel fiber to Purkinje cell synapses show increased mGluR-mediated LTD (Huber et al., 2002; Koekkoek et al., 2005). NMDA receptor-mediated LTD is unaffected and mGluR dependent synaptic translation is increased in FMRP-deficient synapses (Huber et al., 2002). Previously, LTP was shown to be unaffected in the hippocampus of Fmr1-KO mice (Godfraind et al., 1996; Paradee et al., 1999; Li et al., 2002; Larson et al., 2005), however recent findings suggest that hippocampal LTP elicited by threshold levels of theta burst afferent stimulation is impaired in Fmr1-KO mice (Lauterborn et al., 2007). LTP in the neocortex is reduced through GluR1 type of AMPA receptor activation (Li et al., 2002). An anterior piriform cortex shows attenuated LTP in FMRP-deficient brain (Larson et al., 2005). LTP seems to be abolished in the anterior cingulate cortex of Fmr1-KO mice (Zhao et al., 2005). In fact, mGluR5-mediated synaptic plasticity is totally absent in the neocortex of Fmr1-KO mice (Wilson and Cox, 2007). Furthermore, the defect in neocortical LTP appears to be caused by compromised calcium signaling (Meredith et al., 2007). In Fmr1-KO brain, LTP is also reduced in the amygdala (Zhao et al., 2005).

These data indicate that FMRP plays a role particularly in mGluR5 dependent synaptic plasticity and inhibits protein synthesis at synaptic sites (Bear et al., 2004). FMRP is located at the postsynaptic sites as well as translated in synaptoneurosomes after
mGluR activation (Weiler et al., 1997; Zalfa et al., 2003). The rapid synthesis of FMRP during initial synaptic activation that is required for mGluR-mediated LTD leads to the ubiquitination and rapid proteosomal degradation of FMRP at the postsynaptic site (Hou et al., 2006). This means that FMRP is normally rapidly degraded to release its binding partner mRNAs which are to be translated upon activation. mGluR5 activation can no longer increase translation in the absence of FMRP, since protein synthesis is already at the maximal level (Nosyreva and Huber, 2006). In the absence of FMRP, target mRNAs may be made readily available for translation in dendritic spines and this could result in the maintenance of synapses in an activity-independent manner (Galvez et al., 2003) (Fig. 10).

**Figure 10** FMRP contributes to the formation of synaptic connections during the brain development. In the absence of FMRP, synapse stabilization is impaired which leads to increased number of immature dendritic spines. Translation of certain mRNAs in the postsynaptic transport granules can be repressed by FMRP. Knockout, *Fmr1*-KO. Adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Bagni and Greenough, copyright 2005.

The following observations may in part explain the enhanced LTD in the absence of FMRP. AMPA receptor expression seems to be impaired in the *Fmr1*-KO brain, since mGluR-dependent (not mGluR5) translation of AMPA receptors and PSD-95 are dysregulated (Muddashetty et al., 2007). Furthermore, an excessive mGluR mediated AMPA receptor internalization occurs in the absence functional FMRP (Nakamoto et al., 2007). The reduced number of mGluR5s appears to be dependent on constitutive Homer proteins in the postsynaptic density of *Fmr1*-KO mice neurons (Giuffrida et al., 2005). In FMRP-deficient cells, the cAMP cascade is altered resulting in reduced production of this second messenger molecule, which may affect mGluR-dependent or mGluR-independent pathways (Kelley et al., 2007). All of these factors may in part contribute to altered synaptic plasticity in the FMRP-deficient brain.

In the light of the currently available data, the role of FMRP in mGluR-mediated synaptic plasticity involves mRNA transport to synaptic sites, mRNA stabilization, and dynamic regulation of local protein synthesis at synaptic sites (Fig. 11).
Figure 11 Function of FMRP in the postsynaptic protein synthesis and synaptic plasticity. 

**a** Translation of FMRP through activation of mGluR receptors can lead to repression of translation of certain synaptic mRNAs. Phosphorylation of FMRP upon mGluR activity may result in phosphorylation of FMRP and release of some mRNAs in a control synapse.

**b** In FXS spine, lack of FMRP may lead to abnormal translational regulation of cytoskeleton regulator proteins, which in turn, has an impact on spine morphology. The absence of FMRP may lead to exaggerated internalization of ionotropic glutamate receptors that affects synaptic plasticity. AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; INT, internalization mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate. Adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Bagni and Greenough, copyright 2005.

**7.7. FXS and BDNF signaling**

BDNF regulates *Fmr1* mRNA expression, since cultured hippocampal neurons display a transient decrease in *Fmr1* mRNA levels after BDNF administration. Furthermore, transgenic mice overexpressing the full-length TrkB receptor have decreased hippocampal expression of *Fmr1* mRNA and FMRP (Castrén et al., 2002). In addition, TrkB expression is increased in the neocortex of adult *Fmr1*-KO mice (Selby et al., 2007). Finally, BDNF infusion can overcome the impaired hippocampal LTP in young adult *Fmr1*-KO mice (Lauterborn et al., 2007). These observations suggest a connection between FMRP expression and BDNF signaling, which may be occurring temporally and in a brain region-dependent manner.
7.8. Mouse models and other animal models to study FXS

An animal model for FXS was required to more thoroughly investigate the neuronal defects seen in patients. Fortunately, the *FMR1* gene is well conserved between species and the murine *Fmr1* nucleotide and amino acid sequences are 95% and 97% homologous to human gene (Ashley et al., 1993). Murine *Fmr1* is expressed with the same temporal tissue specificity as the human version (Hinds et al., 1993; Hergersberg et al., 1995). Consequently, a mouse model for FXS, *Fmr1*-KO, was generated by inserting a neomycin cassette into exon 5 of mouse *Fmr1* gene by homologous recombination which resulted in the loss of FMRP in all tissues (The Dutch-Belgian fragile X consortium, 1994). Although, this model is not an exact representation of the trinucleotide repeat expansion mutation in FXS, it does cause a total loss of FMRP.

An initial characterization of the mouse model revealed macroorchidism, hyperactivity, and a learning deficit all of which are also hallmarks of FXS (The Dutch-Belgian fragile X consortium, 1994). Further characterization, has revealed several symptoms consistent with the modeled disease including spatial learning defect, attention and arousal deficits, mild hyperactivity, altered sensorimotor integration, stress response, susceptibility to audiogenic seizures, and autistic like behavior (Kooy et al., 1996; D’Hooge et al., 1997; Dobkin et al., 2000; Musumeci et al., 2000; Peier et al., 2000; Van Dam et al., 2000; Chen and Toth, 2001; Mineur et al., 2002; Nielsen et al., 2002; Brennan et al., 2006; Markham et al., 2006; Mineur et al., 2006; Moon et al., 2006) (Table 1).

**Table 1 Phenotypes observed in Fmr1-KO mouse model of FXS**

<table>
<thead>
<tr>
<th>Phenotype in Fmr1-KO mouse</th>
<th>Similar phenotype observed in FXS</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal stress response</td>
<td>+</td>
<td>Lauterborn, 2004; Markham et al., 2006</td>
</tr>
<tr>
<td>Alterations in GABAergic system</td>
<td>not shown</td>
<td>Gruss and Braun, 2004; El Idrissi et al., 2005; D’Hulst et al., 2006; Gantois et al., 2006; Selby et al., 2007</td>
</tr>
<tr>
<td>Altered anxiety-related behavior</td>
<td>+</td>
<td>Spencer et al., 2005</td>
</tr>
<tr>
<td>Altered attention and arousal</td>
<td>+</td>
<td>Moon et al., 2006</td>
</tr>
<tr>
<td>Altered sensorimotor integration</td>
<td>+</td>
<td>Chen and Toth, 2001; Nielsen et al., 2002; Yun et al., 2006</td>
</tr>
<tr>
<td>Autistic like behavior</td>
<td>+</td>
<td>Spencer et al., 2005; Mineur et al., 2006; Moon et al., 2006</td>
</tr>
<tr>
<td>Dendritic spine dysmorphism</td>
<td>+</td>
<td>Comery et al., 1997; Braun and Segal, 2000; Nimchinsky et al., 2001; Irwin et al., 2002; Galvez et al., 2003; Galvez et al., 2005; McKinney et al., 2005; Grossman et al. 2006b</td>
</tr>
<tr>
<td>Hyperactivity</td>
<td>+</td>
<td>The Dutch-Belgian fragile X consortium, 1994</td>
</tr>
<tr>
<td>Learning deficit</td>
<td>+</td>
<td>The Dutch-Belgian fragile X consortium, 1994; Kooy et al., 1996; D’Hooge et al., 1997; Paradee et al., 1999; Dobkin et al., 2000; Peier et al., 2000; Van Dam et al., 2000; Mineur et al., 2002; Brennan et al., 2006</td>
</tr>
<tr>
<td>Macroorchidism</td>
<td>+</td>
<td>The Dutch-Belgian fragile X consortium, 1994</td>
</tr>
<tr>
<td>Susceptibility to audiogenic seizures</td>
<td>+ (seizures)</td>
<td>Musumeci et al., 2000; Chen and Toth, 2001</td>
</tr>
</tbody>
</table>
Learning deficits, are an important part of the FXS phenotype, and have been assessed in *Fmr1*-KO using Morris water maze test and radial arm maze both of which gave similar results of defects in reversal phase of the tests suggesting a deficit in spatial learning (The Dutch-Belgian fragile X consortium, 1994; Kooy et al., 1996; D’Hooge et al., 1997; Dobkin et al., 2000; Van Dam et al., 2000; Mineur et al., 2002). There have been some mouse strain dependent difficulties in replicating these results (Fisch et al., 1999; Paradee et al., 1999). In the acquisition of leverpress escape/ avoidance task, which is another type of learning test, *Fmr1*-KO mice perform poorly compared to WT mice (Brennan et al., 2006). In contextual fear conditioning, there is evidence that the *Fmr1*-KO mice perform poorly in this type of learning, although the deficit is probably very mild (Paradee et al., 1999; Peier et al., 2000; Van Dam et al., 2000). The acoustic startle response in *Fmr1*-KO mice is altered, which is a sign of a deficit in brain mechanisms of sensorimotor integration (Chen and Toth, 2001; Nielsen et al., 2002). Interestingly, the development of the startle response is impaired after postnatal week 3 or 4 in *Fmr1*-KO mice (Yun et al., 2006). Some FXS individuals are affected by epileptic seizures and a similar phenotype is also apparent in *Fmr1*-KO mice, which show susceptibility to audiogenic seizures (Musumeci et al., 2000; Chen and Toth, 2001). *Fmr1*-KO mice exhibit autistic like behavior with social withdrawal consistent with FXS, which is one of the best-characterised causes for autism (Spencer et al., 2005; Belmonte and Bourgeron, 2006; Mineur et al., 2006; Moon et al., 2006). In addition, impaired inhibitory control, attention, and arousal regulation are a part of the *Fmr1*-KO as well as the FXS phenotype (Moon et al., 2006). Increased anxiety is one of the features of FXS and recently, the stress response in *Fmr1*-KO mice was shown to be dysregulated by an impaired glucocorticoid negative feedback (Hessl et al., 2004; Lauterborn, 2004; Markham et al., 2006).

Neuroanatomical gross-morphology of the *Fmr1*-KO mouse brain is normal compared to the mild alterations observed in FXS brains (The Dutch-Belgian fragile X consortium, 1994; Kooy et al., 1999). However, structural abnormalities in the GABAergic inhibitory circuits of the adult *Fmr1*-KO mouse neocortex have been found (Selby et al., 2007) alongside with other alterations apparent in an FMRP-deficient GABAergic system (Gruss and Braun, 2004; El Idrissi et al., 2005; D’Hulst et al., 2006; Gantois et al., 2006). The dendritic spine deficit, characteristic of the FXS phenotype, is also present in the visual cortex, the barrel region of somatosensory cortex, olfactory bulb mitral cells and in cultured hippocampal neurons of *Fmr1*-KO mice (Comery et al., 1997; Braun and Segal, 2000; Nimchinsky et al., 2001; Irwin et al., 2002; Galvez et al., 2003; Galvez et al., 2005; McKinney et al., 2005) (Fig. 6). The hippocampal CA1 region of the *Fmr1*-KO brain displays obviously immature spine phenotype, although it is somewhat morphologically different compared to the phenotype in the neocortex (Grossman et al., 2006b). In *Fmr1*-KO mouse, dendritic spine dysmorphism displays a developmentally dynamic pattern. The deficit in spine morphology is present in 1-week-old mice and then disappears at the age of 4 weeks (Nimchinsky et al., 2001). Furthermore, the dendritic spine deficit is again present in 2-month-old *Fmr1*-KO mice brain, suggesting dynamic role for FMRP in regulating dendritic spine shape and synapse formation (Galvez and Greenough, 2005). There is evidence that stabilization, which normally removes excess dendritic spines during development, is impaired in the FMRP-deficient brain (Greenough and Chang, 1988; Galvez et al., 2003; Galvez et al., 2005). This may suggest the occurrence of abnormal increases in excitatory synapses in the FXS brain.

