Neural progenitor cell differentiation and migration

- Role of glutamate signaling, brain-derived neurotrophic factor, and hypoxia/acidosis

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ACADEMIC DISSERTATION

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“Your brain never stops developing and changing. It's been doing it from the time you were an embryo, and will keep on doing it all your life. And this ability, perhaps, represents its greatest strength.”

- James Trefil (from *Are We Unique?*, 1997)

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I. **Jansson LC**, Wigren H-K, Nordström T, Åkerman KE. Functional α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors in differentiating embryonic neural progenitor cells. *Neuroreport* 2011; 22, 282-287.

II. **Jansson LC**, Louhivuori L, Wigren H-K, Nordström T, Louhivuori V, Castrén ML, Åkerman KE. Effect of glutamate receptor antagonists on migrating neural progenitor cells. *Eur J Neurosci* 2013; 37, 1369-1382.

III. **Jansson LC**, Wigren H-K, Nordström T, Louhivuori L, Louhivuori V, Castrén ML, Åkerman KE. Brain-derived neurotrophic factor increases the motility of a particular *N*-methyl-D-aspartate /GABA-responsive subset of neural progenitor cells. *Neuroscience* 2012; 224, 223-234

IV. Nordström T, **Jansson LC**, Louhivuori LM, Åkerman KEO. Effects of acute hypoxia/acidosis on intracellular pH in differentiating neural progenitor cells. *Brain Res* 2012; 1461, 10-23.

ABSTRACT

Multipotent neural progenitors are the cells from which all of the mammalian central nervous system (CNS) develops. During development the cells proliferate actively and differentiate into all the different cell types of the adult brain. Neurogenesis continues in the adult brain but to a much lesser extent than during development and is restricted to the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampus. Adult neurogenesis is influenced by many different factors, including insults to the brain and neurodegenerative diseases. Neurotransmitters acting through neurotransmitter receptors have been implicated as regulators of neurogenesis. The main excitatory neurotransmitter glutamate is linked to neural progenitor cell proliferation, differentiation, neurite extension, and cell migration. Glutamate is also involved in the pathogenesis of several neurological disorders. Other factors linked to brain pathogenesis, such as hypoxia and acidosis, are also known to influence neural progenitor cells. Elucidating the mechanisms governing stem cell behavior during normal and pathological conditions will aid in the development of cell-based therapies for treating insult or disease within the CNS. The aim of this thesis was to study the role of glutamate receptor agonists and antagonists in neural progenitor cell differentiation and migration to increase the understanding of how this neurotransmitter influences these cells. In addition, the effects of brain-derived neurotrophic factor (BDNF) and the reactivity of the cells to components associated with ischemic stroke were studied.

In these studies we utilized the neurosphere model to study neural differentiation and cell migration by combining gene expression analysis and immunocytochemical staining with Ca^{2+} and time-lapse imaging. We found that the neurosphere-derived cells initially mainly expressed and responded to stimuli through metabotropic glutamate receptor 5 (mGluR5) and that receptor expression and the functional response of the receptor corresponded to the distribution of radial glial cells. Ionotropic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate (KA) receptors were also present during early differentiation and expressed mainly by neuron-like cells. The expression of mGluR5 decreased and the expression and functional maturity of AMPA/KA receptors increased with time in differentiating culture. Pharmacological block of glutamate receptor function revealed that radial glial process extension and neuronal motility are regulated through both mGluR5 and AMPA/KA receptors, but that the receptors have opposing effects on these cellular characteristics. After prolonged differentiation a small subpopulation of neuronal cells responding to stimulation with *N*-methyl-D-aspartate (NMDA) and γ-amino butyric acid (GABA) appeared. This subpopulation of cells was responsive to motogenic and neurogenic actions mediated by BDNF. BDNF was shown to enhance neuronal differentiation and to promote cell migration by lengthening periods of active movement and shortening stationary periods. In addition, we found that, by measuring membrane potential and intracellular pH, two distinct cell populations with different endogenous properties and which reacted differently to hypoxic, acidic, and alkalinizing conditions could be identified. These two cell populations corresponded to radial glial and neuron-like cells.

Taken together, the results presented in this thesis provide evidence that the neurosphere model is a suitable tool to study neural progenitor cell differentiation and migration and various factors influencing these processes *in vitro*. This study contributes new information regarding neural progenitor cell characteristics and their behavior when differentiated in the presence of or challenged with factors influencing neurogenesis, both during normal and pathological conditions. These findings may be useful in developing treatment programs for neurological disorders.

ABBREVIATIONS

AC	adenylyl cyclase
ADAR	adenosine deaminase acting on RNA
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ATP	adenosine triphosphate
BCECF-AM	bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CGE	caudal ganglionic eminence
CNPase	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CTZ	cyclothiazide
DAG	diacylglycerol
DAPI	4’,6-diaminodino-2-phenylindole
DHPG	(S)-3,5-dihydroxyphenylglycine
$DiBAC_4(3)$	bis-(1,3-dibutylbarbituric acid)-trimethine oxonol
EAATs	excitatory amino acid transporters
EBSS	Earle’s balanced salt solution
EGF	epidermal growth factor
Fura-2-AM	fura-2 acetoxymethyl ester
GABA	γ-amino butyric acid
GFAP	glial fibrillary acidic protein
GLAST	glial glutamate-aspartate transporter
GLT-1	glial glutamate transporter-1
HBM	HEPES-buffered media
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
iGluRs	ionotropic glutamate receptors
INM	interkinetic nuclear migration
iPS	induced pluripotent stem cell
IP_3	inositol 1,4,5-trisphosphate
IPCs	intermediate progenitor cells
IZ	intermediate zone
KA	kainate
LGE	lateral ganglionic eminence
LTD	long-term depression
LTP	long-term potentiation
MAP-2	microtubule-associated protein-2
MGE	medial ganglionic eminence
mGluRs	metabotropic glutamate receptors
MPEP	2-methyl-6-(phenylethynyl)-pyridine
mRNA	messenger ribonucleic acid
MZ	marginal zone
NeuN	neuron-specific nuclear protein
NMDA	*N*-methyl-D-aspartate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH_i	intracellular pH
PhTx	philanthotoxin
PIP_2	phosphatidylinositol 4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
$p75^{NTR}$	p75 neurotrophin receptor
RMS	rostral migratory stream

RNA	ribonucleic acid
rpm	rotations per minute
SGZ	subgranular zone
SVZ	subventricular zone
Trk	tropomyosin-related kinase
TRPC	canonical transient receptor potential
VGLUTs	vesicular glutamate transporters
VZ	ventricular zone

1 INTRODUCTION

For a long time it was believed that neurons and glia originate from different progenitor cells and that neurogenesis in mammals was restricted to the developing brain. It is now known that all the cells of the developing brain originate from multipotent neural progenitor cells and that neurogenesis continues throughout adult life, although in a very restricted manner. The identification of multipotent neural progenitors in the adult brain initiated efforts to understand the behavior of these cells, both during development and in adult neurogenesis, in more detail. Various neurodegenerative diseases and traumatic insults to the central nervous system have devastating consequences, affecting the lives of millions worldwide. The lack of cure for these conditions has, together with progress made within the field of stem cell research, inspired and challenged scientists trying to develop cell-based therapies for various neurological disorders for decades already. Old questions are answered but new questions continue to arise. This thesis addresses some of the questions regarding neural progenitor cell differentiation and migration and the endogenous properties of these cells during development and under circumstances associated with pathological conditions.

2 REVIEW OF THE LITERATURE

2.1 Stem cells

Stem cells are by definition cells that through self-renewal are able to give rise to identical daughter cells and that have the ability to generate differentiated cell types with more restricted potential. There are a variety of different stem cell populations, as presented in Figure 1, with varying potential for differentiation. Totipotent stem cells are the only cells that can truly give rise to all the cell types of the organism, including extraembryonic tissues, and they exist only in the early embryo. Pluripotent stem cells are able to differentiate into all cell types of the three germ layers; the mesoderm, endoderm, and ectoderm, but cannot produce a whole new individual as pluripotent cells cannot differentiate into extraembryonic tissues (Thomson *et al.,* 1998; reviewed by Smith, 2001; Weissman *et al.,* 2001). Pluripotent stem cells can be derived from the inner cell mass of the blastocyst of many species, including mice and humans (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.,* 1998), or generated from differentiated cells by retroviral introduction of a defined set of transcriptor factor genes to the cell genome (induced pluripotent stem cells, iPS) (Takahashi and Yamanaka, 2006; Takahashi *et al.,* 2007). Multipotent stem cells are more restricted in their differentiation potential than pluripotent stem cells. They exist in various tissues of the body and can give rise to the cells of that tissue. For example, hematopoietic stem cells in the bone marrow gives rise to the various cells of the circulation system; mesenchymal stem cells generate muscle, fat, cartilage, and bone; epidermal stem cells gives rise to cells of the skin; and neural stem cells differentiate into all the cells of the neuroectodermal lineage. In addition, unipotent stem cells like the spermatogonial stem cells of the testes, which can only generate a single cell type, exist (reviewed by Weissman *et al.,* 2001; Lanza *et al.,* 2009). The mechanisms governing stem cell self-renewal and differentiation are tightly governed by both cell-intrinsic and cell-extrinsic factors within the stem cell niche (reviewed by Alvarez-Buylla and Lim, 2004; Jones and Wagers, 2008).

2.1.1 Neural stem/progenitor cells

Multipotent stem cells deriving from the developing or adult mammalian central nervous system (CNS) are termed neural stem or progenitor cells (reviewed by Gage, 2000). The developing brain originates from neural progenitor cells in a manner that is tightly controlled both temporally and spatially. Small populations of neural progenitor cells persist in restricted regions of the brain during postnatal development and into adulthood (reviewed by Kriegstein and Alvarez-Buylla, 2009). Neural progenitor cells can be isolated from the ventricular (VZ) and subventricular (SVZ) zones of the brain (Davis and Temple, 1994; Vescovi *et al.,* 1999) and the lumen of the spinal cord (Kalyani *et al.,* 1997, 1998) during development, and from the SVZ of the lateral ventricles (Lois and Alvarez-Buylla, 1993; Doetsch *et al.,* 1999; Gritti *et al.,* 1999; Johansson *et al.,* 1999; Doetsch *et al.,* 2002) and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus in the adult (Kaplan and

Bell, 1984; Seri *et al.*, 2001). These cells have the ability to self-renew and the capacity to differentiate into all the major cell types of the CNS; i.e. neurons, astrocytes, and oligodendrocytes (reviewed by Kriegstein and Alvarez-Buylla, 2009). Neural progenitor cells can be propagated *in vitro* in an undifferentiated state in the presence of the mitogens basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Reynolds and Weiss, 1992; Doetsch *et al.*, 1999; Gritti *et al.*, 1999). The cells typically form free-floating cellular aggregates of proliferating cells termed neurospheres (Reynolds and Weiss, 1992). Growth factor withdrawal induces spontaneous neural cell differentiation (Reynolds and Weiss, 1992; Doetsch *et al.*, 1999).