The mouse model for the *Fmr1* autosomal homolog *Fxr2*, *Fxr2*-knockout, has been generated, and shows no pathological defects in brain or testes (Bontekoe et al., 2002). Its
behavioral phenotype includes a learning defect in the Morris water maze task and mild hyperactivity in the open-field test, less contextual conditioned fear, impairment in the rotarod test, less sensitivity to a heat stimulus, and reduced prepulse inhibition (Bontekoe et al., 2002). These features are partially consistent with those of Fmr1-KO mice, and suggest a similar but not identical function for the Fmr1 gene and the Fxr2 gene. Similarities are restricted to impaired spatial learning and hyperactivity which are seen in both of these models. The double knockout mouse Fmr1/Fxr2 exhibits an exaggerated phenotype suggesting that these homologous genes cooperate in regulating locomotor activity, sensorimotor gating and cognitive processing (Spencer et al., 2006). The other autosomal homolog of FMR1, FXR1, has also been knocked out in mice (Mientjes et al., 2004). Fxr1-knockout mice display a surprisingly different phenotype compared to Fmr1-KO mice, as they have abnormalities in muscle development, which leads to early neonatal death by respiratory or cardiac failure. This implies that the Fxr1 gene may have a similar function to the Fmr1 gene in the regulation of mRNA transport and/or translation but in muscle instead of neurons. Recently, a conditional Fmr1-KO mouse model was created by lox P sites flanking the promoter and the first exon of Fmr1 but the characterization of this model is still incomplete (Mientjes et al., 2006).

In Drosophila melanogaster, a structurally and functionally well-conserved ortholog for the human FMR1 gene, the dFMR1 gene, exists (Wan et al., 2000). Several groups have independently established null alleles of the dFMR1 gene, which have provided great deal of additional insight into the molecular biology of FXS (Zhang et al., 2001; Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002). As in humans, a mutation in the dFMR1 gene in Drosophila causes neuroarchitectural and behavioral defects (Zarnescu et al., 2005; Zhang and Broadie, 2005).

To summarize, many animal models created to study FXS, have been and will continue to be very useful in elucidating the molecular, physiological, cognitive, and behavioral phenotypes caused by a deficit in FMRP.

7.10. Regulators of G-protein signaling (RGS)s and FXS

RGS proteins are a large family of similar proteins (>30 members), which regulate G-protein coupled receptor-mediated signaling through direct binding to activated Gα subunits (Hollinger and Hepler, 2002; Willars, 2006). This binding leads to inhibition of downstream signaling by neurotransmitters and Ca^{2+} induced stimulation of the GTPase activity of the Gα subunits. Some of the RGS proteins can also act as effector antagonists and guanine nucleotide dissociation inhibitors (Willars, 2006). In addition to regulating the G-protein signaling, structurally diverse RGS proteins can have other functions by interacting with phospholipids, effectors, scaffolds, and receptors (Willars, 2006). RGS proteins play a part in processes such as cell growth, differentiation, cell motility, and intracellular trafficking (Hollinger and Hepler, 2002). The members of RGS protein family that are enriched in the telencephalon include RGS2 and RGS4 (Gold et al., 1997; Ingi et al., 1998; Ingi and Aoki, 2002; Grillet et al., 2003; Ebert et al., 2006). The main difference between these two members of the same protein family is that RGS4 binds to Gqα and Gzi subunits, whereas RGS2 almost exclusively and potently binds to Gqα subunits (Hepler et al., 1997; Heximer et al., 1997; Ingi et al., 1998). However, RGS2 regulates presynaptic Ca^{2+} channel activity by inhibiting Gzi subunit activity (Han et al., 2006). In the brain, RGS2 has been implicated in the control of various stress responses,
and in the proper development of dendritic spines and synapses in the hippocampal CA1 region (Oliveira-Dos-Santos et al., 2000). In some cells, RGS4 binds to the Gzq/11 subunit to modulate the frequency of IP3-mediated Ca^{2+} oscillations (Xu et al., 1999; Luo et al., 2001). In synaptic transmission, RGS4 plays a role as a GTPase activator for numerous G-protein coupled neurotransmitter receptors including δ- and μ-opiate, adrenergic α2A, muscarinic M1-3, mGluR1 and 5, and 5-HT1A/2A (Traynor and Neubig, 2005). Moreover, RGS4 is especially implicated as one of the susceptibility genes for schizophrenia since there is evidence that it can be absent or downregulated in the brain of schizophrenic patients (Mirnics et al., 2001; Levitt et al., 2006). FXS and schizophrenia exhibit some commonalities (Sobesky et al., 1994). Interestingly, RGS4 seems to inhibit G-protein coupled signaling through mGluR5, which is aberrant in FXS (Saugstad et al., 1998; Bear et al., 2004). Furthermore, Homer proteins can stimulate RGS4 expression and mGluR5 aberrances have been associated with Homer proteins in FXS (Shin et al., 2003; Giuffrida et al., 2005). Therefore, since RGS4 and mGluR5 appear to be closely associated, a connection between them may exist in FXS pathology.

### 7.11. Treatment for FXS patients

The therapy for FXS includes both behavioral and physical components of the symptoms of individual patients including treatments with stimulants, 2-adrenergic agonists, antidepressants, anticonvulsants, and antipsychotics (Berry-Kravis and Potanos, 2004). There is no treatment to replace the absent FMRP expression, yet. Inhibitors of methylation and histone deacetylases have activated the FMR1 gene to some extent (Chiurazzi et al., 1999) but it seem that long full-mutation transcripts are poorly translated (Feng et al., 1995). Furthermore, attempts to reactivate the FMR1 gene could affect other genes in vivo with unpredictable consequences. Regardless of the setbacks in FMR1 gene activation, our increased understanding about FMRP function in synaptic plasticity has brought possibilities for new pharmacological interventions in FXS. Since FMRP is a suppressor of mGluR5 activity mediated translation at postsynaptic sites, an exaggerated mGluR dependent protein synthesis is prone to occur in the absence of FMRP (Bear et al., 2004). Activity of mGluR5 can be prevented by administration of a specific antagonist 2-methyl-6-(phenylethynyl) pyridine (MPEP). The efficiency of MPEP in rescuing phenotypes in FMRP-deficient animal models has been already demonstrated. In Fmr1-KO mice, MPEP treatment restored open-field activity to normal and reduced audiogenic seizure susceptibility (Yan et al., 2005). MPEP improved the neurite branching defect in zebrafish model of FXS (Tucker et al., 2006). In the Drosophila model of FXS, MPEP ameliorated the memory defect in experience-dependent courtship behavior and abolished the defect in neuronal mushroom bodies (McBride et al., 2005).

Recently, a new candidate for therapy in FXR has emerged since inhibition of PAK activity in Fmr1-KO mice seems to ameliorate a large part of the functional, neuroanatomical, and behavioral phenotypes in these mice (Hayashi et al., 2007). Fully or partially rescued phenotypes include aberrant dendritic spines, reduced neocortical LTP, altered locomotor activity, steroetypy, and altered trace fear conditioning. FMRP and PAK interaction appears to be mediated through the KH2 domain.

It should be noted that simple stimulatory environment and normal social contacts may be an important part of improving the individual FXS patients’ condition, since an
enriched environment improves behavioral and even neuroanatomical phenotypes in *Fmr1*-KO mice (Restivo et al., 2005).

The GABAergic system seems to be affected when FMRP is lacking. In the neocortex of the *Fmr1*-KO mouse, GABAA receptor expression is decreased as well as expression of some GABAergic neuron subtypes. Drugs acting on GABAA receptors are readily available and they have been applied to ameliorate symptoms for example in Down’s syndrome. Therefore, agonists for GABAA receptors may provide therapeutic intervention in FXS (D’Hulst and Kooy, 2007).

These data indicate that some of the symptoms in FXS are a consequence of a defect in mGluR5-mediated signaling. In addition to pharmacological blockade of mGluR5, signaling pathways downstream of mGluR5 could also provide a target for drug interventions in FXS. Inhibition of PAK may provide a novel means for drug intervention in FXS.
8. AIMS OF THE STUDY

The overall aim was to investigate the intracellular mechanisms, which influence fate determination of neural stem cells (NSCs). This was studied by:

1. *In vitro* and *in vivo* survival, proliferation, and differentiation of NSCs in fragile X syndrome

2. Embryonic and early postnatal formation of the FMRP-deficient neocortex

3. The effect of impaired BDNF/TrkB signaling on *in vitro* survival, proliferation, and differentiation of NSCs
9. MATERIALS AND METHODS

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

FVB Fmr1-KO and their wild-type (WT) littermates were used in publications I, II, III (The Dutch-Belgian fragile X consortium, 1994). C57BL/6J Fxr2-KO mice and their WT littermates were used in publication I (Bontekoe et al., 2002). Heterozygous transgenic mice overexpressing a truncated TrkB.T1 receptor under the Thy1 promoter and their WT littermates were used in publication IV (Saarelainen et al., 2000b). Animals were genotyped from tail samples with PCR amplification by specifically designed primers for each transgenic animal strain.

Animals were provided with food and water ad libitum and kept on a 12/12 h light/dark cycle at room temperature (~23°C) in an accredited animal facility in accordance with National Institutes of Health guidelines. All animal experiments were conducted according to the guidelines of The Society for Neuroscience and were approved by the Experimental Animal Ethics Committee of the National Laboratory Animal Center, Finland.

9.2. Mouse brain tissue (I, II, III, IV)

Before the sacrificing, the mice were anaesthetized with CO\textsubscript{2}. Adult mice were sacrificed with cervical dislocation and the pups by decapitation. Brain tissue was obtained at the following embryonic and postnatal (P) ages in each publication: E13 (II), E15 (IV), E16 (III), E17 (II, III), P0 (III), P5 (III), P6 (II), P7 (IV), P10 (I), and adult (I, II). Fresh brain tissue was used for generating NSC cultures (II, IV) but was fixed in 4% paraformaldehyde (PFA) for frozen or paraffin sectioning (I, II, III).

9.3. Human brain tissue (II)

Fetal human brain tissue was obtained from terminated pregnancies and the age of the fetus was determined by intrauterine ultrasound examinations. Human NSC cultures were generated from an 18-week-old fragile X fetus with a methylated repeat expansion of 276 to 300 trinucleotide repeats in the 5’ UTR region of the FMR1 gene and from control fetuses at 7, 12, and 18 weeks of gestation.

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. Fully informed consents were obtained. A mutation in the FMR1 gene was detected by polymerase chain reaction (PCR) and Southern analysis.
9.4. BrdU injections to analyze cell proliferation (II, III)

Pregnant heterozygous Fmr1 female mice received a single series of four intraperitoneal injections of BrdU (50-100 mg/kg; Sigma) at E13 or E14 on a single day during 12-h time span to label S-phase cohort of newborn cells.

9.5. Tissue processing (I, II, III)

All brains were collected and fixed in 4% paraformaldehyde overnight at 4°C. Brains were processed either for frozen sectioning or paraffin sectioning. For frozen sectioning, brains were washed twice in PBS after fixation and then cryoprotected in 30% sucrose solution over night at 4°C before the freezing. Brains were cut into 14 μm (I) or 20 μm (II, III) coronal sections with a microtome or a vibratome and thaw-mounted onto object glasses. For paraffin sectioning, brains were washed twice in PBS after fixation. Then brains were dehydrated through the following alcohol series: 2 h in both 70% ethanol and in 96% ethanol, and 100% ethanol over night at 4°C. This was followed by treatment with xylene (twice for 2 h) before immersion in paraffin over night at 60°C. The next day, brains were molded into paraffin and then cut with a rotatory microtome into 20 μm (III) coronal sections onto object glasses.

9.6. NSC culturing (II, IV)

NSCs were isolated and propagated from the walls of the lateral ventricles of embryonic and postnatal brain with a previously described method (Clarke et al., 2000). NSCs were derived from animals of different ages: E13 (II), E15 (IV), P6 (II), and P7 (IV). Tissue was dissected from the brains, incubated in Hank's balanced salts solution containing 1.33 mg/ml trypsin, 2 mM glucose, 0.7 mg/ml hyaluronidase and 0.2 mg/ml kynurenic acid (30 min, 37 °C), and dissociated mechanically. Cells were centrifuged at 200 x g for 5 min, resuspended in 0.9 M sucrose in 0.5× Hank's balanced salts solution, and centrifuged for 10 min at 750 x g. The cells were then resuspended in 2 ml Earl's balanced salts solution and centrifuged through a gradient of the same salt solution with 4% bovine albumin at 200 x g for 7 min. Dissociated cells were plated in the DMEM-F12 culture medium (Life Technologies) containing 2 mM L-glutamine, 15 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, B27 supplement, 20 ng/ml epidermal growth factor (EGF, Life Technologies), and 10 ng/ml basic fibroblast growth factor (FGF2, PeproTech EC Ltd, London, UK). Mitogens EGF (20 ng/ml) and FGF2 (10 ng/ml) were added every third day, and 50% of the medium was refreshed every 3 to 4 days. The resulting clusters of NSCs/NPCs termed neurospheres were passaged every 7 to 9 days with papain treatment (0.5 mg/ml, Sigma).

9.7. Transduction of NSCs/NPCs with lentivirus vectors (IV)

Viral transduction has been described as viable method to express transgenes in NSCs (Englund et al., 2000; Falk et al., 2002; Consiglio et al., 2004). Mouse NSCs/NPCs from dissociated neuropsheres were transduced with a recombinant lentivirus (LV) expressing.
TrkB.T1 tagged with Flag and green fluorescent protein (GFP) driven by the CMV promoter (lenti-CMV-Flag-TrkB.T1-ires-GFP; TiG4) and control viruses expressing enhanced GFP (lenti-CMV-EGFP; G3) or Flag- and GFP-tagged full-length TrkB receptor (lenti-CMV-Flag-TrkB.FL-ires-GFP; FiG4). The multiplicity of infection was 10 to 40.

9.8. In utero electroporation and transgene delivery into the fetal mouse brain (III)

In utero electroporation was carried out as described earlier (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). Previously described expression plasmids, FMRP with I304N mutation in the KH2 domain with EGFP (FMRPmt-EGFP) (1.3 μg/μl), and overexpressing FMRP with EGFP (FMRP-EGFP) (1.3 μg/μl) were delivered into the mouse brain (Castrén et al., 2001). The expression plasmid encoding EGFP (1 μg/μl) was used as a control. Brains were collected at E17.

9.9. In situ hybridization (I, II)

In situ hybridization was done as described previously (Wisden and Morris, 1994) with complementary oligonucleotide probes for mouse Rgs4, Rgs2, and Fmr1 mRNA. The probes were 3' end-labeled with [α-32P]dATP (2000 Ci/mmol, New England Nuclear, Boston, MA). Sense and scramble oligonucleotide sequences were used as negative controls. Hybridization was performed overnight (42°C) on postfixed sections with 1 x 10^3 to 3 x 10^3 cpm/ml of labeled probe in buffer containing 50% formamide, 4 x standard saline citrate (SSC), 10% dextran sulfate, and 10 mM DTT. After overnight hybridization, sections were briefly immersed in 1 x SSC at RT followed by a wash for 30 min at 50°C in 1 x SSC. This was followed by washes in 1 x SSC, 0.1 x SSC, 70% ethanol, and 94% ethanol for 3 min each at RT. Hybridized sections were exposed to Kodak Biomax MR films (I) (Kodak, France) or Hyperfilm-ßmax films (II) (Amersham Biosciences/GE Healthcare) for 3 to 6 weeks. In publication I, quantification of autoradiograms was performed with MCID image analysis system (Imaging Research, St. Catharines, Ontario, Canada), and optical densities were converted to specific radioactivity (nCi/g) with 14C microscales (ARC, St. Louis, MO).

9.10. NSC proliferation in vitro (II, IV)

9.10.1. Clonal analysis of NSCs/NPCs (IV)

For clonal analysis, NSCs/NPCs were derived from E15 embryos overexpressing TrkB.T1 transgenic and their WT littermates. Neurospheres at the second or seventh passage were cultured for 7 days and dissociated with papain into a single cell suspension. After dilution, single progenitors were plated in 10-μl aliquots at a clonal density of 1-2 cells per well into 96-well plates containing 100 μl of culture medium containing normal concentrations of mitogens (EGF and FGF2). In addition to normal mitogens, some of the
Cell cultures contained BDNF (10 ng/ml; PeproTech). After 24 h, plates were scored and the wells containing a single cell were marked. Fresh culture medium (40 µl) was added to the plates following 11 days in culture. Plates were finally screened for the presence of spheres at day 13 from the start of the culture.

For low-density analysis, 20 cells/100 µl/well were plated onto a 96-well plate. The number of spheres was counted after 4 days and the size of the spheres was measured after 6, 8 and 10 days in vitro.

9.10.2. $^3$H-thymidine incorporation (II, IV)

Cell proliferation was studied by measuring the incorporation of $^3$H-thymidine during the S-phase of the cell cycle. Cells were plated at a density of 100 000 cells/ml in DMEM-F12 culture medium supplemented with mitogens (EGF and FGF-2). After one day of incubation with $^3$H-thymidine (0.5 µCi/ml, Amersham Biosciences/GE Healthcare), cells were precipitated with 50 µl/ml trichloric acid for 20 min at 4°C, solubilized in 80 µl of 1 M NaOH for 20 min at room temperature (RT), and neutralized with 250 µl of 1 M HCl. The radioactivity of the complete lysate was measured with scintillation fluid (OptiPhase HiSafe 3) in a liquid scintillation counter (1450 MicroBeta, Wallac).

9.10.3. Cell viability: MTT assay (IV)

MTT is a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], which forms a formazan dye only in metabolically active cells. This allows a quantitative analysis of viable cells in the presence of MTT. Cells were plated at a density of 10 000 cells/100 µl onto 96-well tissue culture plates and cultured as floating neurospheres. After specific times in culture, cells were incubated with MTT (1 mg/ml; Sigma) for 2 h in a cell culture incubator (37°C, 5% CO$_2$, humid). The reaction was stopped by adding 100 µl of a solution composed of 20% sodium dodecyl sulphate in 50% N,N-dimethyl formamide, pH 4.7. Cells were incubated overnight to completely solubilize the formazan dye crystals. The absorbance value of the solution was measured in a spectrophotometer at 570 nm. Assays were performed with eight replicates.

9.11. Differentiation of NSCs in vitro (II, IV)

In general, neurospheres and papain-treated single cells (100 000 cells/ml) were plated on cover glasses coated with poly-D-lysine (10 µg/ml; Sigma) and laminin (5 µg/ml; GIBCO/BRL) in culture medium without mitogens. In publication II, plated cells were allowed to differentiate for 1, 5, 7, 14, 21 days in culture. For apoptosis studies in publication IV, cells were differentiated at the same density on poly-D-lysine-coated cover glasses for 4 or 7 days. When assessing the number of cells positive for proliferation marker Ki67 (IV), dissociated cells were allowed to attach for 6 h on poly-D-lysine-coated cover glasses before fixation.
9.12. RNA extraction and real-time quantitative PCR (IV)

Total RNA was extracted with Trizol reagent (Life Technologies Ltd), treated with DNAse I at 37°C for 45 min, and inactivated at 65°C for 10 min. RNAs were quantified according to optical density. cDNA was synthesized from 1 µg of the total RNA with oligo(dT)12-18 primers and Multi Scribe reverse transcriptase enzyme (Applied Biosystems). Real-time quantitative PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems) using the GeneAmp Gold RNA PCR reagent kit (Applied Biosystems) with 10 ng of total cDNA for each sample in 25 µl of PCR reaction mixture. The product was amplified up to 33 PCR cycles, after uracil removal (2 min at 50°C) and polymerase activation (9.5 min at 96°C). One PCR cycle consisted of denaturation for 30 s at 94°C and annealing/extension for 1.5 min at 62°C. The amplification specificity of the PCR products was confirmed with agarose gel electrophoresis. Quantitative results were provided and analyzed by Sequence Detection Systems 1.9.1 (Applied Biosystems) analysis software. The baseline for each reaction was equalized by determining the mean value of the 3 to 15 cycles of the lowest measured data points for each sample and subtracting this from each reading point. Glyceraldehyde-3-phosphate dehydrogenase and 36B4 acidic riboprotein were used as internal references and to normalize the data. All assays were run in triplicate.

9.13. Ca²⁺ imaging (II)

Intracellular Ca²⁺ measurements were performed as described previously (Larsson et al., 2005). After differentiation overnight, neurosphere-derived cells were incubated (20 min at 37°C) with 4 µM fura-2 acetoxymethyl-ester in a Na⁺-Ringer solution (pH 7.4) consisting of 137 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 10 mM glucose, 1 mM probenecid, and 20 mM HEPES. After incubation, the cover glasses were attached to the bottom of a thermostat heated perfusion chamber (37°C). Between 10 to 50 of the single layer of cells at the edge of the neurospheres, were simultaneously monitored by 340 and 380 nm light excitation using a filter changer under the control of an InCytIM-2 system (Intracellular Imaging, Cincinnati, OH) and dichroic mirror (DM430, Nikon). The emission was measured through a 510-nm barrier filter with an integrating charge-coupled device camera. Cells attached to cover glasses were bathed at a rate of 2 ml/min with the Na⁺-Ringer solution containing the different substances tested when specified. When the high K⁺ concentration solution was used to stimulate the cells, the Na⁺-Ringer solution was mixed with a Ringer solution in which the NaCl had been replaced with KCl to achieve the proper extracellular K⁺ concentration (70 mM).


Apoptosis was detected using the terminal transferase-mediated dUTP nick end labelling (TUNEL) method. The DeadEnd™ Colorimetric TUNEL system in situ apoptosis detection kit (Promega) was employed according to the manufacturer's instructions. Fixed neurosphere sections, differentiated NSCs, and differentiated neurosphere cells were
rinsed with PBS and permeabilized with ice-cold 100% methanol before the TUNEL labeling.

9.15. Immunohistochemistry (II, III, IV)

In publication II and IV, cultured cells on the cover glasses were fixed with 4% PFA in PBS, pH 7.4, for 10 min and permeabilized with ice-cold 100% methanol for 20 min. After blocking with PBS containing 20% normal goat serum (II, IV) or rabbit serum (IV) for 20 min, cells were incubated with primary antibodies from 1 h at 37°C to over night at 4°C, and secondary antibodies from 45 min to 1 h at RT (Table 2). For the immunocytochemical staining of neurosphere cells in publication IV, neurospheres were cultured for 8 to 10 days after dissociation, fixed with 4% PFA in PBS, pH 7.4 at RT for 1 h, and cryoprotected by an overnight incubation in 10% sucrose solution at 4°C. The next day, neurospheres were suspended in Tissue-Tek mounting compound (Sakura Finetek, Zoeterwoude, The Netherlands) and frozen quickly in dry ice. 14-µm microtome sections of neurospheres were processed for immunocytochemistry. For Ki67 staining in publication IV, cells were carefully fixed with 2% PFA for 5 min and 4% PFA for 15 min at RT before permeabilization. When biotinylated anti-Flag was used (IV), endogenous biotin background staining was eliminated with Endogenous Biotin-Blocking kit (Molecular Probes) according to the manufacturer's instructions. Cover glasses were rinsed and mounted with SlowFade antifading reagent (Molecular Probes) (II, IV).