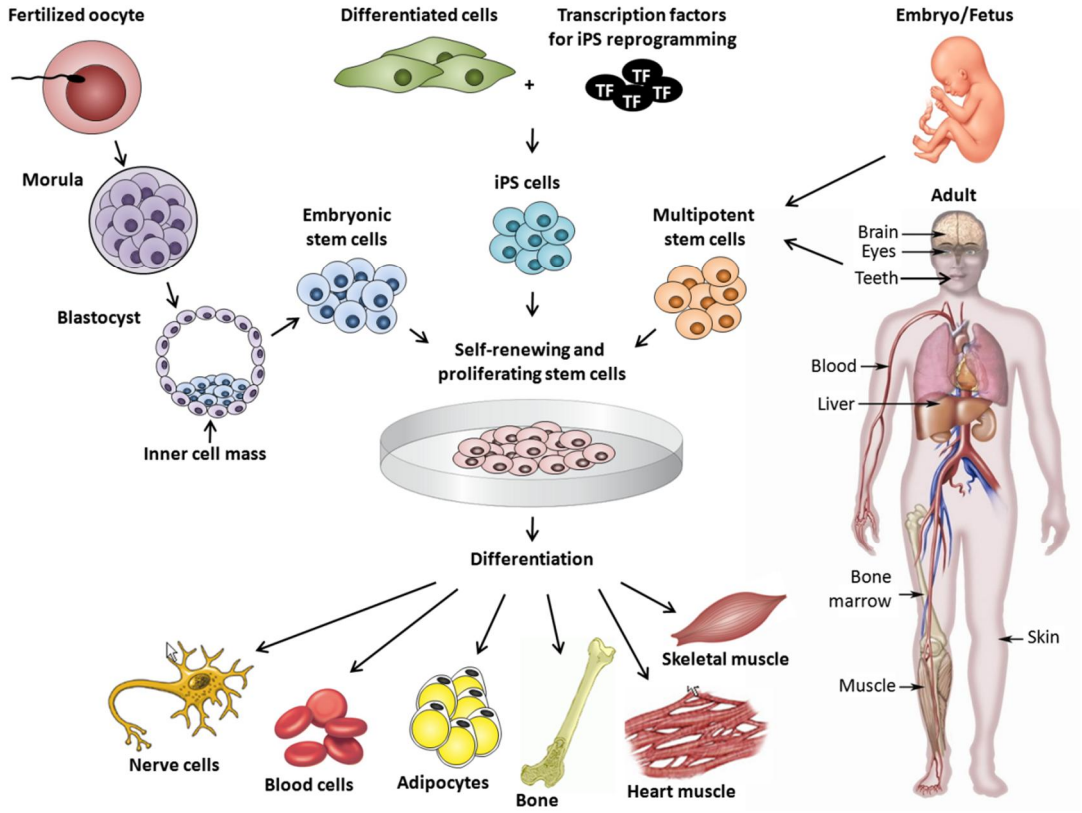

Figure 1. Origin of stem cell populations. Pluripotent stem cells can be derived from the inner cell mass of the blastocyst or by reprogramming of differentiated cells. Multipotent stem cells can be derived from various tissues of the body at various stages of development. iPS, induced pluripotent stem cells; TF, transcription factor.

2.2 Development of the mammalian cortex

The mammalian neocortex is a complex but highly organized structure responsible for cognition, sensory perception, and motor function. The six-layered cortex contains a wide range of different neurons and supporting glial cells, a total of around 50-60 billion in the adult human cortex (Pelvig *et al.*, 2007), located in distinct areas and layers with common birth dates and similar morphological features, gene expression profiles, physiological functions, and projection patterns.

2.2.1 Early corticogenesis

During early embryonic development the ectoderm forms out of the pluripotent stem cells of the blastocyst. The neural plate forms out of the ectoderm on the dorsal side of the embryo. The neural plate then folds and closes, generating the neural tube. All the structures of the forebrain are generated from the neuroepithelial cells lining the ventricular walls in the most anterior part of the neural tube. The ventricular system forms out of the cavity of the neural tube. The embryonic telencephalon consists of the dorsal pallium (roof) and the ventral subpallium (base). The neocortex derives mainly from the anterior and lateral parts of the dorsal pallium but cortical interneurons originate in the ganglionic eminences of the ventral subpallium. Early cortical development consists of several distinct stages. Initially the neuroepithelium of the neural plate acquire an anterior/posterior identity. Dorsal/ventral patterning of the neural tube then results in the generation of regional subdomains of progenitor cells, which differ markedly based on their gene expression profiles and gradients of morphogens, thus enabling the production of the various different cell types of the forebrain (neuronal specification). (Reviewed by Marín and Rubenstein, 2003; Wilson and Houart, 2004; Ayala *et al.*, 2007; Bystron *et al.*, 2008).

At the onset of cortical neurogenesis, pictured in Figure 2, a single layer of homogenous neuroepithelial cells line the ventricular cavity. Symmetric division of neuroepithelial cells increases the thickness and surface area of the embryonic cerebral wall, forming the VZ. As neurogenesis continues the neuroepithelial cells transform into radial glial cells. The radial glial cells express molecular markers like glial glutamate-aspartate transporter (GLAST), RC2, and brain lipid binding protein that distinguish them from neuroepithelial cells (Hartfuss *et al.*, 2001; Malatesta *et al.*, 2003; reviewed by Götz and Huttner, 2005; Bystron *et al.*, 2008; Kriegstein and Alvarez-Buylla, 2009). Radial glial cells in the developing cortex of non-human primates and humans also express glial fibrillary acidic protein (GFAP) (Levitt and Rakic, 1980; Zecevic *et al.*, 2005). In the developing CNS of rodents the expression of GFAP is, however, low or absent (Sancho-Tello *et al.*, 1995; Hartfuss *et al.*, 2001; Imura *et al.*, 2003). The SVZ, containing proliferating intermediate progenitor cells (IPCs) and which is the major contributor of cells to the cortex during late neurogenesis, then forms immediately above the VZ (Haubensak *et al.*, 2004; Zecevic *et al.*, 2005; reviewed by Bystron *et al.*, 2008). Cortical neurons are born as a result of asymmetric division of radial glial cells, either directly or through neurogenic IPCs. The IPCs, in turn, divide symmetrically, producing either two new IPCs or two neurons (Noctor *et al.*, 2004, 2008). The radial glial cells function as neural progenitors of the brain and are capable of producing neurons and glial cells of remarkable diversity depending on developmental stage and location within the brain (Anthony *et al.*, 2004; reviewed by Kriegstein and Alvarez-Buylla, 2009).

The first wave of neurons migrating out of the proliferative zones form the preplate while a second wave of migrating neurons splits the preplate into two layers, the deep subplate and the superficial marginal zone (MZ) in between which the cortical plate forms (Luskin and Shatz, 1985). The MZ forms the future layer I of the cortex and successive waves of

migrating postmitotic neurons arriving at the cortical plate form the layers II-VI of the cortex (layer VI being the innermost layer) in an inside-out manner so that neurons that are born later migrate past earlier-born neurons. The time when a neuron is born correlates tightly with its laminar fate (Rakic, 1974) and earlier-born cells are more plastic than later-born cells. The subplate becomes separated from the SVZ by the intermediate zone (IZ), which in the mature brain contains myelinated afferent and efferent axons of the cortex. (Reviewed by Ayala *et al.*, 2007; Molyneaux *et al.*, 2007; Bystron *et al.*, 2008).

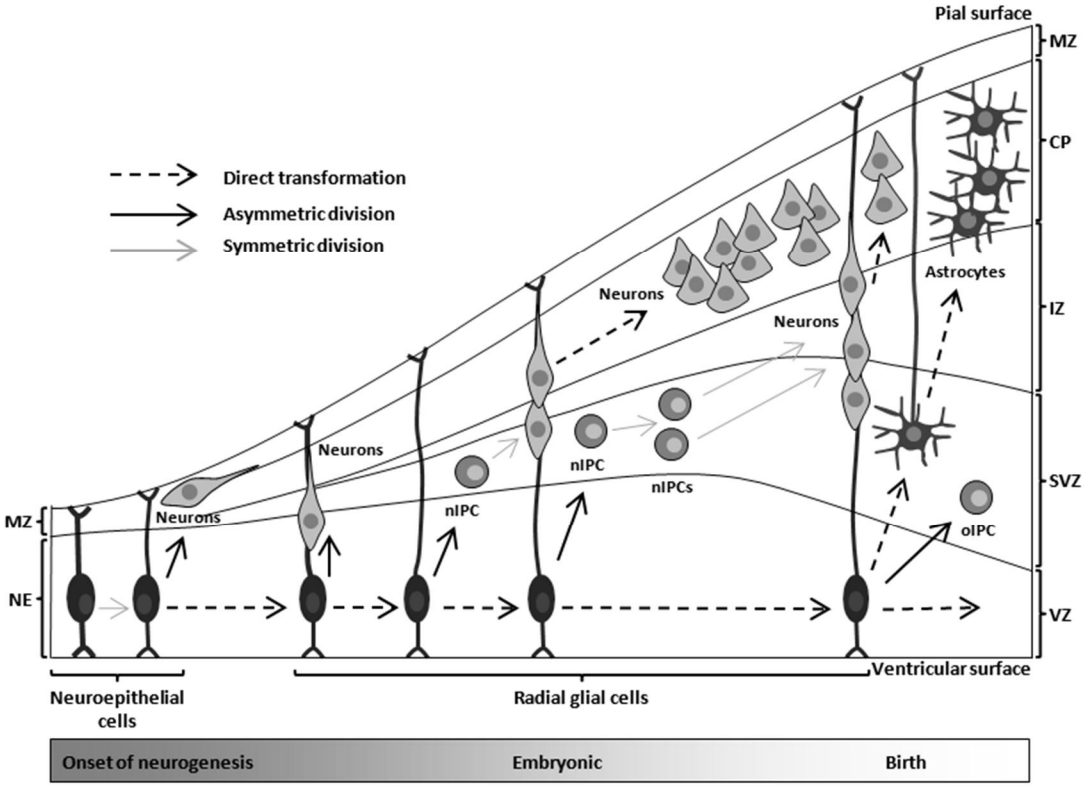

Figure 2. Cortical neurogenesis. Neuroepithelial cells transform into radial glial cells that generate neurons, either directly or through intermediate progenitor cells. Later in development astrocytes and oligodendrocytes are produced. CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; NE, neuroepithelium; nIPC, neurogenic intermediate progenitor cell; oIPC, oligodendrocytic progenitor cell; SVZ, subventricular zone; VZ, ventricular zone. Adapted from Kriegstein and Alvarez-Buylla, 2009.

2.2.2 Cortical neurons

There are two main classes of cortical neurons, excitatory glutamatergic projection neurons and inhibitory GABAergic local circuit interneurons, both of which there are a variety of neuronal subtypes with different morphological, molecular, and physiological properties. The glutamatergic neurons originate from the neuroepithelial cells lining the wall of the lateral ventricles, see Figure 3A (Rakic, 1974; reviewed by Molyneaux *et al.*, 2007). The GABAergic interneurons, in turn, derive from the medial (MGE), lateral (LGE), and caudal (CGE) ganglionic eminences of the ventral telencephalon from where they migrate to the cortex through the MZ, IZ, or SVZ, and to the olfactory bulb, as depicted in Figure 3A (De Carlos *et al.*, 1996; Anderson *et al.*, 1997; Tamamaki *et al.*, 1997; Lavdas *et al.*, 1999;

Wichterle *et al.,* 1999; Anderson *et al.,* 2001; Jiménez et al, 2002; Nery *et al.,* 2002; Polleux *et al.,* 2002; Yozu *et al.,* 2005; Taniguchi *et al.,* 2011). Parvalbumin- and somastotatin-expressing cortical interneurons derive primarily from the MGE, the major source for cortical interneurons, while calretinin-expressing cells derive from the CGE (Wichterle *et al.,* 1999; Anderson *et al.,* 2001; Xu *et al.,* 2004). The LGE produces mainly striatal medium spiny neurons and olfactory bulb interneurons but also some cortical cells (Anderson *et al.,* 2001; Stenman *et al.,* 2003). In humans interneurons are also generated locally in the SVZ of the dorsal telencephalon (Letinic *et al.,* 2002; Zecevic *et al.,* 2005).

Both glutamatergic and GABAergic cells are found in each of the six cortical layers but glutamatergic neurons are much more abundant than GABAergic neurons, which comprise only about one fifth of the cortical neurons (Hendry *et al.,* 1987). Glutamatergic neurons project their axons to various cortical and subcortical structures. The GABAergic interneurons, in turn, make local connections within the cortex. (Reviewed by Markram *et al.,* 2004; Molyneaux *et al.,* 2007)

2.2.3 Modes of cortical migration

Radial glial cells of the VZ have two processes (Figure 2 and Figure 3B), a shorter one that extends to the ventricular surface and a longer one that extends to the pial surface (Noctor *et al.,* 2001, 2008). The pial process of the radial glial cells grows longer as the cortical plate thickens. As radial glial cells divide they undergo so-called interkinetic nuclear migration (INM), see Figure 3B. During INM the cell body travels up and down within the VZ, along the radial process, depending on the phase of the cell cycle so that the S phase occurs when the cell body is located closer to the pial surface and the M phase when the cell body is closer to the ventricular surface (Haydar *et al*., 2000; reviewed by Götz and Huttner, 2005). IPCs located in the SVZ may have multiple processes but these are not in contact with either the ventricular or the pial surface and the cells do not undergo INM (Noctor *et al.,* 2004).

After the last cell division newborn postmitotic glutamatergic neurons exit the proliferative zones and migrate along the pial process of the radial glial cells to their laminar destination in the cortex through the process of radial migration, see Figure 3B (Rakic, 1974; Haydar *et al*., 1999; Miyata *et al.,* 2001; Noctor *et al.,* 2001; reviewed by Marín and Rubenstein, 2003). This mode of migration is also known as locomotion and was suggested already in the late 19th century by Santiago Ramón y Cajal. It is the main mode of cortical migration and characterized by saltatory motion with sporadic pauses. When neuronal migration ceases at the end of cortical development the radial glial cells lose contact with the ventricular surface and migrate to the cortical plate through a process called somal translocation. (Nadarajah *et al.,* 2001). During somal translocation (Figure 3B), which is radial glia-independent, the long leading process is shortened as the cell body moves closer to the pial surface in a relatively smooth and continuous motion. The cells also have a short trailing process, the remains of the ventricular process. (Brittis *et al.,* 1995; Miyata *et al.,* 2001; Nadarajah *et al.,* 2001). As the

cells reach the cortical plate they differentiate into cortical astrocytes, see Figure 2 (Noctor *et al.*, 2004, 2008).

GABAergic interneurons deriving from the ganglionic eminences migrate to the cortex and the olfactory bulb by tangential migration, see Figure 3A (Lois and Alvarez-Buylla, 1994; De Carlos *et al.*, 1996; Anderson *et al.*, 1997; Tamamaki *et al.*, 1997; Lavdas *et al.*, 1999; Wichterle *et al.*, 1999; Anderson *et al.*, 2001; Jiménez et al, 2002; Nery *et al.*, 2002; Polleux *et al.*, 2002; Yozu *et al.*, 2005; Taniguchi *et al.*, 2011). This non-radial form of migration occurs by cells migrating on or in close association with each other, along axonal fibers, or independently of any specific substrate (reviewed by Marín and Rubenstein, 2003). The cells migrating towards the cortex by tangential migration are transiently directed towards the ventricle before reaching their final destination in the cortex through radial migration, see Figure 3A (reviewed by Kriegstein and Noctor, 2004; Ayala *et al.*, 2007). The migratory route to the olfactory bulb along which neuroblasts, immature dividing neurons (Menezes *et al.*, 1995), migrate tangentially is known as the rostral migratory stream (RMS) (Doetsch and Alvarez-Buylla, 1996; Lois *et al.*, 1996; Kornack and Rakic, 2001; Pencea *et al.*, 2001).

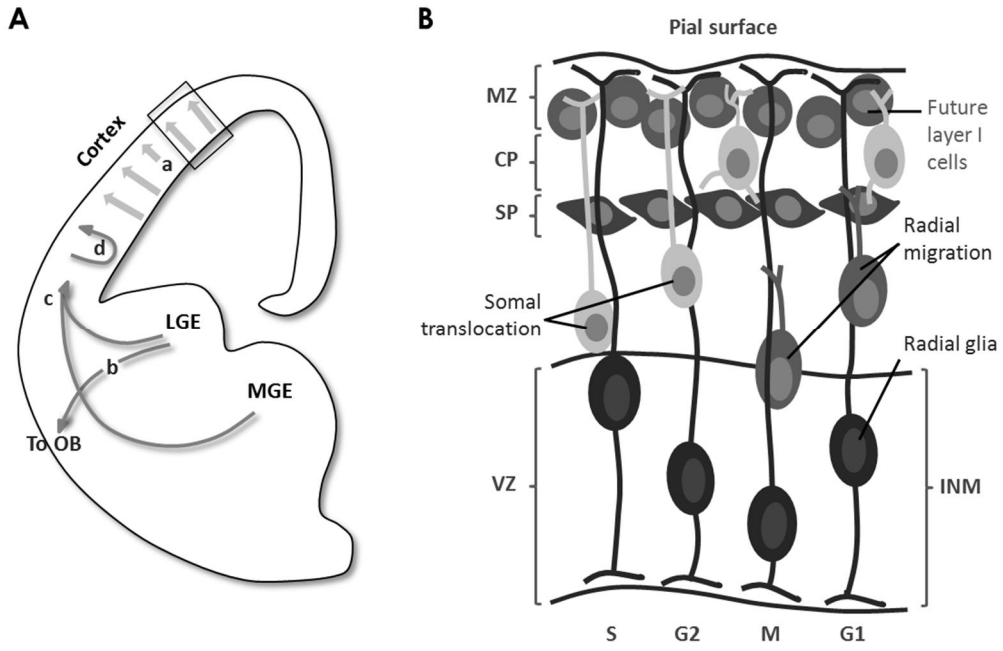

Figure 3. Cortical migration. (A) Cell migration to the cortex and the olfactory bulb. (a) Radial migration of newborn glutamatergic neurons from the dorsal VZ to the cortex. (b, c) GABAergic interneurons originate in the ganglionic eminences and migrate tangentially to the olfactory bulb (b) and cortex (c). (d) The cortical interneurons reach their final destination in the cortex after a transient stage of ventricle-directed migration followed by radial migration. (B) Modes of cortical migration in the developing cortex (indicated by the boxed area depicted in A). Radial glial cells extend processes to the ventricular and pial surfaces and undergo INM as they divide. Postmitotic neurons reach their laminar destination through the radial glia-dependent process of radial migration, also known as locomotion. At the end of corticogenesis radial glial cells lose contact with the ventricular surface and migrate to the cortical plate in a radial glia-independent process known as somal translocation. CP, cortical plate; INM, interkinetic nuclear migration; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, marginal zone; OB, olfactory bulb; SP, subplate; VZ, ventricular zone. Adapted from Ayala *et al.*, 2007.

2.3 Neurogenesis in the adult brain

In the adult mammalian brain neurogenesis is restricted to two sites, the SVZ (Lois and Alvarez-Buylla, 1993; Doetsch *et al.,* 2002) and the SGZ (Kaplan and Bell, 1984; Seri *et al.,* 2001, 2004), as shown in Figure 4. The origin of the progenitor cells of the adult brain have not been conclusively determined but lineage-tracing studies suggest that they originate from the radial glial cells of the developing brain (Merkle *et al.,* 2004; Seri *et al.,* 2004; reviewed by Ihrie and Alvarez-Buylla, 2008). The neural progenitors are maintained in and regulated by the microenvironment of the stem cell niche, which also directs the differentiation of progenitor cell progeny (reviewed by Jones and Wagers, 2008).

The primary stem cells of the SVZ, see Figure 4A, correspond to GFAP-positive, slowly dividing astrocytic type B cells (Doetsch *et al.,* 1997, 1999). The cells express some of the properties of both radial glial and neuroepithelial cells. GFAP-negative type C cells are generated from type B cells through asymmetrical division and they function as IPCs, or transit-amplifying progenitor cells, are highly proliferative, and are found in clusters next to chains of migrating neuroblasts (Doetsch *et al.,* 1997, 1999; reviewed by Kriegstein and Alvarez-Buylla, 2009). Out of these type C cells immature neuroblasts, type A cells, are born, which then migrate along the RMS to the olfactory bulb in tightly associated chains of neuronal precursors ensheathed by GFAP-positive astrocytes (Doetsch and Alvarez-Buylla, 1996; Lois *et al.,* 1996; Doetsch *et al.,* 1997; Peretto *et al.,* 1997, 1999; Kornack and Rakic, 2001; Pencea *et al.,* 2001). As the migrating neuroblasts reach the olfactory bulb they start to migrate radially until they reach their final destination in the bulb where they differentiate into local, mainly GABAergic, granule and periglomerular neurons (Lois and Alvarez-Buylla, 1994; Doetsch *et al.,* 1997, 1999).

Astrocytic GFAP-positive type B cells, or type I progenitors, function as primary stem cells also in the adult SGZ, see Figure 4B (Kaplan and Hinds, 1977; Bayer *et al.,* 1982, 1985; Seri *et al.,* 2001; Filippov *et al.,* 2003; Seri *et al.,* 2004). Type B cells produce immature GFAP-negative type D cells, or type II progenitors, which by division of D1 cells (corresponds to type A neuroblasts of the SVZ) generate D2 cells that through maturation yield D3 cells, which differentiate into mature hippocampal granule neurons (Kaplan and Hinds, 1977; Bayer *et al.,* 1982, 1985; Seri *et al.,* 2001; Filippov *et al.,* 2003; Seri *et al.,* 2004). The cells migrate only a short distance to the granule cell layer (Seri *et al.,* 2004) from where they project axons to the hippocampus, contributing to learning and memory (Kempermann *et al.,* 1997; Markakis and Gage, 1999; Shors *et al.,* 2001).

Neurogenesis is low in the adult brain during normal physiological conditions and declines further with age. Adult neurogenesis is affected, either positively or negatively, by an enriched environment, exercise, sleep deprivation, hormone levels, stress, mood disorders, drug and alcohol abuse, and various pathological conditions such as stroke, seizures, and neurodegenerative disease (Gould *et al.,* 1992; Kempermann *et al.,* 1997; Parent *et al.,* 1997; Tanapat *et al.,* 1999; Eisch *et al.,* 2000; Seri *et al.,* 2001; Arvidsson *et al.,* 2002; Guzman-Marin *et al.,* 2005; reviewed by Fuchs and Gould, 2000; Gage, 2000; Ming and Song, 2005; reviewed by Grote and Hannan, 2007).