In publications II and IV, the immunoperoxidase reaction was developed using ammonium nickel sulphate (0.015%)-intensified 0.3% 3,3′-DAB as a chromogen, giving a blue-to-black granular reaction end product (0.005% H₂O₂).

For BrdU detection in publication II, sections were treated with 1% H₂O₂ to remove the endogenous peroxidase activity and immunohistochemical staining was performed with biotin-streptavidin detection reagents and conjugated peroxidase (Boehringer Mannheim) or fluorescent detection.
Table 2  Antibodies in the immunohistochemical stainings. * = dilution made according to manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Provider</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-actNotch1 (rabbit)</td>
<td>1:100</td>
<td>Abcam</td>
<td>III</td>
</tr>
<tr>
<td>anti-BLB P (rabbit)</td>
<td>1:1000</td>
<td>Chemicon</td>
<td>III</td>
</tr>
<tr>
<td>anti-BrdU-kit (mouse)</td>
<td>*</td>
<td>Amersham/GE Healthcare</td>
<td>II, III</td>
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<td>III</td>
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<td>IV</td>
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<td>II, IV</td>
</tr>
<tr>
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<td>1:500</td>
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<td>BabCO</td>
<td>II, IV</td>
</tr>
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</table>

In publication III, frozen sections were incubated in 1:1 ethanol/chloroform solution to remove fat from the brain tissue and re-hydrated through a descending alcohol series before the Nissl staining. For Nissl staining, sections were incubated in cresyl violet solution (Sigma) for 30 to 60 s and washed briefly twice with distilled H₂O. Sections were immersed in 70% ethanol for 5 min and in 96% ethanol for 10 min, and dehydration was done by immersing the sections in 100% ethanol and xylene both twice for 5 min. Sections were mounted with DePex medium (BDH Laboratory Supplies, England). Paraffin sections were deparaffinized sequentially three times in xylene for 10 min, and thereafter, re-hydrated in 100% ethanol, 96% ethanol, 70% ethanol and distilled H₂O for 5 min each. For antigen retrieval, sections were boiled in 10 mM citrate buffer (pH 6.0) for 15 min. Permeabilization and background blocking was done by treating sections with a solution of 0.5% Triton-X 100 and 20% normal goat serum in PBS for 1 h at RT. Thereafter, sections were incubated with primary antibodies over night at 4°C and with secondary antibodies for 1 h at RT (Table 2). Sections were mounted with Gel Mount media (Sigma). The colorimetric staining of sections was performed with the Vectastain ABC kit (Vector Laboratories) and the DAB-substrate kit for peroxidase including nickel (Vector Laboratories) according to manufacturer’s instructions.

Nuclei were counterstained with 4’,6-diaminodino-2-phenylindole (DAPI) (II, III, IV) (0.1 µg/ml, Boehringer Mannheim Biochemica) or hematoxylin (II, III).
9.16. Brightfield, epifluorescence and laser scanning confocal imaging (II, III, IV)

Live cell imaging was performed by using a CC-12 digital camera (Soft Imaging System, Lakewood, USA) under an Olympus IX70 (Olympus, Tokyo, Japan) inverted microscope. Immunocytochemically stained cells were imaged with an epifluorescence Axioplan 2 microscope system (Zeiss, Jena, Germany) connected to a high-resolution AxioCam camera (Zeiss) and AxioVision 4.1 (Zeiss) software. Alternatively, high-resolution digital camera with epifluorescence microscope Olympus Provis (Olympus) or Zeiss N HBO100 (Zeiss) were used. Laser scanning confocal microscopy was performed with the LSM 5 Pascal system (Zeiss) or Leica TCS SP2 AOBs (Leica). To obtain high resolution images from brain sections and to perform stereological analyses, a digital MicroFire S99808 camera (Optronics, CA, USA) attached to Olympus BX51 epifluorescence microscope (Olympus) were applied.

9.17. Analysis of the imaging data (II, III, IV)

To count cell numbers, determine the diameters of cells and neurospheres, and to measure neurite lengths, images were analyzed using AIDA (version 3.44, Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany) or Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD, USA) or Neurolucida Software (NeuroBright Field, VT, USA). In \textit{in vitro} experiments, measurements were performed from images of uniformly plated cells on cover glasses or from plated neurospheres with uniform size and/or propagation period (II, IV). Differentiated viable and apoptotic neurons in publications IV, were counted directly under a microscope from cover glasses after the cells were fixed and immunostained. Brightness and contrast were optimized before counting.

For semiquantitative analyses of brain sections in publication III, cells were counted in the dorsolateral neocortex as described previously with minor modifications (Fukumitsu et al., 2006). The coronal somatosensory neocortical level at E16 was approximately 860 μm posterior to the rostral border of the olfactory bulbs. At E17, the analyzed cortical level was approximately 1.3 mm posterior to the rostral border of the olfactory bulbs. Cells in the neocortical sections were counted from images of cortical columns with defined dimensions or from confocal images with defined size.

9.18. Stereological analyses (II, III)

Stereological analyses of brain sections were performed with the Stereo Investigator software (MicroBrightField, Inc., Vermont, USA) attached to Olympus BX51 (Olympus) epifluorescence microscope with MicroFire S99808 digital camera (Optronics). In publication II, randomly selected serial sections through the anterior part of the lateral ventricles of WT and \textit{Fmr1}-KO embryonic brains at E17 were analyzed stereologically to assess BrdU-labeled newborn cells. The final counting was performed with 100x oil objective. In publication III, the stereological assessment of ER81-positive cells at P5 was done on cortical coronal serial sections spanning Bregma 1.18 mm to -0.94 mm approximately. Optical dissector counting frame dimensions were 60 μm x 60 μm x 20
µm (20 µm is z-axis) and the sampling grid dimensions were 130 µm x 120 µm both of which were optimized for cell counting which was performed with a 60x oil objective (Olympus).


Data was analysed using Student's t-test (I, II, III, IV), ANOVA (II, IV), and the Mann-Whitney test (II, IV). P values ≤ 0.05 were considered to be statistically significant.

10. RESULTS

10.1. Expression of Rgs mRNAs in the Fmr1-KO mouse brain (I)

We examined the expression of genes encoding Rgs proteins, which are tightly regulated modulators of G-protein signaling (Hollinger and Hepler, 2002), and thus can have an effect in mGluR mediated signaling as well as intracellular Ca\(^{2+}\) release. The expression of neuronal transcripts of the Rgs family, Rgs2 and Rgs4, were investigated in the brain of Fmr1-KO and Fxr2-KO mice by in situ hybridization. We initially chose to study the expression of these Rgs mRNAs at P10 when the dendritic spine abnormalities are present in the Fmr1-KO brain. At this time point, we found abundant expression of Rgs4 mRNA in the distinct neocortical and hippocampal regions of the WT mouse brain, which was consistent with previous observations (I, Fig. 1A) (Ingi and Aoki, 2002). We found that the Rgs4 mRNA levels were significantly decreased in the CA1 region of the hippocampus and the retrosplenial cortex of the Fmr1-KO brain compared to the control (I, Fig. 1A). This may suggest a developmental delay, since the temporally uniform expression pattern of Rgs4 in the postnatal piriform cortex was unchanged in Fmr1-KO mice brain (I, Fig. 1B). In the adult mouse retrosplenial cortex and hippocampus, Rgs4 mRNA levels showed no significant differences between Fmr1-KO and WT mice, although, a trend toward decreased Rgs4 levels in the adult Fmr1-KO brain could be observed. Another Rgs family transcript, Rgs2 mRNA was expressed predominantly in the hippocampal pyramidal neuron layer, in the granule neuron layer of the dentate gyrus, and in the superficial layers of the neocortex. The expression pattern of Rgs2 was normal in the brain of P10 Fmr1-KO mice suggesting Rgs4-specific alterations in the absence of FMRP (I, Fig. 2) (Ingi and Aoki, 2002).

To examine whether Rgs4 mRNA expression was specifically reduced in the FMRP-deficient brain, we studied the expression of Rgs4 in brain sections of the Fxr2-KO mice, which lack FMRP homolog FXR2P and have a partially congruent behavioral phenotype with Fmr1-KO mice (Bontekoe et al., 2002). FMRP and FXR2P may be able to fill each others place, since they have highly overlapping expression patterns in the brain and interact in the same protein complexes (Zhang et al., 1995; Bakker et al., 2000). Rgs4 levels in the brain of Fxr2-KO mice were normal (I, Fig 3) further supporting a specific defect in Rgs4 expression in the absence of FMRP.
10.2. Differentiation of mouse and human NSCs in the absence of FMRP (II)

FMRP expression is highest during early murine development (Abitbol et al., 1993). We found substantially high FMRP expression in the embryonic as well as adult mouse brain at the periventricular region, which contains self-renewing NSCs throughout the mouse life span (II, Fig. 1A).

We compared the differentiation of NSCs derived from *Fmr1*-KO mice to WT NSCs. After 5 days of differentiation on coated cover glasses, the neurospheres derived from *Fmr1*-KO brains at E13 and P6 produced 3-fold and 4.5-fold more TuJ1-positive neurons than the WT neurospheres did (II, Fig. 1B). Furthermore, TuJ1-positive *Fmr1*-KO cells exhibited a reduced number of primary neurites compared with WT cells after 1 day of differentiation (II, Fig. 1C and D). The maximum length of the neurites was significantly shorter and the cell-body volumes were smaller in *Fmr1*-KO cells than in WT cells after differentiation for 1 or 5 days (II, Fig. 1D). When observing the glial differentiation of NSCs, we found that the number of GFAP-expressing cells was significantly reduced among the differentiated cells derived from E13 or P6 *Fmr1*-KO mice compared with the respective controls (II, Fig. 1E). The number of apoptotic TUNEL-positive cells was increased in differentiating TuJ1-negative *Fmr1*-KO cells reflecting the reduced amount of differentiated GFAP-positive astrocytes among these cells (Fig. 1F). Proliferation was unaltered in the neurosphere cultures derived from WT and *Fmr1*-KO brains (II, Fig. 1G and H). The data suggest that the absence of FMRP directed the differentiation of mouse NSCs toward neuronal lineage.

We also isolated and propagated human NSCs from postmortem tissue of a FXS fetus and control fetal brains (II, Fig 4A). The FXS derived NSCs showed no immunoreactivity for FMRP in contrast to control human NSCs (II, Fig. 4B). The differentiation of these NSCs could be induced by removal of mitogens (II, Fig 4A), and upon differentiation FMRP labeling was predominantly present in GFAP-negative neuronal cells (II, Fig. 4C) (Devys et al., 1993). We compared the differentiation of NSCs generated from the 18-week-old fetal FXS brain with age-matched control cells. During the first day of differentiation, FXS neurosphere cells generated shorter radial processes and more TuJ1-positive cells than controls (II, Fig. 4D). After 14 days of differentiation, 5.3-fold more TuJ1-positive cells and 70% less GFAP-positive cells were generated from FXS neurosphere cells than from the age-matched control neurosphere cells (II, Fig. 5A and B). The increased number of TuJ1-positive and reduced number of GFAP-positive cells differentiated from FXS NSCs were also observed when compared to control NSCs derived from human fetal brains at different developmental stages. Consistent with the morphological alterations of newborn TuJ1-positive cells derived from *Fmr1*-KO mouse neurospheres, the average neurite length and cell-body volume of the TuJ1-positive cells derived from FXS neurospheres were significantly reduced compared with the age-matched fetal control (II, Fig. 5C).