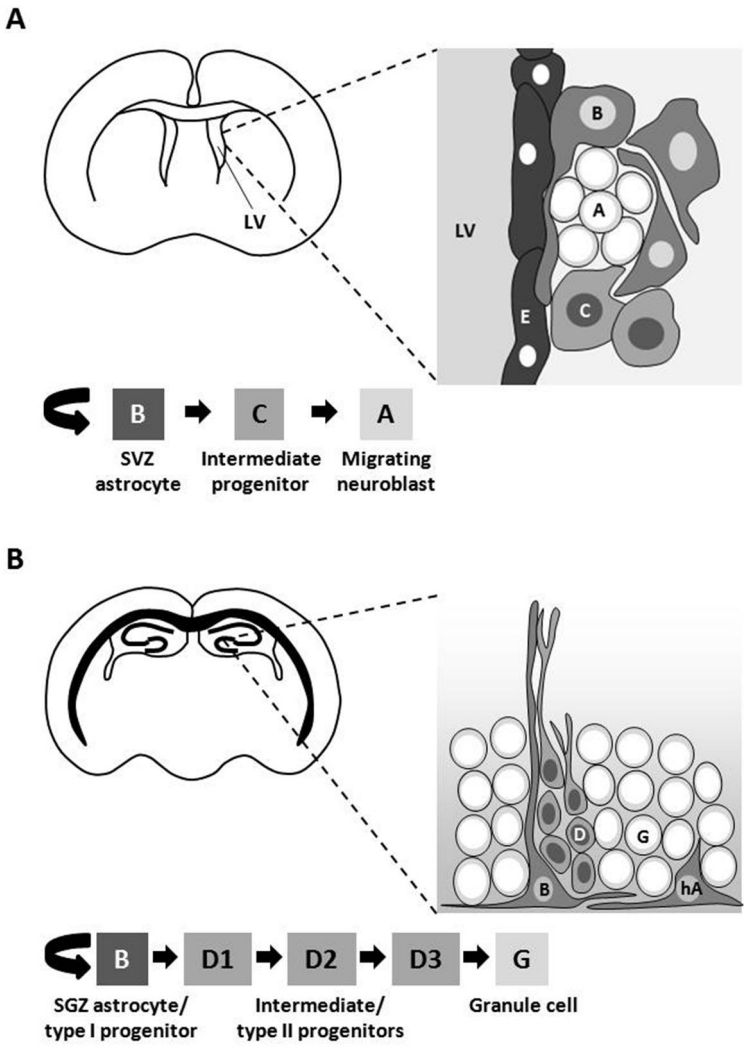

Figure 4. Neurogenesis in the adult brain. The architecture of the neurogenic zones and progression of cell differentiation in the SVZ (A) and the SGZ (B). E, ependymal cell; hA, horizontal astrocyte; LV, lateral ventricle; SGZ, subgranular zone; SVZ, subventricular zone. Adapted from Ihrie and Alvarez-Buylla, 2008.

2.4 Neurotransmitters and neurotransmitter receptors in the developing brain

When neurons in the developing brain reach their final location in the cortex they start to extend neurites to establish synaptic connections with other cells, forming a complex electrical network (reviewed by Spitzer, 2006). Neurotransmitters and their receptors mediate and modulate excitatory and inhibitory signal transmission in the brain but they do not only relay messages, they also affect cell proliferation, differentiation, migration, neurite extension, and integration of newborn neurons into the neural network, both in the developing and adult brain (Komuro and Rakic, 1993; LoTurco *et al.*, 1995; Behar *et al.*, 1998, 1999, 2000; Maric *et al.*, 2000; Lopez-Bendito *et al.*, 2003; Nguyen *et al.*, 2003; Deisseroth *et al.*, 2004; Cuzon *et al.*, 2006), see also Table 1. Neural progenitor cells in the developing brain express functional neurotransmitter receptors already early during neurogenesis, before terminal cell division and synaptogenesis (Komuro and Rakic, 1993; LoTurco *et al.*, 1995; Behar *et al.*, 1998; 1999, 2000; Maric *et al.*, 2000; Lopez-Bendito *et*

al., 2003; Di Giorgi-Gerevini *et al.,* 2004; Nguyen *et al.,* 2003, 2004; Cuzon *et al.,* 2006). Neurotransmitters and their receptors thus seem to have important roles in neuronal development. Two of the most important neurotransmitters during development are glutamate and γ-amino butyric acid (GABA), but also glycine, acetylcholine, serotonin, and dopamine play roles in neurogenesis (Ohtani *et al.,* 2003; Crandall *et al.,* 2007; reviewed by Nguyen *et al.,* 2001).

2.4.1 Glutamate

Glutamate is the major excitatory neurotransmitter in the CNS and essential in behavioral functions such as memory and learning (reviewed by Nakanishi, 1992; Dingledine *et al.,* 1999; Mattson, 2008). In addition to its role at the synapse in signal transmission and neuronal plasticity glutamate is also a key player in neurogenesis. While all the glutamate receptor subtypes seem to be of importance for the various processes of cell proliferation, differentiation, and migration, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate (KA) receptors and metabotropic glutamate receptors (mGluRs) seem to be more involved in regulating cell proliferation and *N*-methyl-D-aspartic acid (NMDA) receptors in promoting neuronal differentiation and migration (LoTurco *et al.,* 1995; Behar *et al.,* 1999; Haydar *et al.,* 2000; Maric *et al.,* 2000; Luk *et al.,* 2003; Brazel *et al.,* 2005; Di Giorgi-Gerevini *et al.,* 2005; Nakamichi *et al.,* 2008), see also Table 1. Glutamate has been proposed to regulate neurogenesis non-synaptically, either directly through ionotropic and/or metabotropic glutamate receptors expressed by neural progenitors, or through nearby cells by activating them to secrete molecules affecting neurogenesis, such as neurotrophic factors (Zafra *et al.,* 1991; Lee *et al.,* 2002; Bai *et al.,* 2003; reviewed by Schlett, 2006; Mattson, 2008). As it turns out, the production of neurotrophic factors like brain-derived neurotrophic factor (BDNF) in neurons can be stimulated by glutamate (Zafra *et al.,* 1991; Mackowiak *et al.,* 2002) while BDNF in turn promotes neurogenesis (Lee *et al.,* 2002). Excess glutamate can, however, decrease neurogenesis and blocking of glutamate receptor function can increase cell proliferation (Cameron *et al.,* 1995; reviewed by Fuchs and Gould, 2000). Receptor activation by glutamate causes local or global changes in the intracellular Ca^{2+} concentration of the target cell. Ca^{2+} is a versatile second messenger and a key regulator of neuronal differentiation, triggering various cellular functions such as cell migration and neurite extension (reviewed by Berridge *et al.,* 2000; Zheng and Poo, 2007).

2.4.1.1 Glutamate transport and metabolism

Glutamate in neurons is synthesized from glutamine by the enzyme glutaminase and packed into synaptic vesicles, where the glutamate concentration can be as high as 100 mM, by vesicular glutamate transporters (VGLUTs), see Figure 5. The VGLUTs (VGLUT1-3) pack glutamate into synaptic vesicles through the mechanism of an electrochemical H^+ gradient. (Reviewed by Meldrum, 2000; Takamori, 2006). VGLUT1 and VGLUT2 are expressed by glutamatergic neurons and astrocytes while VGLUT3 exist in both glutamatergic and non-

glutamatergic neurons, including GABAergic neurons, astrocytes, oligodendrocytes, and neural progenitors (Bezzi *et al.*, 2004; Boulland *et al.*, 2004; Montana *et al.*, 2004). The expression of VGLUT1-3 is developmentally regulated (Boulland *et al.*, 2004). Glutamate is released into the synaptic cleft, where its concentration is in the millimolar range following excitatory stimulation (reviewed by Meldrum, 2000), thus activating ionotropic or metabotropic glutamate receptors on the postsynaptic membrane. Glutamate is rapidly cleared from the synaptic cleft, mainly through uptake by astrocytes surrounding the synaptic cleft, but some pre- and postsynaptic uptake by neurons also occurs (Rothstein *et al.*, 1996; reviewed by Takamori, 2006; Niciu *et al.*, 2012)

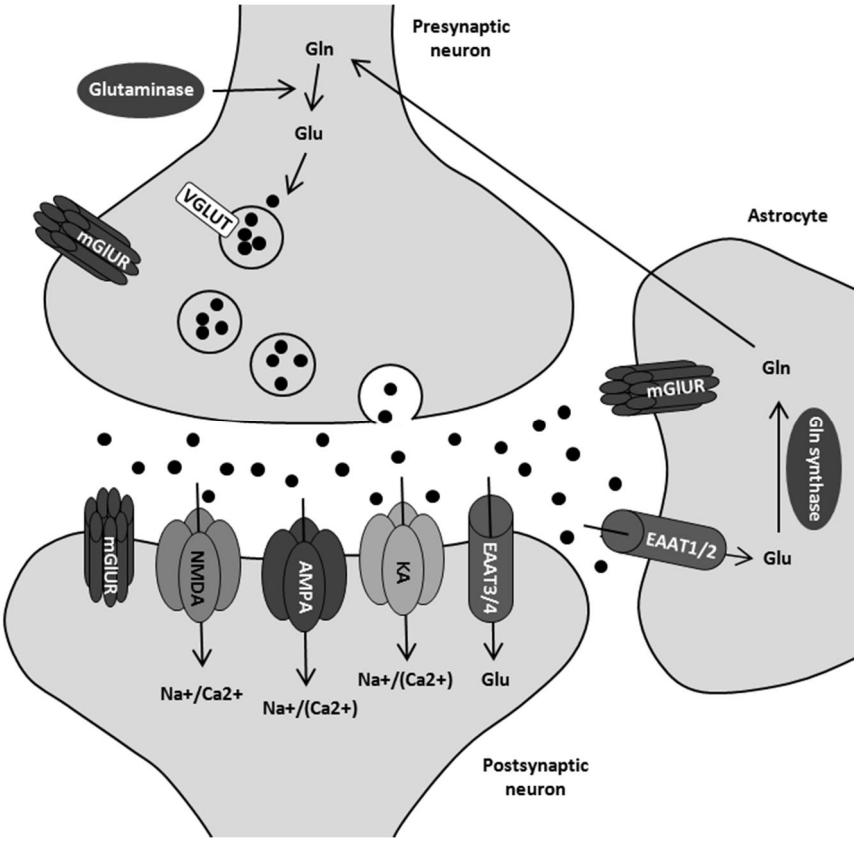

Figure 5. Glutamatergic metabolism, transport, and neurotransmission. Glutamate (Glu) is synthesized from glutamine (Gln) by glutaminase, packed into vesicles by vesicular glutamate transporters (VGLUTs), and released into the synaptic cleft following synaptic activity. Glutamate activates ionotropic (AMPA, KA, and NMDA) or metabotropic (mGluR) glutamate receptors, relaying fast synaptic neurotransmission. Glutamate is cleared from the synaptic cleft though uptake by excitatory amino acid transporters (EAATs), converted back to glutamine by glutamine synthase, released back to the extracellular space, and taken up by neurons. AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; KA, kainate; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate. Adapted from Niciu *et al.,* 2012.

Glutamate uptake is mediated by the action of one or several of the five identified Na^+-dependent excitatory amino acid transporters (EAATs) located in the plasma membrane of astrocytes and neurons, as seen in Figure 5 (reviewed by O'Shea, 2002; Niciu *et al.*, 2012). EAAT1 (corresponds to GLAST in rodents) and EAAT2 (glial glutamate transporter-1,

GLT-1, in rodents) are located mainly on glial cells in the forebrain, cerebellum, and spinal cord but are expressed transiently and to a limited extent by neurons during development (Storck *et al.*, 1992; Furuta *et al.*, 1997b; Plachez *et al.*, 2000). EAAT2/GLT-1 is responsible for most of the glutamate uptake. Both EAAT1/GLAST and EAAT2/GLT-1 are found in the brain from early neurogenesis, in regionally distinct patterns, and their expression increases as the brain matures (Furuta *et al.*, 1997b; Hartfuss *et al.*, 2001). In particular, EAAT1/GLAST and EAAT2/GLT-1 are expressed by radial glial cells of the embryonic proliferative zones (Sutherland *et al.*, 1996; Furuta *et al.*, 1997b; Hartfuss *et al.*, 2001). EAAT1/GLAST is also found in cortical neural progenitor cells of the adult brain (Hartfuss *et al.*, 2001). EAAT3 (EAAC1 in rodents) is a neuron-specific transporter that is largely expressed in the CNS but its expression decreases upon brain maturation (Furuta *et al.*, 1997a, b). EAAT4 is mainly present on cerebellum neurons with some expression in the forebrain and the expression increases as neurogenesis proceeds (Furuta *et al.*, 1997a, b; Dehnes *et al.*, 1998; Massie *et al.*, 2001). EAAT5 is expressed only in the retina (Arriza *et al.*, 1997; Pow and Barnett, 2000). A cysteine/glutamate anti-porter located on glial cells also aids in recycling glutamate. Glutamate that has been taken up from the synaptic cleft by astrocytes is converted back to glutamine by the enzyme glutamine synthase and released to the extracellular space from where it is taken up by neurons, allowing the glutamine/glutamate cycle to start over, see Figure 5. Glutamate is also newly synthesized from glucose. (Reviewed by Niciu *et al.*, 2012).

2.4.1.2 Glutamate excitotoxicity

The concentration of glutamate in the extracellular fluid is low, only up to 2 µM in the adult brain (reviewed by Meldrum, 2000), and tightly regulated. The low glutamate concentration in the extracellular fluid and the rapid clearance of glutamate from the synaptic cleft are important mechanisms in avoiding excitotoxic cell death. High concentrations of glutamate may cause excitotoxicity through sustained, excessive activation of glutamate receptors followed by an increase in intracellular Ca^{2+}, activation of caspases, lipases, and proteases, inflammatory cascades, production of free radicals, and changes in gene expression patterns (Olney, 1969; Olney and Sharpe, 1969; Choi, 1987; Choi *et al.*, 1987; Bonfonco *et al.*, 1995; Portera-Cailliau *et al.*, 1997; reviewed by Lau and Tymianski, 2010). Cell death caused by glutamatergic excitotoxicity is a contributing factor to neuronal loss in cerebral ischemia, traumatic brain injury, neurodegenerative disease, and aging (Portera-Cailliau *et al.*, 1997; reviewed by Mattson, 2008; Lau and Tymianski, 2010). In addition, failure of the glutamatergic signaling system to function normally has been linked to various psychiatric disorders (reviewed by Mattson, 2008). High glutamate levels are, however, found in the developing brain and in neurogenic zones of the adult (Miranda-Contreras *et al.*, 1998, 1999, 2000; Behar *et al.*, 1999; Haydar *et al.*, 2000). As cells in these regions express functional glutamate receptors it seems that neural progenitor cells and immature neurons are not as sensitive to glutamate as are mature neurons.

2.4.2 Glutamate receptors

There are two classes of glutamate receptors, ionotropic ligand-gated ion channels and metabotropic G-protein coupled receptors. The ionotropic glutamate receptors (iGluRs) are divided into NMDA and non-NMDA, AMPA and KA, receptors and the metabotropic receptors into group I, II, and III mGluRs, depending on their selective agonists, see Figure 6. The expression profile of the glutamate receptors and the specific subunit composition of the iGluRs yield an extraordinary diversity of glutamate receptors in the brain, which is of great importance as they shape postsynaptic signals, synaptic plasticity, and gene transcription. The iGluRs are expressed mainly in the CNS, on neurons as well as on glial cells, and mediate the vast majority of fast excitatory neurotransmission in the brain. In addition, Ca^{2+} entry through iGluRs is important during development and for synaptic plasticity underlying behavioral processes and learning. Metabotropic glutamate receptors, in turn, exhibit a modulatory role on synaptic activity and plasticity by activating second messenger pathways and downstream factors. (Reviewed by Hollmann and Heinemann, 1994; Dingledine *et al.,* 1999; Niciu *et al.,* 2012).

2.4.2.1 AMPA and KA receptors

The AMPA receptors are one of the major types of ionotropic glutamate receptors and mediate fast, within 1 millisecond, excitatory synaptic transmission in the CNS with rapidly decaying currents. The AMPA receptors are permeable mainly to Na^+ but also flux some Ca^{2+} and they exhibit substantial molecular and functional diversity. Binding of two molecules of glutamate is required for optimal function of the receptor. (Reviewed by Hollmann and Heinemann, 1994; Dingledine *et al.,* 1999; Niciu *et al.,* 2012). The AMPA receptor subunits GluA1-4 (Collingridge *et al.,* 2009), previously known as GluR1-4 (Boulter *et al.,* 1990) or GluRA-D (Keinänen *et al.,* 1990), see Figure 6, form tetrameric complexes of heteromeric subunits with electrophysiological properties, Ca^{2+} permeability, and receptor desensitization depending on the specific subunit composition of the receptor (Sommer *et al.,* 1990; reviewed by Hollmann and Heinemann, 1994; Dingledine *et al.,* 1999; Niciu *et al.,* 2012). Alternative splicing and ribonucleic acid (RNA) editing increases the number of subunit variants (Sommer *et al.,* 1990).

The AMPA receptor subunits are encoded by four different genes, *Gria1-4,* and alternative splicing of the gene products leads to the existence of two forms of each subunit, so called "flip" and "flop" variants of which the "flip" variants desensitizes slower and to a lesser extent than the "flop" variants (Sommer *et al.,* 1990). The different splice variants also influence receptor sensitivity to allosteric modulators (Miu *et al.,* 2001). During development the "flip" variant is more highly expressed while both variants appear at equal levels in the mature brain. In addition, C-terminal splice variants, which influence protein binding and receptor targeting, of GluA2 and GluA4 exists. (Reviewed by Dingledine *et al.,* 1999; Kew and Kemp, 2005). Most AMPA receptor complexes in the CNS contain GluA1 and GluA2 subunits, with GluA2 typically rendering AMPA receptors impermeable to Ca^{2+} as a

consequence of Q/R-editing of *Gria2* pre-messenger RNA (mRNA) (Burnashev *et al.,* 1995; Geiger *et al.,* 1995; Whitney *et al.,* 2008). This Q/R-editing is almost 100% in the adult CNS and necessary for survival, as shown by the postnatal death of mice lacking the RNA-editing enzyme adenosine deaminase acting on RNA 2 (ADAR2) (Higuchi *et al.,* 2000). $ADAR2^{-/-}$ mice exhibit reduced expression of GluA1 and GluA2 receptor subunits in brain extracts and altered AMPA receptor channel properties in glutamatergic neurons, including increased receptor desensitization and Ca^{2+} permeability, leading to seizures and premature death (Higuchi *et al.,* 2000). Human neural progenitor cells have been shown to express unedited Ca^{2+}-permeable AMPA receptors while mature glutamatergic neurons derived from human neural progenitors mostly express edited Ca^{2+}-impermeable AMPA receptors (Whitney *et al.,* 2008). The Q/R-editing of *Gria2* pre-mRNA also controls the number of AMPA receptors by influencing receptor assembly and trafficking. AMPA receptors are diffusely detected on dendrites during development but are later found mainly on postsynaptic membranes. The AMPA receptors are synthesized and assembled in the lipid bilayer of the endoplasmic reticulum and traffic rapidly between the intracellular compartments and the plasma membrane. The AMPA receptors are inserted into the plasma membrane through vesicle-mediated fusion and undergo clathrin-dependent internalization (Man *et al.,* 2000). The receptors can also diffuse laterally within the plasma membrane, away from and into the synaptic cleft, a process regulated by dendritic spine morphology (Ashby *et al.,* 2006; reviewed by Newpher and Ehlers, 2008). Receptor trafficking is highly dynamic, dependent on the subunit composition of the receptor, and essential for synaptic plasticity. GluA1 insertion into the plasma membrane is mainly activity-dependent while GluA2 insertion is constitutive. (Reviewed by Dingledine *et al.,* 1999; Groc and Choquet, 2006; Greger *et al.,* 2007; Rao and Finkbeiner, 2007).

The KA receptors consist of the GluK1-5 subunits, see Figure 6, two related subunit families formerly known as GluR5-7 (GluK1-3) and KA1-2 (GluK4-5), which combine to form heteromeric tetramers (Alt *et al.,* 2004; Collingridge *et al.,* 2009; reviewed by Hollmann and Heinemann, 1994; Lerma *et al.,* 2001). The GluK1-3 subunits can also form functional homomeric tetramers while GluK4-5 requires the presence of GluK1, GluK2 or GluK3 to form functional channels. Alternative splicing and RNA editing of KA receptor subunits increases subunit variants, affecting ion permeability and functional properties of the receptor complexes, and varies during development. (Burnashev *et al.,* 1995; Alt *et al.,* 2004; reviewed by Dingledine *et al.,* 1999; Lerma *et al.,* 2001). The KA receptors are fast acting, mostly Ca^{2+}-impermeable glutamate receptors that bind glutamate with a higher affinity than AMPA receptors. KA receptors, like AMPA receptors, desensitize rapidly upon glutamate binding but recovers from desensitization slower than AMPA receptors. Unlike AMPA receptors, synaptic KA receptors typically display slow kinetics due to neuropilin and tolloid-like (NETO) 1/2 auxiliary subunits. The KA receptors are widely expressed in the CNS on both pre- and postsynaptic membranes. (Reviewed by Dingledine *et al.,* 1999; Lerma *et al.,* 2001; Copits and Swanson, 2012).

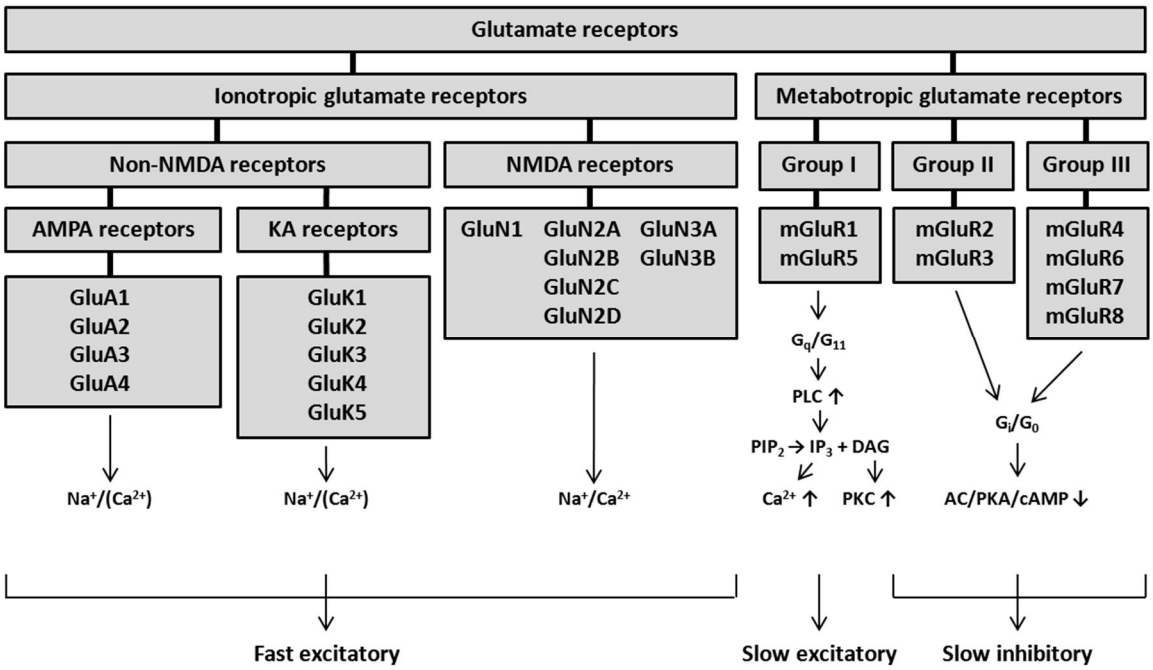

Figure 6. Glutamate receptor subtypes and mechanisms of signal transduction. The ionotropic glutamate receptor family consists of AMPA, KA, and NMDA receptors with various AMPA, KA, and NMDA receptor subunits. The metabotropic glutamate receptors consist of the mGluR1-8 subunits, which are divided into group I, II, and III mGluRs. Ionotropic glutamate receptors are permeable to cations and metabotropic glutamate receptors couple through G proteins. Group I mGluRs activate phospholipase C (PLC) with subsequent formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-biphosphate (PIP_2), leading to Ca^{2+} release from intracellular stores and protein kinase K (PKC) activation. Group II and III mGluRs inhibit adenylyl cyclase (AC) and protein kinase A (PKA) and reduces cyclic adenosine monophosphate (cAMP) levels. AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; KA, kainate; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate. Adapted from Kew and Kemp, 2005.