10.3. Ca$^{2+}$ responses of differentiating NSCs derived from FMRP-deficient brain (II)

An intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) response to acetylcholine (ACh) during the first day of NSC differentiation has been shown previously (Ma et al., 2000). Almost all of the mouse cells we measured responded to ACh (II, Fig. 2A). Cells were treated with glutamate with or without extracellular Ca$^{2+}$ to assess the presence of mGluRs and ionotropic glutamate
receptors. Elevated K⁺ concentration (70 mM) was used to demonstrate the presence of voltage-gated Ca²⁺ channels in these cells. Three different populations in both Fmr1-KO and WT cells could be distinguished based on their response to neurotransmitters. Approximately 30% of the cells responded to glutamate in the absence of Ca²⁺ with a metabotropic type of response but showed a minor response to K⁺ (type I cells) (II, Fig. 2A). Another cell population, which did not display metabotropic [Ca²⁺] response to glutamate, responded to increased K⁺ concentration with a robust rise in [Ca²⁺] (type II cells) (II, Fig. 2B). The remaining cell population had minor or no metabotropic responses to glutamate and K⁺ (type III cells). Interestingly, type I cells usually displayed oscillations with a frequency of 4 to 6 spikes/min in response to ACh and glutamate. The oscillatory response for 100 µM ACh was significantly accentuated in the Fmr1-KO mouse cells compared with WT cells (II, Fig. 2C). These cells also exhibited metabotropic response to glutamate (II, Fig. 2D). To investigate which mGluR subtype was responsible for the [Ca²⁺], elevation in type I cells, antagonists for the mGluR1 and mGluR5 subtypes were administered with different concentrations of glutamate. The challenge of cells with low concentrations of glutamate in the presence of 10 µM of the mGluR5 antagonist MPEP abolished the [Ca²⁺] response, but this was not seen with an antagonist for mGluR1 or with high glutamate concentration (30 µM) (II, Fig. 2E and F).

An ACh-induced [Ca²⁺] response was also seen in the majority of the neurosphere-derived human cells during the first day of differentiation. The typical response comprises a peak and a stable phase similar to that seen in mouse cells. While the ratio between the initial peak and the stable phase was found to be intact in cells derived from FXS neurospheres, a significant fraction of these cells responded to ACh with [Ca²⁺], oscillations during the stable phase and the response was almost absent in control cells (II, Fig. 5D and E).

10.4. The production of new cells in the Fmr1-KO brain (II)

To investigate NSCs in vivo, BrdU was injected into the embryonic brain of Fmr1-KO and WT mice and the production of new, BrdU-positive cells was measured using immunohistochemistry. The total number of BrdU-positive cells 4 days after BrdU administration was not significantly altered in the developing neocortex of Fmr1-KO mice when compared with WT mice (II, Fig. 3A). However, a relatively high number of BrdU-positive cells was observed in the SVZ of the Fmr1-KO telencephalon compared with the corresponding region in WT brains (II, Fig. 3B and C).

10.5. Formation of the neocortex in the presence of the dominant negative form of FMRP (III)

We studied the effects of FMRP with the I304N mutation by in utero electroporation of the expression plasmid FMRPmt-EGFP (Castrén et al., 2001) into embryonic mouse brain at E14. Three days later, the number of FMRPmt-EGFP-positive cells was significantly higher in the VZ/SVZ and in the IZ than the number of EGFP-positive cells after transfection of a control plasmid encoding EGFP (III, Fig. 1A) or after transfection of wild-type FMRP-EGFP plasmid. These FMRPmt-EGFP-positive cells in the VZ/SVZ and in the IZ also exhibited more immunoreactivity for nestin than EGFP-control cells (Fig.
1B and C). The expression of FMRPmt-EGFP greatly increased the proportion of nestin/EGFP-positive cells amongst the transfected cells in the VZ/SVZ when compared to controls (III, Fig. 1D). The mutated FMRP caused only minor alterations in the proportion of nestin/EGFP-positive cells in the IZ, in the cortical plate (CP), or in the marginal zone (MZ) (III, Fig. 1D). These findings agree with our previous studies which showed altered neurogenesis in vitro and an increase of newborn cells in the SVZ of the Fmr1-KO mouse (E17) in vivo (II).

10.6. Differentiation of glutamatergic neurons in the neocortex of Fmr1-KO mice (III)

We decided to study neocortical glutamatergic neurogenesis in Fmr1-KO mice, based on previous observations of alterations in glutamatergic differentiation of FMRP-deficient NPCs in vitro and enlarged newborn cell population in the embryonic SVZ of knockout mice (II). Glutamatergic neuron diversity is generated from radial glial NPCs and from intermediate progenitor cells (IPCs) in a manner that is strictly spatially and temporally controlled. IPCs (basal progenitors), which are actually the progeny of surface dividing NPCs of the VZ, proliferate at non-surface positions within the VZ and SVZ, and they express Tbr2 when they are already committed to glutamatergic fate (Englund et al., 2005). When assessing Tbr2-positive cell populations in the Fmr1-KO neocortex and comparing them with WT, we found an increase in the density of Tbr2-positive cells at E17 (III, Fig. 2A and B) but not at E16. However, in the Fmr1-KO neocortex at E16, Tbr2-positive cells in the BrdU-positive cell cohort born at E13 were significantly increased (III, Fig. 2C-E). These findings suggested that the proliferation, fate specification, or differentiation of NPCs/IPCs into the glutamatergic lineage is abnormal in the embryonic neocortex of Fmr1-KO mice (Englund et al., 2005; Hevner et al., 2006).

10.7. Formation of the layered structure of the developing Fmr1-KO mouse neocortex (III)

Newborn neurons acquire their specific laminar positions in the developing neocortex according to their birth date. We wanted to find out if the putatively aberrant Tbr2-positive cell population born around E13-E14 caused any alterations in cortical neuron lamination or positioning. Analysis of neocortex formation from Nissl-stained Fmr1-KO brain sections at E16 showed that the cell density in the CP of Fmr1-KO mice was significantly increased when compared to controls (III, Fig. 3A and B), but the thickness and the cell number were almost intact (III, Supplemental Fig. 2A and B) thus suggesting that the alterations seen in the SVZ could affect cell lamination. When analyzing the structure and lamination of the anterior somatosensory neocortex at P5, an increase in the overall density of cells in the Fmr1-KO neocortex was observed compared to WT (III, Fig. 3C-E). The cell density in layer V was significantly higher in the Fmr1-KO neocortex than in WT controls, and the same trend was also seen in layer II/III (III, Fig. 3F). The relative thickness of the layer V was not affected; however, we could observe a slight but significant decrease in the relative thickness of layer VI (III, Fig. 3G). To further investigate the alteration seen in layer V formation in the neocortex of Fmr1-KO mice, we assessed the expression of the transcription factor ER81, which is a specific marker for
layer V neurons (de Launoit et al., 1997; Yoneshima et al., 2006). Quantitative analysis revealed an increase in the number of ER81-positive cells in the Fmr1-KO neocortex at P5 compared to WT controls (III, Fig. 3H, I, and Supplemental Fig. 3A and B). This suggested alterations in the development of ER81-expressing cortico-cortical and subcerebral projection neurons in the Fmr1-KO neocortex (Hevner et al., 2003).

10.8. Radial glia in the developing neocortex of the Fmr1-KO mouse (III)

Tbr2-positive cells were increased in the SVZ of the Fmr1-KO mouse brain and this may also involve alterations in the differentiation of radial glia. When we assessed the expression of known radial glial markers, we observed that the expression of BLBP was not changed at E16 in the Fmr1-KO mouse neocortex when compared to controls (III, Fig. 4A), whereas the expression of GLAST was slightly but not significantly increased (III, Fig. 4B). In the neonatal neocortex of Fmr1-KO mice, actNotch1 showed a trend towards higher expression than in the WT brain (III, Fig. 4C). These findings suggest that the radial glia is not significantly altered during the early development of Fmr1-KO neocortex.

10.9. Expression of TrkB.T1 receptor in NSCs/NPCs derived from transgenic TrkB overexpressing mice (IV)

Neurospheres consist of NSCs/NPCs with a limited capacity to proliferate in the presence of mitogens in vitro. In the absence of mitogens, we could induce cells in neurospheres to differentiate into neurons, astrocytes and oligodendrocytes (IV, Fig. 1A and B). NPCs express both full-length TrkB (TrkB.TK) and TrkB.T1 (Ahmed et al., 1995; Barnabe-Heider and Miller, 2003) and we found that TrkB.T1 and TrkB.TK had similar mRNA expression levels in proliferating neurosphere NPCs derived from P7 mice. Transgenic mice that overexpress Flag-tagged TrkB.T1 under the neuron-specific Thy1 promoter have been previously characterized (Saarelainen et al., 2000b). Neurospheres derived from the transgenic mice co-expressed the transgene with nestin (IV, Fig. 1C). These neurospheres did not express markers for differentiated neuronal cells (IV, Fig. 1D). The Thy1 antigen, which has been previously shown to be expressed in several tissue-derived stem cells (Fiegel et al., 2003), was shown here to be expressed in NPCs. Furthermore, the TrkB.T1 transgene was co-expressed with the proliferation marker Ki67 in neurospheres (IV, Fig. 1D). Upon differentiation, the TrkB.T1 transgene was expressed in the majority of neurosphere-derived cells (IV, Fig. 1E). Consistent with the previously observed neuron-specific expression of TrkB.T1 transgene in vivo, the expression of the transgene was neuron-specific in differentiated cells (IV, Fig. 1F-H) (Saarelainen et al., 2000b). When studied by quantitative real-time PCR, TrkB.T1 expression (transgene and endogenous combined) was significantly higher in neurosphere cells derived from P7 mice overexpressing TrkB.T1 than in WT cells and the ratio of TrkB.TK expression to TrkB.T1 expression was significantly decreased in the transgenic cells (IV, Fig. 2A-C).
10.10. Formation of neurospheres by NSCs/NPCs derived from mice overexpressing TrkB.T1 (IV)

We compared the properties of neurosphere-forming cells derived from WT mice with those of cells derived from mice overexpressing TrkB.T1. Dissociated neurosphere cells were propagated in the presence of mitogens at clonal density and the absolute numbers of neurospheres were counted after 13 days in vitro. Both types of NPCs formed neurospheres in single cell as well as in low-density cultures, whereas sphere formation was significantly reduced in the presence of the TrkB.T1 transgene (IV, Fig. 3A). The reduced number of Ki67-positive cells was observed in the presence of the TrkB.T1 transgene after plating the dissociated neurosphere cells onto a coated matrix in the absence of mitogens (IV, Fig. 3B). To investigate whether TrkB.T1 overexpression had an effect on the survival of neurosphere NPCs, we counted the number of apoptotic cells in transgenic and WT neurospheres. The number of TUNEL-positive cells in transgenic neurospheres after 8 days of passaging was significantly increased (IV, Fig. 3C). These data suggest that the transgenic TrkB.T1 neurospheres contained less NPCs, and upon differentiation had increased number of cells that were not in the active cell cycle. In addition, overexpression of TrkB.T1 reduced the survival of NPCs. However, exogenous BDNF was able to abolish the differences in the number of neurosphere-forming cells and Ki67-positive cells between WT and transgenic TrkB.T1 cells (IV, Fig. 3D and E), indicating that overexpressed TrkB.T1 inhibited the interaction of endogenous BDNF with the TrkB.TK receptor.

10.11. Proliferation of NSCs/NPCs overexpressing TrkB.T1 (IV)

The transgenic TrkB.T1 cells from dissociated neurospheres generated larger neurospheres than WT cells when observed after 6, 8, and 10 days of proliferation in culture thus suggesting that the transgenic spheres propagated more readily (IV, Fig. 4A). Furthermore, the increase in the average diameter of transgenic TrkB.T1 neurospheres derived from P7 mice was significant within 3 days of the onset of neurosphere formation (IV, Fig. 4B and C). The increased proliferation and growth of neurospheres overexpressing TrkB.T1 compared with WT neurospheres were shown by $^3$H-thymidine incorporation and MTT assays (IV, Fig. 4D and E). The effects of culture media mitogens were synergistic in transgenic cultures. FGF-2 had no effect on growth alone, whereas EGF alone induced cell growth to 40% of the increase seen in the presence of both growth factors.