2.4.2.2 NMDA receptors

NMDA receptors are the other major type of ionotropic glutamate receptors. They are highly permeable to Ca^{2+} and are also permeable to Na^+. NMDA receptor activation by glutamate causes a longer membrane depolarization compared to that of AMPA receptors. The NMDA receptor family consists of seven subunits, GluN1, GluN2A-D, and GluN3A-B (formerly NR1, NR2A-D, and NR3A-B), see also Figure 6 (Monyer *et al.*, 1992; Ciabarra *et al.*, 1995; Collingridge *et al.*, 2009; reviewed by Hollmann and Heinemann, 1994). Additional splice variants increase subunit diversity, affecting receptor expression and function (Nakanishi *et al.*, 1992). The GluN1 gene *Grin1* encodes three alternatively spliced exons, resulting in a total of eight different functional transcripts with different properties and distribution. The NMDA receptor is a heterotetramer and for the NMDA receptors to be functional the GluN1 subunit must be present in the receptor complex in two copies (Monyer *et al.*, 1992). In addition to GluN1 the receptor complex contains GluN2 or GluN2 and GluN3 subunits, which have more regulatory roles in the receptor complex. Also GluN1/GluN3 receptor complexes exist but they are not activated by glutamate or NMDA but instead form excitatory glycine receptors (Chatterton *et al.*, 2002). (Reviewed by Dingledine *et al.*, 1999; Yamakura and Shimoji, 1999).

The NMDA receptors have the highest affinity for glutamate but for the NMDA receptor to be activated by glutamate the presence of its modulator glycine is required (Kleckner and Dingledine, 1988). Glycine itself has no action on the NMDA receptor but it promotes the action of the receptor by functioning as a co-agonist by binding to a specific site on the GluN1 subunit. The binding site for glutamate, in turn, is located on the GluN2 subunit. The NMDA receptor requires binding of two molecules of glycine and two molecules of glutamate for optimal activation. Activation of the NMDA receptor is voltage-dependent due to voltage-dependent binding of and receptor inhibition by extracellular Mg^{2+} within the pore. Mg^{2+} blocks the influx of ions when bound to the receptor, thus regulating receptor function. At normal resting potential extracellular Mg^{2+} blocks most NMDA receptors. Receptor activation through membrane depolarization, the function of the NMDA receptor being at its greatest at moderately depolarized potentials, is facilitated by AMPA/KA receptor activation and occurs when the receptor block by Mg^{2+} is decreased. As the receptor is activated the pore becomes permeable to ions, especially Ca^{2+}, which flows in through the pore unit and trigger various Ca^{2+}-dependent and -independent signaling cascades within the cell, regulating the presence of AMPA receptors in the synaptic membrane, synaptic strength, and gene expression, all which together modulate long-term potentiation (LTP) and -depression (LTD). (Reviewed by Dingledine *et al.*, 1999; Yamakura and Shimoji, 1999; Rao and Finkbeiner, 2007; Lau and Tymianski, 2010; Niciu *et al.*, 2012).).

2.4.2.3 Metabotropic receptors

The mGluRs are seven-transmembrane spanning G-protein coupled receptors that are localized on pre- and postsynaptic membranes on both neurons and glial cells. mGluRs modulate synaptic activity and plasticity, either via second messenger pathways or by utilizing slow excitatory or inhibitory potentials. Glutamate binding occurs on the extracellular N-terminal domain while the intracellular C-terminal domain regulates receptor activity. Some mGluRs are also active in the absence of glutamate due to a constitutive activity and their function thus depends on receptor expression levels. (Reviewed by Pin *et al.*, 2003; Kew and Kemp, 2005; Niciu *et al.*, 2012). Metabotropic glutamate receptors are associated with learning, modulating LTP and LTD, but also with excitotoxicity in neurodegenerative diseases where mGluRs can both increase NMDA-mediated neurotoxicity and function in a neuroprotective manner (Bruno *et al.*, 1995, 2001; Blaabjerg *et al.*, 2003a, b; Di Giorgi-Gerevini *et al.*, 2004, 2005; reviewed by Pin *et al.*, 2003; Lau and Tymianski, 2010).

The mGluR family consists of the mGluR1-8 subunits, which combine to form homodimers. Additional splice variants of the gene transcripts increase receptor subunit diversity. The subunits are divided into three groups based on their sequence homology, pharmacology, and downstream signaling, see Figure 6 (reviewed by Hollmann and Heinemann, 1994; Schoepp *et al.*, 1999; Pin *et al.*, 2003; Kew and Kemp, 2005; Niciu *et al.*, 2012). Group I of the metabotropic receptors consist of mGluR1 and mGluR5 that couple through G_q/G_{11}, activating phospholipase C (PLC) with subsequent production of inositol 1,4,5-trisphosphate

(IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-biphosphate (PIP_2), IP_3 which releases Ca^{2+} from intracellular stores and DAG which activates protein kinase C (PKC) (Blaabjerg *et al.*, 2003a, b; reviewed by Berridge *et al.*, 2000; Pin *et al.*, 2003; Kew and Kemp, 2005; Niciu *et al.*, 2012). Group I mGluRs can also activate Ca^{2+}-permeable channels such as canonical transient receptor potential channels (TRPCs) (Kim *et al.*, 2003; Berg *et al.*, 2007) and generate oscillating Ca^{2+} waves associated with growth and proliferation of cells of the developing cortex (Weissman *et al.*, 2004; reviewed by Berridge *et al.*, 2000). Group I mGluRs are present mainly on the postsynaptic membrane of excitatory glutamatergic synapses. mGluR2 and mGluR3 forms group II, and mGluR4 and mGluR6-8 forms group III. Both group II and III mGluRs couple to inhibitory G_i/G_o proteins that decrease cyclic adenosine monophosphate (cAMP) by inhibiting adenylyl cyclase (AC) and subsequently protein kinase A (PKA), resulting in altered gene expression, inhibition of voltage-gated Ca^{2+} channels, and activation of K^+ channels. Group II and III mGluRs are mainly present on presynaptic membranes on both glutamatergic and GABAergic neurons as well as on glial cells. (Reviewed by Pin *et al.*, 2003; Kew and Kemp, 2005; Niciu *et al.*, 2012).

2.4.3 Glutamate and glutamate receptor expression

Glutamate is found at high levels in the developing mouse brain. During early cortical development the glutamate concentration in the proliferative zones is high, reflecting the high proliferation rate of the cells, and decreases with time as proliferation slows. Later in development glutamate is found in increasing concentrations in structures close to the target destinations of cortical neurons, functioning as a chemoattractant for young neurons, especially during late stages of development. (Behar *et al.*, 1999; Haydar *et al.*, 2000).

2.4.3.1 Ionotropic receptor expression

Glutamate acting on ionotropic glutamate receptors regulates neural development in the embryonic brain as well as mediates neurogenesis in the adult (Bardoul *et al.*, 1998; Gray and Sundstrom, 1998; Sadikot *et al.*, 1998; Haydar *et al.*, 2000; Bai *et al.*, 2003; Luk *et al.*, 2003; Brazel *et al.*, 2005). The AMPA/KA receptors are the first iGluRs to be expressed during embryonic development (reviewed by Nguyen *et al.*, 2001). Functional AMPA/KA receptors, as determined using Ca^{2+} imaging and electrophysiology, are highly expressed by cells in the proliferative zones of the embryonic rodent brain already during early neurogenesis, before terminal cell division (LoTurco *et al.*, 1995; Haydar *et al.*, 2000; Maric *et al.*, 2000). Glutamate acting on AMPA/KA receptors increases the proliferation of cortical neural progenitor cells (Gray and Sundstrom, 1998; Haydar *et al.*, 2000; Bai *et al.*, 2003; Brazel *et al.*, 2005). The NMDA receptors become functional later during development compared to AMPA/KA receptors (LoTurco *et al.*, 1991, 1995; Sadikot *et al.*, 1998). Cells in the proliferative zones of embryonic and perinatal brain express *Grin1* and *Grin2* subunit mRNA but the NMDA receptors only show functional Ca^{2+} responses and depolarizing currents when the cells have differentiated into young postmitotic neurons, around the time when

neuronal migration starts, with receptor activation promoting cell migration from the VZ to the cortical plate (LoTurco *et al.,* 1991, 1995; Behar *et al.,* 1999; Maric *et al.,* 2000). Glutamate acting through NMDA receptors mediates neural fate determination and promotes neuronal differentiation and migration by decreasing cell proliferation, both in embryonic and postnatal cells (Behar *et al.,* 1999; Nacher *et al.,* 2001; Hirasawa *et al.,* 2003; Deisseroth *et al.,* 2004; reviewed by Nguyen *et al.,* 2001; Schlett, 2006). NMDA receptors are thus considered proneural (Nacher *et al.,* 2001; Hirasawa *et al.,* 2003). The developmental expression of functional AMPA/KA vs. NMDA receptors, with AMPA/KA receptors appearing early in development while NMDA receptors only become functional after a certain level of neuronal maturity, may reflect or regulate the transition from proliferating cells to postmitotic neurons (LoTurco *et al.,* 1995; Kitayama *et al.,* 2003).

Expression of the glutamate receptor subunits is spatially and temporally regulated. The GluA1-3 AMPA receptor subunits are expressed in the developing cortical plate (Métin *et al.,* 2000) and later during development *Gria1*, *Gria3*, and *Gria4* mRNA expression is seen in distinct layers of the cortex while *Gria2* mRNA is expressed in all layers of the cortex (Keinänen *et al.,* 1990). In addition, the AMPA receptor subunits are widely expressed in all other structures of the brain, including the hippocampus where GluA1-3 are highly expressed in the hippocampal pyramidal layer and the dentate gyrus and GluA4 is expressed to a lesser extent and mainly by GABAergic interneurons, and the cerebellum where GluA1-4 expression occurs in distinct cell populations (Monaghan *et al.,* 1984; Keinänen *et al.,* 1990; Pellegrini-Giampietro *et al.,* 1991; Petralia and Wenthold, 1992; Geiger *et al.,* 1995; Arai *et al.,* 1997; Pfeiffer *et al.,* 2009).

The GluN1 subunit of the NMDA receptors is highly and ubiquitously expressed in both developing and adult brain with an increased expression upon brain maturation (Monyer *et al.,* 1992; Buller *et al.,* 1994; Monyer *et al.,* 1994; Petralia *et al.,* 1994a; Behar *et al.,* 1999; Bendel *et al.,* 2005; Henson *et al.,* 2008). The expression of the subunits GluN2 and GluN3 is also developmentally regulated and GluN2 expression co-localizes with GluN1 expression to a large extent (Petralia *et al.,* 1994b). The *Grin2b* and *Grin2d* subunits are the most highly expressed *Grin2* subunits in the embryonic brain (Behar *et al.,* 1999; Monyer *et al.,* 1994). *Grin2b* mRNA is almost exclusively expressed in the forebrain while *Grin2d* mRNA expression is highest in the cerebellum and brainstem. Expression of *Grin2a* and *Grin2c* subunit mRNA is only detected in the postnatal brain where *Grin2a* mRNA is distributed widely in the neocortex and hippocampus and *Grin2c* mRNA is found in the cerebellum and brainstem (Buller *et al.,* 1994; Monyer *et al.,* 1994). During development *Grin3a* mRNA expression is found initially in the spinal cord and thalamus and later in the cortex and hippocampus, with the expression declining as the brain matures (Ciabarra *et al.,* 1995; Bendel *et al.,* 2005). *Grin3b* mRNA is expressed in the neocortex and hippocampus, cerebellum, brainstem, and the alpha motor neurons of the spinal cord (Nishi *et al.,* 2001; Bendel *et al.,* 2005).

Table 1. Glutamate and GABA receptor function in neural progenitor cells and their derivatives.

Receptor	Material	Findings	Reference
AMPAR/ KAR	Rat E16 cortical slices and VZ cells	KA application decreases DNA synthesis.	LoTurco *et al*., 1995
	Adult rat DG granule cell progenitors	KA increases neuronal differentiation.	Gray and Sundstrom, 1998
	Mouse E13-E14 organotypic slices	Glutamate increases neocortical VZ cell proliferation by shortening cell cycle, decreases SVZ cell proliferation, and decreases the generation of migrating postmitotic neurons.	Haydar *et al*., 2000
	Organotypic slice cultures from rat E15 neocortex (IZ)	AMPAR activation leads to neurite retraction in tangentially migrating neurons of the IZ.	Poluch *et al*., 2001
	Hippocampal DG cells in intact adult rat	Administration of AMPAR potentiators increases cell proliferation.	Bai *et al*., 2003
	Adult rat hippocampal granule cells	AMPAR block reduces seizure-induced cell proliferation in DG.	Jiang *et al*., 2004
	SVZ-derived NPCs from perinatal (P0) rat	KAR activation increases cell proliferation. KAR activation prevents NPC apoptosis but only when the NMDA receptor is antagonized.	Brazel *et al*., 2005
	Organotypic hippocampal slice cultures of P6-P8 rat	AMPAR block increases the number of postmitotic neurons derived from pre-existing neuronal progenitor cells in the dentate granule cell layer.	Poulsen *et al*., 2005
	NPCs from human fetal (13-16 weeks) cortical tissue	AMPAR activation induces neuronal differentiation and increases NPC-derived neuronal dendritic arbor formation.	Whitney *et al*., 2008
NMDAR	Mouse P10 cerebellar granule cells	NMDAR activation increases cell migration and receptor antagonism decreases migration.	Komuro and Rakic, 1993
	Adult rat hippocampus DG	NMDAR activation decreases and receptor block increases cell proliferation. NMDAR block increases neuron numbers.	Cameron *et al*., 1995
	Striatal progenitor cells of GABAergic interneurons in intact P35-P42 rats	NMDAR antagonism during E15-E18 decreases number of striatal neurons in the adult rat.	Sadikot *et al*., 1998
	Mouse E13-E18 cortical cells and slice cultures (VZ/SVZ/CP)	Glutamate/NMDA stimulates cell migration with a larger effect on VZ/SVZ cells than on CP cells. NMDAR antagonists block migration of cells from VZ/SVZ to the CP.	Behar *et al*., 1999
	Hippocampal DG cells in intact adult rat	NMDAR antagonism suppresses neurogenesis in dentate granule cell layer and SGZ following ischemia.	Arvidsson *et al*., 2001
	Hippocampal SGZ cells in intact adult rat	NMDAR blockade increases SGZ cell proliferation and number of radial glia and granule neurons.	Nacher *et al*., 2001
	Slice and primary cultures from E17 rat cortex	NMDAR blockade increases cell proliferation in primary cultures and VZ of slice cultures and delays neuronal maturation and migration.	Hirasawa *et al*., 2003
	Intact adult rat hippocampus DG, SVZ	NMDAR activation reduces cell proliferation in DG cell layer and block of NMDAR increases cell proliferation. NMDAR activation or block does not affect cell proliferation in the SVZ.	Kitayama *et al*., 2003
	Striatal rat E15-E18 progenitor cells	NMDAR antagonism reduces striatal neuron numbers and reduces proliferation in the LGE when applied *in utero*. In cell cultures NMDAR blockade reduces BrdU uptake while NMDAR activation increases cell proliferation.	Luk *et al*., 2003

NMDAR	Rat hippocampal NPCs in culture and in intact adult rat, and perinatal (P0) hippocampal cells in culture	NMDA activation increases neurogenesis (increased number of MAP-2-positive cells) while antagonizing NMDAR function reduces neurogenesis.	Deisseroth *et al*., 2004
	Adult rat hippocampal granule cells	NMDAR block reduces seizure-induced cell proliferation in DG.	Jiang *et al*., 2004
	Organotypic hippocampal slice cultures of P6-P8 rat	NMDAR block increases the number of postmitotic neurons derived from pre-existing neuronal progenitor cells in the dentate granule cell layer.	Poulsen *et al*., 2005
	NPCs from human fetal (10-16 weeks) cortex	NMDAR activation increases NPC proliferation.	Suzuki *et al*., 2006
	Intact mice treated *in utero* and primary progenitor cell cultures from E15 rat	NMDAR block impairs cell proliferation.	Gandhi *et al*., 2008
mGluR	Mouse E13-E18 cortical cells and slice cultures (VZ/SVZ/CP)	mGluR activation stimulates cell migration.	Behar *et al*., 1999
	Adult mouse hippocampal DG cells in intact mice	Block of group II mGluR function increases cell proliferation in the DG.	Yoshimizu and Chaki, 2004
	Organotypic hippocampal slice cultures of P7 rat	Block of mGluR1 reduces number of immature neurons.	Baskys *et al*., 2005
	SVZ-derived NPCs from perinatal (P0) rat	Group II mGluR activation increases cell proliferation. mGluR3 activation prevents NPC apoptosis but only when the NMDA receptor is antagonized.	Brazel *et al*., 2005
	Mouse E20 forebrain NPCs, adult hippocampal DG, and SVZ cells in intact mouse	mGluR5 activation increases cell proliferation. mGluR5 knockout or blockade reduces number of progenitor cells in SVZ and DG. mGluR3 block reduces cell numbers.	Di Giorgi-Gerevini *et al*., 2005
	NPCs from SVZ of adult mice	mGluR1b block decreases cell proliferation and apoptosis and promotes neuronal differentiation. mGluR5 block increases apoptosis and decreases number of cells undergoing neuronal differentiation.	Castiglione *et al*., 2008
	Intact mice treated *in utero* and primary progenitor cell cultures from E15 rat	mGluR5 activation is necessary for striatal but not cortical progenitor cell proliferation with mGluR5 blockade reducing striatal progenitor cell proliferation, the effects of which are restricted to the VZ. Block of mGluR5 during the proliferative period (E15-E18) decreases neuronal numbers in the striatum.	Gandhi *et al*., 2008
	Cortical NPCs from E15.5 mouse brain	Group III mGluR agonist decreases and antagonist increases cell proliferation. Activation of group III mGluRs decreases neuronal differentiation and increases astrocytic differentiation.	Nakamichi *et al.,* 2008
	Human fetal (10 weeks) NPC cell line from ventral midbrain	mGluR7 activation decreases cell proliferation and increases astrocytic differentiation of NPCs.	Vernon *et al.,* 2011
	NPCs from human fetal (13 weeks) cortex	mGluR5 activation promotes and mGluR5 blockade decreases NPC proliferation.	Zhao *et al.,* 2011
	Adult hippocampal NPCs of mice	mGluR5 activation promotes cell proliferation.	Nochi *et al*., 2012
	Cortical NPCs from E15 rat brain	mGluR5 activation promotes and mGluR5 blockade decreases NPC proliferation. Hypoxic conditions increase mGluR5 expression.	Zhao *et al.,* 2012

GABAR	Rat E16 cortical slices and VZ cells	GABA application decreases DNA synthesis.	LoTurco *et al*., 1995
	Rat E14-E21 cortical neurons	GABA stimulates neuronal migration.	Behar *et al*., 1996
	Rat E18-E19 cortical cells (VZ/CP)	GABA stimulates or arrests migration of cortical cells depending on GABAR type and signaling mechanism.	Behar *et al*., 1998
	Mouse E13-E18 cortical cells and slice cultures (VZ/SVZ/CP)	GABA stimulates cell migration.	Behar *et al*., 1999
	Rat E18 cortical slices	$GABA_AR$ activation promotes cell migration from the VZ/SVZ to the IZ. $GABA_BR$ activation promotes cell migration to the CP.	Behar *et al*., 2000
	Mouse E13-E14 organotypic slices	GABA increases neocortical VZ cell proliferation by shortening cell cycle and decreases SVZ cell proliferation. GABA decreases the generation of migrating postmitotic neurons.	Haydar *et al*., 2000
	Rat E17-E18 cortical neurons	GABA stimulates neuronal migration.	Behar *et al*., 2001
	Rat E15 cortical slices	$GABA_BR$ antagonism causes a change in the cortical distribution of MGE-derived cells, with more cells in VZ/SVZ and fewer in LIZ, CP, MZ, and reduce length of the leading process of the cells.	Lopez-Bendito *et al*., 2003
	NPCs from perinatal (P0-P3) rat striatum	$GABA_AR$ activation inhibits cell cycle progression in NPCs and organotypic slices.	Nguyen *et al*., 2003
	Mouse E13.5-E15.5 telencephalic slices	GABA promotes entry of tangentially migrating cells from MGE to cortex.	Cuzon *et al*., 2006
	Perinatal (P0-P3) mice and neocortical slice cultures	$GABA_AR$ block reduces motility of tangentially migrating GABAergic interneurons and activation increases migration.	Inada *et al*., 2011

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AMPAR, AMPA receptor; BrdU, bromodeoxyuridine; CP, cortical plate; DG, dentate gyrus; DNA, deoxyribonucleic acid; E, embryonic; GABA, γ-amino butyric acid; GABAR, GABA receptor; IZ, intermediate zone; KA, kainate; KAR, KA receptor; LGE, lateral ganglionic eminence; LIZ, lower intermediate zone; MAP-2, microtubule associated protein-2; MGE, medial ganglionic eminence; mGluR, metabotropic glutamate receptor; MZ, marginal zone; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; NPCs, neural progenitor cells; P, postnatal; SGZ, subgranular zone; SVZ, subventricular zone; VZ, ventricular zone.