We wanted to see whether the properties of the NSCs/NPCs derived from the transgenic mice overexpressing TrkB.T1 were attributable to the increase in TrkB.T1 expression. Therefore, we generated NSCs/NPCs with viral-mediated (LV-mediated) overexpression of TrkB.T1 (Englund et al., 2000; Falk et al., 2002). For the LV construct, TiG4, encoding mouse TrkB.T1, GFP, and Flag-tag we achieved transduction efficiency (10%) similar to that in previous studies (Falk et al., 2002) (IV, Fig. 6A). The TrkB.T1 transgene was expressed in undifferentiated NSCs/NPCs (IV, Fig. 6B). Upon differentiation, the LV-transduced cells gave rise predominantly to TuJ1-negative and GFAP-positive glial cells and a few TuJ1-positive cells (IV, Fig. 6C and D). Despite the low transduction efficiency, $^3$H-thymidine incorporation (IV, Fig. 6C), average diameter of neurospheres (IV, Fig. 6F), and cell growth in the MTT assays were all significantly increased after virus-mediated overexpression of TrkB.T1 compared with controls (IV,
Fig. 6G). The data consistently show that overexpression of TrkB.T1 increases proliferation of NSCs/NPCs in both a transgenic mouse model and after viral-mediated transgene delivery.

10.12. Increased neurogenesis of differentiating NSCs/NPCs derived from mice overexpressing TrkB.T1 (IV)

We studied differentiation of transgenic TrkB.T1 NSCs/NPCs in vitro. After differentiation, TrkB.T1 expression continued to be significantly higher in TrkB.T1 overexpressing cells than in WT ones. Both the number of TuJ1-positive neurons derived from NPCs overexpressing TrkB.T1 and the apoptosis of these TuJ1-positive cells were increased compared with WT after 4 days and 7 days of in vitro differentiation (IV, Fig. 5A and B). This suggested increased turnover of newborn neurons, which overexpress TrkB.T1.

11. DISCUSSION

Since isolation of human ES cells in 1998, stem cells have been a hot topic in research with their wide array of possible therapeutic applications (Thomson et al., 1998). The CNS in particular, is an attractive target for stem cell therapy and CNS tissue specific NSCs have been under vigorous investigation (Muller et al., 2006). Neuropathological conditions such as Alzheimer’s disease, Parkinson’s disease, and spinal cord injury are putative targets for NSC therapy. However, studying genetically mutated NSCs derived from diseased brain can provide valuable information about the properties of NSCs in general and also allow the design of possible drug interventions. The present studies show alterations in the proliferation, survival and differentiation of NSCs/NPCs in two different but related models of impaired learning. In models of FXS (II, III), which is a common form of inherited mental retardation, the differentiation of NSCs and early brain development were altered when FMRP was absent or functionally inactive. These alterations were associated with group I mGluR5 action and glutamatergic neuronal lineage. Furthermore, mRNA levels of RGS4 (I), which can modify G-protein-mediated signaling through mGluR5, were down regulated in the Fmr1-KO mouse brain. The findings show novel pathological features in NSCs and early brain development of the FXS. In the model with overexpression of the TrkB.T1 receptor (IV), which affects the BDNF signaling, NSC proliferation, survival and differentiation were altered. This illustrates the importance of BDNF/TrkB signaling in directing NSCs to proliferate, to die by apoptosis, or to differentiate into neuronal cells. Interestingly, FXS and BDNF/TrkB signaling have been linked to each other (Castrén et al., 2002; Selby et al., 2007). In summary, FMRP and BDNF/TrkB signaling are important to the normal development of neurons and neuronal networks in the brain and already have an impact at the level of NSCs and NPCs.
11.1. The properties of NSCs in FXS

FXS is a developmental disorder, however very little is known about the actual mechanism of the disease during early development. The absence of functional FMRP is the cause of the disease (Pieretti et al., 1991) and the function of this protein in differentiated neurons is beginning to be elucidated (Galvez et al., 2003; Bagini and Greenough, 2005; Galvez et al., 2005). Several studies have reported neuroanatomical alterations in the human FXS brain, which include some developmentally transient features such as increased hippocampal volume in young adults (Kates et al., 2002; Lee et al., 2007). In addition, the structure of the visual pathway seems to be altered in the adult FXS brain (Kogan et al., 2004b). These alterations correlate with the cognitive performance of the FXS patients (Kogan et al., 2004a; Gothelf et al., 2007). The mouse model, however, lacks some of the neuroanatomical features seen in FXS patients’ brains (Kooy et al., 1999), although the GABAergic system seems to be altered in the neocortex of adult Fmr1-KO mice (D’Hulst et al., 2006; Selby et al., 2007). Specifically, the number of GABAA receptors is reduced together with the overall number of GABAergic interneurons. More careful analysis shows that the number of parvalbumin expressing GABAergic neurons is decreased, their morphology is changed, and the remaining cells are mostly located at the deeper neocortical layers (Selby et al., 2007). This finding suggests that the neuroanatomy of the adult Fmr1-KO neocortex is altered where the GABAergic system is concerned. These data show that in the absence of functional FMRP, neuroanatomy is altered and it affects the cognitive phenotype. On the other hand, whether the dysfunction of FMRP causes the neuroanatomical alterations or whether it is only a secondary effect is still unclear. Our studies provide new data about the developmental aspects of FXS brain anatomy. We show that the glutamatergic system is also altered in the Fmr1-KO mouse brain. This suggests that the altered neuroanatomy is caused by the absence of FMRP.

Here, we report altered morphology of human and mouse FMRP-deficient NSC-derived differentiated neurons in vitro. These young neurons displayed a decreased number of neurites, which were shorter than normal. In addition, these neurons had a smaller soma size compared to control. This immature morphology suggests a delayed maturation of FMRP-deficient neurons differentiated from NSCs. Immature neuronal morphology is, in fact, one of the key features in the FXS and Fmr1-KO mouse brain. FMRP-deficient neocortical and hippocampal pyramidal neurons display an immature dendritic spine phenotype, and the morphology of GABAergic interneurons in the neocortex of Fmr1-KO mice is altered (Irwin et al., 2001; Nimchinsky et al., 2001; Irwin et al., 2002; Grossman et al., 2006b; Selby et al., 2007). Altered neurite and spine phenotypes have also been observed in cultured hippocampal neurons (Braun and Segal, 2000). In Fmr1-KO mice, the aberrant spine phenotype is present during first few postnatal weeks and in the adult brain (Nimchinsky et al., 2001; Galvez and Greenough, 2005). However, at the age of 4 weeks, Fmr1-KO mice neocortical neurons do not exhibit an aberrant dendritic spine phenotype (Nimchinsky et al., 2001). Thus, this feature appears to be postnatally dynamic. Why this is occurring and whether it occurs in the human FXS brain is not known. Our data suggest that this feature may already be prevalent at the level of NSCs and in early differentiating neurons when functional FMRP is absent.

In addition to the immature morphology of neurons derived from the FMRP-deficient NSCs, the overall production of neurons from these NSCs in vitro is increased compared to controls. On the other hand, the glial cell population is reduced and is proposed to exhibit increased apoptosis during early differentiation of FMRP-deficient
NSCs. During early neocortical development induction of gliogenesis has been proposed to be initiated by factors secreted by newborn neurons. Cardiotrophin-1 could be one of these factors secreted by differentiating neurons, since it has been proposed to be a strong candidate to initiate glial differentiation through activation of the JAK-STAT pathway and the GFAP promoter during neocortex formation (Barnabe-Heider et al., 2005; Miller and Gauthier, 2007). It could be that FMRP-deficient newborn neurons do not mature sufficiently or fast enough to secret cardiotrophin-1 and provide trophic support for glia. This is, however, only speculation since the evidence of a connection between cardiotrophin-1 and FMRP is lacking. Signaling by WNT/β-catenin promotes neural proliferation cooperation with FGF2 (Hirabayashi et al., 2004; Israsena et al., 2004). Interestingly, FGF2 mRNA is a target for FMRP and in the absence of FMRP, FGF2 may be overexpressed thus contributing to the increased production of neurons (Miyashiro et al., 2003). However, in our experimental system, we also included FGF2 in the propagation culture media of NSCs/NPCs, which may make interpretation of FGF2 function complicated. A novel mechanism promoting the glial differentiation of NSCs/NPCs through TrkB.T1 receptor activation has been found recently (Cheng et al., 2007). This pathway involves G-protein coupled activation of protein kinase C (PKC), thus suggesting that PKC activation is promoting glial differentiation. The mRNA of one subtype of PKCs is a target for FMRP regulation thus indicating a possible connection with increased neuronal differentiation in the absence of functional FMRP (Miyashiro et al., 2003). Until our observations, there were no other reports of neuron overproduction in the FMRP-deficient brain or in FMRP-deficient neuronal cell cultures until our observations in vitro and in vivo, however, a recent report showed that FMRP is crucial for the maintenance and repression of the differentiation of germ line stem cells (GSC)s in Drosophila (Yang et al., 2007). This suggests that in the absence of functional FMRP, the differentiation of stem cells may be induced.

Although the proliferation NSCs appears to be unaffected by the defect in FMRP, we observed an abnormally enlarged newborn cell population in the SVZ of the Fmr1-KO mouse neocortex at E17. BrdU labelling showed that these cells had been in active cell cycle during the time of late deeper layer neurogenesis in the neocortex. This alteration was associated with abnormal migration or positioning of newborn cells in the Fmr1-KO neocortex rather than the proliferation of NSCs/NPCs, since the overall number of neocortical BrdU-labeled cells was not changed. Thus, the absence of functional FMRP does not seem to affect the proliferation of NSCs/NPCs.

11.2. Ca\(^{2+}\) oscillations in mGluR5 responsive differentiating FMRP-deficient NSCs

We found that most of the differentiating FMRP-deficient NSCs cells have an aberrant oscillatory intracellular Ca\(^{2+}\) response to ACh. Furthermore, this response was not ACh-specific and could be evoked with other neurotransmitters including glutamate. We were able to associate the oscillating cells with cells that are especially responsive to mGluR5 receptor activation. It is known that oscillating [Ca\(^{2+}\)], release can significantly enhance gene expression (Dolmetsch et al., 1998), thus the absence or lack of FMRP may induce gene expression. The [Ca\(^{2+}\)], signaling is involved in early morphological maturation and the neurotransmitter phenotype acquisition of neurons (Ciccolini et al., 2003). Therefore, aberrant [Ca\(^{2+}\)], release may be causative for the immature phenotype of newborn neurons.
that we observed after differentiation of FMRP-deficient NSCs in vitro. Furthermore, the increased number of neurons differentiated from FMRP-deficient NSCs in vitro may be explained by an accelerated 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 NSCs. Recently, spines and dendrites of neurons in the mature prefrontal cortex have been shown to exhibit similar alterations thus suggesting that alterations in Ca$^{2+}$ signaling may be a general feature of FMRP-deficient neural cells (Meredith et al., 2007). The altered Ca$^{2+}$ signaling in the FMRP-deficient neural cells may substantially intensify overall gene expression in concert with mGluR5 dependent protein synthesis in these cells.

11.3. Embryonic neocortex development of the Fmr1-KO mice

We observed alterations during embryonic neocortex development of Fmr1-KO mice. First, at E17 we found an aberrant cell cohort born at E14 at the SVZ. Second, we showed that the dominant negative FMRP mutation I304N resulted in an increased proportion of nestin-positive cells in the VZ/SVZ at E17. In this experiment, the transfection of undifferentiated VZ NSCs that line the lateral ventricle wall was performed at E14 by in utero electroporation. Therefore, these two separate findings support each other despite the fact that in utero electroporation is a relatively crude method to deliver transgenes into a developing brain. Nevertheless, delivering FMRP with the I304N mutation in the KH2 domain into neocortical NSCs/NPCs, illustrates that the KH2 domain is important for FMRP function in NSCs/NPCs during neocortex formation. Third, we went back to assess the embryonic neocortex of Fmr1-KO mice and found that the Tbr2-positive cell population was increased in the SVZ at E17. Tbr2 is a transcription factor, which is expressed in immature SVZ cells (mainly in IPCs) that are committed to glutamatergic fate (Englund et al., 2005). This was in agreement with our previous observation that cells generated from FMRP-deficient NSCs display intense oscillatory [Ca$^{2+}$]$_i$ responses to glutamate in vitro. The finding that Tbr2 and BrdU double labeling in the Fmr1-KO neocortex increased at E16 when the mice received BrdU at E13, showed that the maturation of the Tbr2-positive cell population was impaired. This may involve exaggerated turning of the apical VZ progenitors into Tbr2 expressing basal progenitors by Ngn2 activity (Miyata et al., 2004). The absence of Ngn2 is known to impair differentiation of neocortical neurons born before E14.5 and results in dysformation of the deeper neocortical layers (Schuurmans et al., 2004). Direct upstream regulators of Ngn2 expression including WNT/β-catenin signaling and Pax6 may be involved in increasing the Tbr2-positive cell population (Scardigli et al., 2003; Hirabayashi et al., 2004; Israsena et al., 2004). The GABAA receptor subtype has been shown to be downregulated in the FMRP-deficient neocortex (D’Hulst et al., 2006). GABAA receptors participate in regulating the migration of NPCs or newborn neurons from VZ/SVZ to IZ, which suggests the involvement of these receptors in the accumulation of Tbr2-positive cells in the SVZ in the absence of FMRP (Behar et al., 2000). All these experiments suggest that the cell population, which is altered in the FMRP-deficient brain, is born during the early phase of the neurogenesis when the deeper layer neurons are generated (Götz and Huttner, 2005; Molnar et al., 2006). Now that we have seen the FMRP-deficient glutamatergic cell population derived from NSCs to be responsive for mGluR5 activation in vitro we can consider whether this is occurring in vivo as well. ES cells and NSCs are known to express
mGluR5s (Melchiorri et al., 2007). In ES cells, mGluR5s promote self-renewal in cooperation with cytokine LIF signaling (Melchiorri et al., 2007) and in NSCs/NPCs mGluR5 activation increases survival and proliferation (Di Giorgi-Gerevini et al., 2005). This suggests that mGluR5 expressing NPCs may survive better than other NPCs in the absence of FMRP. In neurons, synaptic expression of FMRP is group I mGluR-dependent and protein synthesis through group I mGluR activation in synaptoneurosomes is FMRP-dependent (Weiler et al., 1997; Weiler et al., 2004). This kind of interplay suggests alterations in NSC properties when FMRP is functionally absent. The FMRP target mRNAs in NSCs should be mainly affected but unfortunately they are still unknown.

11.4. Early postnatal neocortex formation in the Fmr1-KO mouse brain

In the postnatal neocortex of Fmr1-KO mice, we detected alterations in the structure of the deeper layers. Layer V showed increased density of Nissl-stained cells in the absence of FMRP, and layer VI had a putative lamination defect. The cell density increase seen by Nissl staining was only mild, therefore we decided to use a layer V specific marker ER81 to confirm the observed defect. Consequently, we could indeed see an increase in the density of ER81-positive neurons in layer V of the Fmr1-KO neocortex at P5. This finding can be related to our observations on an aberrant glutamatergic cell population born around E13 to E14 in the neocortex of Fmr1-KO mice, because layer V and VI neurons are born at that time. Furthermore, the increase in certain glutamatergic cell populations in the postnatal FMRP-deficient brain during the first week of postnatal development may be transient, because adult Fmr1-KO brains have grossly normal neuroanatomy (The Dutch-Belgian fragile X consortium, 1994; Kooy et al., 1999). However, recent reports about differences in GABAergic interneuron populations in the adult Fmr1-KO neocortex imply that alterations in the glutamatergic system may persist into adulthood. On the other hand, FMRP expression declines during aging in the mouse brain, which suggests a developmentally dynamic function for FMRP (Singh et al., 2007). This is supported by dynamic alterations in dendritic spine morphology during the first two postnatal months of Fmr1-KO mouse development (Nimchinsky et al., 2001; Galvez and Greenough, 2005). We show that this altered cell population in the Fmr1-KO mouse neocortex includes subcerebrally and cortico-cortically projecting pyramidal neurons (spiny giant pyramidal neurons), which express ER81 (Sugitani et al., 2002; Beggs et al., 2003; Hevner et al., 2003; Yoneshima et al., 2006). Phosphorylation of Ngn2, which is an upstream effector of Tbr2, promotes a dendritic phenotype of pyramidal neurons (Hand et al., 2005). In the adult and developing Fmr1-KO neocortex, dendritic spine abnormalities have also been observed on pyramidal neurons of layer V (Nimchinsky et al., 2001; Irwin et al., 2002; McKinney et al., 2005). This suggests that the enlarged ER81-positive cell population may be the one that exhibits the spine abnormalities. The stabilization of dendritic spines, which means activity dependent elimination of unused synaptic connections (spines), is defective in the absence of functional FMRP (Galvez et al., 2003; Bagni and Greenough, 2005; Galvez et al., 2005). FMRP appears to be a selective factor for synapses that will be preserved and this selection likely occurs through group I mGluR activation. Our observations suggest that this kind of stabilization may actually occur in whole cell populations during neocortex development.

Epileptic seizures caused by exaggerated excitatory activity are part of the phenotype in FXS and in Fmr1-KO mice (Incorpora et al., 2002; Nielsen et al., 2002). Alterations in
the stabilization of dendritic spines suggest increased overall excitatory activity. However, if the overall number of excitatory neurons was increased, the effect might be even more prominent. The reported increase in the density of parvalbumin-positive interneurons and their altered morphology in the neocortical layer V of the adult Fmr1-KO mice may be a compensatory effect to reduce excitatory activity (Selby et al., 2007). Therefore, alterations in the neocortical glutamatergic populations may underlie the epileptic seizure susceptibility in the FMRP-deficient brain.

Further evidence that FMRP may act on specific excitatory neuron populations comes from studies of the visual pathway of FXS patients (Kogan et al., 2004b). This study is based on an observed defect in visual-motor function among FXS individuals (Kogan et al., 2004a). The magnocellular cells in the lateral geniculate nucleus are specifically expressing FMRP and are affected in FXS brains (Kogan et al., 2004b). The lateral geniculate nucleus is formed from layers of magnocellular and parvocellular cells, which are neurons that project to the visual cortex. In the absence of FMRP, this structure is aberrant due to defects in the magnocellular cells. Parvocellular cell layers do not express FMRP and are primarily unaffected by the FMRP-defect. This is evidence of cell population based function of FMRP in neurons.

The mRNA and protein-binding partners of FMRP in the NSCs are unknown. Therefore, we can only use the information available on FMRP-binding mRNAs in the mature brain. Nevertheless, some potentially interesting mRNAs binding to FMRP may affect neocortical formation and/or NSCs differentiation. Two possible candidates are messages encoding FGF2 and IGF-I (via IGFBP-5) (Ye and D'Ercole, 1998; Miyashiro et al., 2003). Both of these proteins have a profound effect on the neocortex formation (Ortega et al., 1998; Hodge et al., 2005). These proteins have an especially strong impact on layer V development, which provides another link between them and FMRP. Thus, developmental neocortical defects in Fmr1-KO mice brain may be related to cell growth associated proteins, which are likely regulated by FMRP action.

11.5. Radial glial markers during the development of the Fmr1-KO neocortex

We found only very subtle alterations in radial glial marker expression in the developing neocortex of Fmr1-KO mice. Radial glial markers, BLBP, GLAST, and actNotch1 are expressed in distinct spatiotemporal patterns during neurogenesis (Hartfuss et al., 2001; Tokunaga et al., 2004). BLBP-positive radial glia is associated with the migration of immature neurons in the neocortex (Feng et al., 1994). GLAST is expressed in NPCs of neocortical proliferative zones (Gal et al., 2006) and is associated with the differentiation of astrocytes (Chaudhry et al., 1995; Ullensvang et al., 1997). ActNotch1 is important for maintaining the proliferative potential of NPCs during neurogenesis and later induction of glial differentiation in the neocortex (Yoon and Gaiano, 2005). In NPC cultures, actNotch1 was shown to increase proliferation at low doses and to induce cell cycle arrest at high doses in a cell density dependent manner (Guentchev and McKay, 2006). In the VZ and the SVZ NPCs, Notch signaling inhibits neurogenesis through C-promoter binding factor 1 (CBF1) -related and non-CBF1-related pathways (Mizutani et al., 2007). The expression of BLBP is directly regulated by CBF1 in radial glia (Anthony et al., 2005). A recent microarray study demonstrated that Hes1, which is a target of Notch1/CBF1 signaling and an inhibitor of proneural genes (Nakamura et al., 2000), is overexpressed in the adult FXS brain (Bittel et al., 2007). Furthermore, a proneural gene
target repressed by Hes1, Ngn2, regulates the transcription of Tbr2 (Schuurmans et al., 2004). Although links between radial glial cells and Tbr2-positive cells exist, we were not able to show statistically significant differences in radial glial marker expression in the Fmr1-KO neocortex. However, we observed trends toward increased GLAST expression at E16 and actNotch1 expression at P0. An increase in GLAST expression may reflect abnormalities in radial glial cells, which are precursors of Tbr2-positive cells whereas increased actNotch1 expression may be related to increased proliferation of Tbr2-positive cells and to increased Hes1 expression postnatally. Nevertheless, the connection between FMRP and Notch-signaling remains to be investigated.

11.6. The significance of decreased expression of RGS4 in the Fmr1-KO brain

We have shown that RGS4 mRNA is decreased in the hippocampal CA1 area and in the retrosplenial cortex of the Fmr1-KO brain. However, a direct link is missing between FMRP and RGS4. RGS4 is a protein that is involved with G-protein signaling and it has been shown to regulate mGluR5 dependent signaling. As RGS4 increases GTPase activity and in this manner is repressing or modulating G-protein coupled signaling, the observed decrease in RGS4 expression levels may lead to aberrant [Ca$^{2+}$] oscillations (Xue et al., 1999; Luo et al., 2001; Hollinger and Hepler, 2002). Homer proteins are involved with RGS4 and mGluR5 receptors at the postsynaptic sites of neurons and phosphorylation of Homers is impaired in the absence of functional FMRP (Shin et al., 2003; Giuffrida et al., 2005). RGS4 expression has developmental patterns in the hippocampal CA1 area and neocortical layer II/III suggesting that reduced RGS4 expression in the Fmr1-KO brain at P10 may be caused by a developmental delay (Ingi and Aoki, 2002). Defects in RGS4 expression have been frequently associated with schizophrenia (Mirnics et al., 2001; Levitt et al., 2006). Also, a group of FXS patients have been found to exhibit schizotypal traits, which suggests a connection between the phenotypes of these two common mental disorders (Sobesky et al., 1994). Thus, RGS4 may prove to be an important molecule in FXS pathology and could provide novel possibilities in drug intervention of this disease.

11.7. BNDF/TrkB function in NSCs

We found that overexpression of TrkB.T1 in NSCs/NPCs can result in increased proliferation and decreased clonal survival. After dissociation and brief plating on coated matrix (6 h), expression of Ki67, which a marker for dividing cells, was reduced in these NSCs/NPCs. Exogenous BDNF was able to rescue the survival of neurosphere forming cells and the expression of Ki67 in TrkB.T1 overexpressing NSCs/NPCs. Upon differentiation, these NSCs/NPCs generated more neurons than control cells but the apoptosis of these newborn neurons was simultaneously increased.

BDNF signaling through TrkB receptors is crucial for the differentiation and survival of maturing neurons (Huang and Reichardt, 2001). The traditional function of the truncated TrkB.T1 isoform was to inhibit BDNF signaling occurring through catalytically active TrkB receptor (Biffo et al., 1995; Eide et al., 1996; Ninkina et al., 1996; Haapasalo et al., 2001). This type of function can be executed through ligand sequestering or a dominant-negative heterodimerization of the receptors. However, TrkB.T1 receptors can
relate signals on their own by regulating the activity of small Rho GTPases (Ohira et al., 2005) and by the neurotrophin-dependent release of intracellular \( \text{Ca}^{2+} \) in astrocytes through phospholipase C (PLC) activation (Rose et al., 2003). Recently, one group reported that TrkB.T1 signaling directs NSCs to glial lineage and inhibits neurogenesis in culture and *in vivo* (Cheng et al., 2007). Inhibition of neurogenesis occurs through a dominant-negative action; however, the induction of glial fate occurs through activation of PKC via G-protein signaling. The activated G-protein sub-unit is still unknown, which raises possibilities that it may be the same one involved with RGS4 and mGlur5. Interestingly, TrkB.T1 can induce the outgrowth of dendritic filopodia in hippocampal neurons by cooperating with p75NTR or induce process growth in neuroblastoma cells (Haapasalo et al., 1999; Hartmann et al., 2004). Evidence in NSCs show that TrkB.T1 does not inhibit catalytic TrkB receptor signaling in NSCs, since Akt is not blocked, which occurs in PC12 cells (Cheng et al., 2007). Our model of TrkB.T1 overexpression is a bit problematic, since the transgene is under a Thy1 promoter, which limits the expression to neurons (Saarelainen et al., 2000a). Despite this, we have shown that the transgene is expressed in NSCs/NPCs but in these cells TrkB.T1 likely functions as a ligand sequestering agent instead of a dominant-negative blocker (Cheng et al., 2007). We have confirmed this by viral-induced overexpression of the TrkB.T1 receptor in NSCs/NPCs, which consequently resulted in increased proliferation of these cells. BDNF is known to reduce NPC proliferation and induce neuron differentiation indirectly by acting in a positive feedback loop with nitric oxide (Cheng et al., 2003). Overexpressed TrkB.T1 may overcome this effect by sequestering BDNF and thus promote proliferation (Fig. 12). The clonal survival of human ES cells seems to be improved by BNDF/TrkB signaling (Pyle et al., 2006), thus ligand sequestering action may explain the reduced clonal survival of TrkB.T1 overexpressing NSCs/NPCs. This may be related to improved survival of malignant tumor cells in the presence of TrkB expression (Douma et al., 2004). Furthermore, similar signaling pathways may apply in brain tumor stem cells and NSCs/NPCs, since they seem to be closely related (Vescovi et al., 2006). Our findings suggest that TrkB.T1 may have a ligand sequestering effect on BDNF induced survival and suppression of self-renewal in a certain subpopulation of NPCs.

The increased neuronal differentiation may require further explaining, since normally TrkB.T1 expression promotes glial differentiation. Our model may somehow activate both mechanisms; aberrant neuronal differentiation via PKC activation and inhibition of neuronal survival via a ligand sequestering action within the same cell population. This same feedback mechanism may function in the dentate gyrus of the adult TrkB.T1 overexpressing mice, where the turnover of newborn granule neurons was increased (Sairanen et al., 2005). Thus, in our model, TrkB.T1 expression in NPCs enlarges the cell population committed to neuronal lineage possible through novel signaling mechanism which is normally active in glial NPCs and involves G-protein coupled receptors and PKC (Cheng et al., 2007). On the other hand, some kind of feedback mechanism may induce precursor proliferation in these cells. In contrast, survival of these newborn neurons is reduced due to the ligand sequestering action of overexpressed TrkB.T1 on BDNF/TrkB signaling in our model. This provides a new insight into BDNF/TrkB signaling in NSCs/NPCs during development.
Figure 12 A model of the proposed impact of TrkB.T1 on BDNF signaling in NSCs/NPCs when TrkB.T1 overexpression is directed to neurons. This figure shows three different systems: NSC pool in intact brain, NSCs in culture, and NSCs during in vitro differentiation. EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; NO, nitric oxide.

The decrease in Ki67-positive cells upon plating of TrkB.T1 overexpressing NSCs/NPCs on a coated matrix may be explained by the anoikis effect, which suppresses tumor growth in cells that have lost contact with the extracellular lamina (Douma et al., 2004). In this case, TrkB.T1 acts by ligand sequestering to inhibit TrkB signalling. TrkB expression has been shown to accompany malignant tumor growth. In addition, blocking endogenous BDNF in NPCs plated on a coated matrix results in a decrease in Ki67 expression in these cells (Barnabe-Heider and Miller, 2003). Therefore, TrkB.T1 may counteract TrkB signaling in malignant tumors thus suggesting a possible role for TrkB.T1 in preventing cancer growth.

11.8. Similarities between FMRP action and BDNF/TrkB signaling

The FXS and Fmr1-KO NSCs produce an increased number of neurons upon differentiation and BDNF signaling through the TrkB receptor promotes the differentiation of neurons (Huang and Reichardt, 2001). Exogenous BDNF and increased TrkB receptor expression reduces expression of mRNA encoding FMRP, which may
affect the actual expression of FMRP (Castrén et al., 2002). The suppression of FMRP mRNA expression by BDNF/TrkB signaling may mimic the loss of functional FMRP and result in partially similar phenotypes in models of FXS and exaggerated BDNF/TrkB signaling.

We observed alterations in the formation of the FMRP-deficient neocortex. BDNF and TrkB receptors are also important for neocortex formation, since alterations occur when BDNF/TrkB signaling is disrupted. BDNF has an apparent role in the neocortical lamination in a specific temporal window (Ohmiya et al., 2002; Fukumitsu et al., 2006). When BDNF is administered to the lateral ventricles of the mouse brain at E13.5, it alters the properties of cells destined to become layer IV neurons to those of layers V and VI whereas blocking the BDNF results in the opposite effect. Only neocortical NPCs that are in the S-phase of the cell cycle at the time of BDNF administration are affected. BDNF accelerates neuronal differentiation by promoting cell cycle completion in affected cells and increases interkinetic nuclear migration of neocortical NPCs in the VZ. Therefore, BDNF has an important role in laminar formation of neocortex. In the absence of TrkB receptor expression, developmental migration in the neocortex is delayed and neuronal and oligodendrocyte differentiation is altered (Medina et al., 2004). Furthermore, the migration of neocortical newborn neurons is enhanced after increased BDNF/TrkB signaling (Behar et al., 1997; Brunstrom et al., 1997; Ringstedt et al., 1998). In the adult Fmr1-KO neocortex, TrkB receptor expression is increased in the parvalbumin expressing interneuron population (Selby et al., 2007). Furthermore, indirectly FMRP-related protein, IGF-I, enhances TrkB receptor expression and BDNF induced ERK1/2 phosphorylation in neocortical neurons (McCusker et al., 2006). Thus BDNF/TrkB signaling may contribute to the phenotype seen during FMRP-deficient neocortex formation. Some FXS patients display schizotypal traits, and BDNF with other neurotrophins has been associated with the pathology of schizophrenia (Sobesky et al., 1994; Shoval and Weizman, 2005). Furthermore, reduced BDNF signaling may cause symptoms present in schizophrenia. As we have shown that expression of RGS4, which is a strong candidate in schizophrenia susceptibility (Mirnics et al., 2001), was reduced in the Fmr1-KO mouse brain, the increased TrkB expression may counteract some symptoms of schizophrenia in FXS (Sobesky et al., 1994; Selby et al., 2007). A recent report showed that hippocampal (CA1) LTP induced by afferent theta burst stimulation was impaired in the Fmr1-KO brain (Lauterborn et al., 2007). This is remarkable, because earlier studies have reported no changes in hippocampal LTP in the absence of functional FMRP (Godfraind et al., 1996; Paradee et al., 1999; Li et al., 2002; Larson et al., 2005). Moreover, exogenous BDNF was able to abolish this defect, which establishes yet another link between FMRP and BDNF. Authors suggest that this type of BDNF action may occur downstream of cytoskeleton rearrangement (Lauterborn et al., 2007). These data suggest that FMRP and BDNF/TrkB signaling have largely intertwined functions in NSCs/NPCs and in the developing brain.

11.9. Therapeutic implications of NSCs in FXS and future aspects

The cognitive pathology of FXS has been suggested to be caused by impaired activation of mGluR5 dependent protein synthesis at the postsynaptic sites of CNS neurons (Bear et al., 2004). This leads to improper synaptic maturation of certain neurons including pyramidal neurons of the neocortex. Consequently LTP and LTD are affected in the neocortex and in the hippocampus resulting in deficits in learning and memory.
Antagonists of mGluR5 have been shown to overcome some of these defects in the adult Fmr1-KO mouse brain suggesting a possible clinical use. However, our results implicate that alterations in the FMRP-deficient brain are present already during very early development. The mGluR5 receptor is already expressed in NSCs, therefore FMRP likely has an important function in regulating mGluR5 induced translation in those cells. However, mRNA targets for FMRP in NSCs/NPCs are unknown and future studies should be directed to identify these messages. Our findings imply that antagonists for mGluR5 receptors may have to be administered already at embryonic stage and this may prove to be impossible. This information may be disappointing to FXS patients, since some of the symptoms of the disorder cannot be overcome with drug treatment at adult ages. Still the mGlur5 antagonists may provide a significant improvement to the performance and life quality of FXS patients. A novel possibility for pharmaceutical intervention in FXS was recently found. Reduced expression of PAK, which is involved in the formation of synaptic structure, was able to abolish the great majority of FXS symptoms in mice lacking FMRP (Hayashi et al., 2007). PAK is expressed during postnatal development, and therefore it may provide an interesting target for drug intervention in the adult FXS brain.

Properties of FMRP in NSCs/NPCs and their differentiation during normal brain development are only partially understood. The signaling pathways involved in neuronal and glial differentiation that should be investigated including WNT, BMP, Notch, LIF related signaling upstream of proneural genes (Ngn1, Ngn2, and Mash1) and inhibitors of proneural genes (Hes1 and Hes5). Direct or indirect connection between these factors and FMRP should be investigated. Interestingly, the mRNAs that target the important functional KH2 domain of FMRP are largely unknown, and solving these targets may provide important information about the action of FMRP in general. Furthermore, FMRP mRNA targets are unknown in the NSCs/NPCs, which are likely important during early differentiation of NSCs/NPCs, since mGluR5 is already expressed in these cells (Melchiorri et al., 2007). The function of TrkB.T1 signaling in regulating the differentiation of NSCs/NPCs is probably an important mechanism of choosing between neural and glial fate. The pathways involved are partially known, however identification of the components of the signaling pathway that promotes the glial fate in NSCs/NPCs, may provide the actual connection between BDNF/TrkB signaling and FMRP (Cheng et al., 2007).

Reduced amount of FMRP, impaired BDNF/TrkB signaling and reduced RGS4 expression are implicated in schizophrenia. This may also provide new applications for treatment of FXS, since schizophrenia is under intense investigation. Furthermore, the relationship between FMRP and BDNF/TrkB signaling should be further investigated, since BDNF/TrkB signaling is such an important survival factor for neurons of the developing and mature brain. To summarize, the early developmental aspects of any given nervous system disorder should be evaluated to design more specific and efficient drugs to be used for treating adult patients.
12. Conclusions

This thesis aimed to elucidate the role of NSCs in fragile X syndrome and to investigate the effects of impaired BDNF/TrkB signaling on NSC survival, proliferation and differentiation. Based on the findings presented in this thesis, following conclusions can be drawn:

1. Mouse and human NSCs lacking FMRP generate more neurons with short neurites and small soma size and less astrocytes than control NSCs.

2. NSCs lacking FMRP give rise to more cells responsive to mGluR activation. The responses to mGluR in differentiating cells correlate with oscillatory intracellular Ca$^{2+}$ responses to neurotransmitters. The oscillatory response to mGluR activation was sensitive to mGluR5 antagonist suggesting alterations in cellular plasticity of a distinct cell population responding to mGluR activation in fragile X syndrome.

3. An increased number of cells expressing Tbr2 accumulates in the embryonic SVZ of Fmr1-KO mice suggesting alterations in glutamatergic neurogenesis. The aberrant cells contribute to alterations in the formation of the deeper neocortical layers in the early postnatal brain of Fmr1-KO mice.

4. RGS4 mRNA expression is decreased in the hippocampus and neocortex of the early postnatal brain of Fmr1-KO mice suggesting a role for FMRP in G-protein signaling.

5. Overexpression of the tyrosine kinase activity deficient TrkB.T1 receptor increases the proliferation of cultured NPCs and simultaneously decreases the survival of the neurosphere-forming cells. Exogenous BDNF abolishes the defects in the survival of neurosphere-forming cells, which suggests that TrkB.T1 may inhibit BDNF/TrkB signaling in NPCs by sequestering the endogenous BDNF. TrkB.T1 overexpression increases neuronal differentiation of NPCs. Neuronal apoptosis is paralleled by increase in neuronal differentiation suggesting a homeostatic feedback loop between the survival of neurons and proliferation rate of NPCs.
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