2.4.3.2 Metabotropic receptor expression

Metabotropic glutamate receptors also have important roles in cortical development and neuronal function, see Table 1. The mGluRs, except for mGluR6, which is only expressed in the retina, are expressed in neural progenitors and/or in neuronal and glial cells in embryonic and adult brain in distinct spatial and temporal patterns (van den Pol *et al.*, 1998; Di Giorgi-Gerevini *et al.*, 2004; Yang *et al.*, 2012; reviewed by Pin *et al.*, 2003; Melchiorri *et al.*, 2007). The group I and II metabotropic glutamate receptors mGluR1 and mGluR2 are not expressed in the neurogenic zones of the embryonic or perinatal forebrain (Di Giorgi-Gerevini *et al.*, 2004; Brazel *et al.*, 2005; Di Giorgi-Gerevini *et al.*, 2005; Castiglione *et al.*, 2008). Pharmacological studies have, however, suggested the expression of mGluR1b in adult SVZ-derived neurospheres (Castiglione *et al.*, 2008). mGluR3 has been identified on neural progenitors of the developing forebrain where mGluR3 activation has a role in supporting cell survival and proliferation (Di Giorgi-Gerevini *et al.*, 2004; Brazel *et al.*, 2005; Di Giorgi-

Gerevini *et al.,* 2005). In contrast, blocking group II mGluR function enhances the proliferation of dentate gyrus cells from the adult hippocampus (Yoshimizu and Chaki, 2004). mGluR5 is expressed at high levels during early neuronal development with its expression concentrated around the ventricles, to the inner marginal zone, and to the hippocampal plate (Di Giorgi-Gerevini *et al.,* 2004; Yang *et al.,* 2012). mGluR5 is also expressed in the progenitor cells of the neurogenic zones in both the developing and adult brain, as well as in the cortex, hippocampus, and striatum of the adult, and seems to be of great importance in regulating cell proliferation, survival, and neurogenesis (Di Giorgi-Gerevini *et al.,* 2004; Yoshimizu and Chaki, 2004; Di Giorgi-Gerevini *et al.,* 2005; Castiglione *et al.,* 2008; Gandhi *et al.,* 2008; Kärkkäinen *et al.,* 2009; Zhao *et al.,* 2011; Nochi *et al.,* 2012; Yang *et al.,* 2012; Zhao *et al.,* 2012). The function of mGluR5 in neural progenitors is, however, still not totally clear as one study found mGluR5 to be required for the proliferation of striatal but not cortical VZ-derived progenitors (Gandhi *et al.,* 2008). The group III mGluRs are also involved in regulating progenitor cell proliferation and differentiation. Activation of group III mGluRs in mouse cortical neural progenitors reduces cell proliferation and neuronal differentiation and increases astrocytic differentiation (Nakamichi *et al*., 2008). mGluR7 has also been shown to regulate cell proliferation and astrocytic differentiation in human neural progenitors of the ventral midbrain (Vernon *et al*., 2011).

2.4.4 GABA

GABA is the main inhibitory neurotransmitter in the adult brain and is also important for cortical neurogenesis by regulating cell proliferation, differentiation, migration, and neurite outgrowth (LoTurco *et al.,* 1995; Behar *et al.,* 1996, 1998, 1999, 2000, 2001; Haydar *et al.,* 2000). GABAergic signaling during development is excitatory, activating voltage-gated Ca^{2+} channels and NMDA receptors (LoTurco *et al.,* 1995; Leinekugel *et al.,* 1997). GABAergic signaling becomes inhibitory later during development (Luhmann and Prince, 1991; LoTurco *et al.,* 1995) due to the developmental upregulation of the K^+/Cl^- co-transporter KCC2, thus leading to a reduced intracellular Cl^- concentration and a change in the $GABA_A$ receptor reversal potential of the cell (Rivera *et al.,* 1999). This developmental switch in GABA signaling is in itself regulated by GABA (Ganguly *et al.,* 2001).

GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase, packed into vesicles by vesicular GABA transporters, although non-vesicular release also occur due to reversal of GABA transporters (Wu *et al.,* 2007), and transported to the synapse (Taylor and Gordon-Weeks, 1991; Ma and Barker, 1995; McIntire *et al.,* 1997). After synaptic release GABA is cleared from the synaptic cleft by GABA transporters on neurons and glial cells and metabolized. (Reviewed by Owens and Kriegstein, 2002).

2.4.5 GABA receptors

In the CNS there are two or three classes of GABA receptors, depending on classification. $GABA_A$ and $GABA_C$ ($GABA_C$ which can also be considered to belong to the $GABA_A$ receptors) are Cl^--permeable ion channels (reviewed by Barnard *et al.*, 1998). $GABA_B$ is a metabotropic receptor, which activates K^+ channels, inactivates voltage-gated Ca^{2+} channels, and inhibits adenylyl cyclase activity through $G_{i/o}$ proteins (LoTurco *et al.*, 1995; Kaupmann *et al.*, 1997, 1998; Kuner *et al.*, 1999; Behar *et al.*, 2001; Lopez-Bendito *et al*., 2003). The $GABA_A$ and $GABA_C$ receptors are pentameric ion channels with $GABA_A$ consisting of at least one α subunit of the α1-6 subunits, one β subunit of the β1-3 subunits, and one of the subunits γ1-3, δ, ε, θ or π, while $GABA_C$ consists of ρ1-2 subunits only (reviewed by Barnard *et al.*, 1998; Owens and Kriegstein, 2002). The metabotropic $GABA_B$ receptors are heterodimers consisting of R1 and R2 subunits, R1 of which there are two splice variants, R1a and R1b (Kaupmann *et al.*, 1997, 1998; Behar *et al.*, 2001; Lopez-Bendito *et al.*, 2003; reviewed by Pin *et al.*, 2003). Proper physiological function of $GABA_B$ receptors requires both subunit types to be present in the receptor complex (Kaupmann *et al.*, 1997, 1998; Kuner *et al.*, 1999).

2.4.6 GABA and GABA receptor expression

GABA is expressed in the developing rodent brain during early neurogenesis (Haydar *et al.*, 2000; Jiménez *et al.*, 2002). Expression is initially found throughout the neuroepithelium but later during development GABA expression in the neurogenic zones decreases while expression increases in the structures of the developing cortical layers (Behar *et al.*, 1996; Tamamaki *et al.*, 1997; Haydar *et al.*, 2000; Lopez-Bendito *et al.*, 2003). GABA, like glutamate, thus functions as a chemoattractant for cortical progenitors migrating towards the cortex (Behar *et al.*, 1996, 1998, 1999, 2001).

GABA receptor subunit expression is developmentally regulated (Ma and Barker, 1995; Kaupmann *et al.*, 1997; Kuner *et al.*, 1999; Behar *et al.*, 2000). $GABA_A$ receptor subunits are expressed by neural progenitors and immature neurons (Ma and Barker, 1995; Sah *et al.*, 1997; Carleton *et al.*, 2003; Nguyen *et al.*, 2003; Brazel *et al.*, 2005; Cuzon *et al.*, 2006) as well as close to target areas of cortical progenitor-derived cells (Ma and Barker, 1995; Behar *et al.*, 1998; Métin *et al.*, 2000; Lopez-Bendito *et al.*, 2003). GABA acting through $GABA_A$ receptors regulates progenitor cell proliferation (LoTurco *et al.*, 1995; Antonopoulos *et al.*, 1997; Haydar *et al.*, 2000). Expression of *R1* subunit mRNA of the $GABA_B$ receptor is detected earlier than expression of *R2* subunit mRNA but expression levels of both subunits are high and to a large extent overlapping in the cortex, hippocampus, and cerebellum later in development (Kaupmann *et al.*, 1998; Kuner *et al.*, 1999). Of the *R1* isoforms the *R1a* splice variant is predominant during development while the *R1b* variant dominates in the mature brain. Both isoforms are found in the neurogenic zones and the cortical plate as well as in tangentially migrating interneurons during development. (Behar *et al.*, 2001; Lopez-Bendito *et al.*, 2003).

GABA expressed in the cortical plate acts on migrating progenitors and promotes radial migration towards the cortical plate. Signaling occurs through distinct GABA receptor types at distinct stages of migration. $GABA_B$ receptors regulate cell migration to the cortical plate while $GABA_A$ receptor activation terminates migration of the cells as they arrive at their destination in the cortex. (Behar *et al.,* 1998, 2000, 2001). $GABA_A$ and $GABA_B$ receptors have also been implicated in regulation of tangential migration of embryonic interneurons to the cortex (Lopez-Bendito *et al.,* 2003; Cuzon *et al.,* 2006). Neuroblasts in the RMS migrating tangentially to the olfactory bulb also express $GABA_A$ receptors, even before the appearance of AMPA and NMDA receptors (Carleton *et al.,* 2003).

2.5 The neurotrophic factor BDNF

Neurotrophic factors are important for nervous system development and maintenance. Neurotrophic factors and their receptors are expressed early during development with differing concentration gradients in different regions of the brain and within the cortex itself (Kirschenbaum and Goldman, 1995; Behar *et al.,* 1997; Brunstrom *et al.,* 1997; Fukumitsu *et al.,* 1998; Polleux *et al.,* 2002; Barnabé-Heider and Miller, 2003; Fukumitsu *et al.,* 2006). Neurotrophic factors regulate various cellular functions such as cell survival, proliferation, differentiation, cell number, neurite extension, target innervation, neurotransmitter and ion channel expression, functional plasticity, and neurogenesis (Kirschenbaum and Goldman, 1995; Behar *et al.,* 1997; Lee *et al.,* 2002; Polleux *et al.,* 2002; Barnabé-Heider and Miller, 2003). Neurotrophic factors also function as motogenic factors for migrating neurons during development (Behar *et al.,* 1997; Brunstrom *et al.,* 1997; Polleux *et al.,* 2002). The mammalian family of neurotrophic factors consists of BDNF, nerve growth factor, neurotrophin-3, and neurotrophin-4, which mediate their actions through high-affinity tropomyosin-related kinase (Trk) receptors (TrkA, TrkB, TrkC) or a low-affinity p75 neurotrophin receptor ($p75^{NTR}$) receptor. The Trk receptors primarily regulate neuronal survival and differentiation. $p75^{NTR}$ activation both supports trophic actions by mediating Trk function and causes apoptosis, thus regulating which neurons survive during development and which neurons die. Both receptor types are of importance in the regulation of neurogenesis. (Chiaramello *et al.,* 2007; Young *et al.,* 2007; reviewed by Huang and Reichardt, 2001; Chao, 2003; Nykjaer *et al.,* 2005; Dicou, 2009).

BDNF regulates neurogenesis, supports neuronal function, regulates neurotransmitter actions and synaptic plasticity in the adult brain, and aids in recovery from cellular damage to the brain (Behar *et al.,* 1997, 1998; Tyler and Pozzo-Miller, 2001; Bagley and Belluscio, 2010; reviewed by Schinder and Poo, 2000; Mattson, 2008; Gottmann *et al.,* 2009). BDNF has specifically been shown to promote neurite formation and extension in GABAergic neurons, to promote excitatory and inhibitory synapse formation (Vicario-Abejón *et al.,* 1998), and to enhance glutamatergic but reduce GABAergic synaptic transmission (Tyler and Pozzo-Miller, 2001; Copi *et al.,* 2005; Caldeira *et al.,* 2007). In addition, BDNF promotes neuronal differentiation of neural progenitor cells and neuroblasts by decreasing cell proliferation and increasing neuronal differentiation (Lee *et al.,* 2002; reviewed by Hagg, 2005; Mattson,

2008). In the developing brain the expression of *Bdnf* mRNA is initially low but increases as neurogenesis proceeds (Maisonpierre *et al.*, 1990). In the adult brain *Bdnf* mRNA is widely expressed in the cortex and hippocampus in glutamatergic projection neurons but not in GABAergic interneurons (Cellerino *et al.*, 1996). The high-affinity receptor for BDNF, *TrkB*, is highly expressed in the brain already during early corticogenesis (Klein *et al.*, 1990). *TrkB* mRNA is expressed by both glutamatergic and GABAergic neurons but its expression is higher in interneurons (Klein *et al.*, 1990; Cellerino *et al.*, 1996). A developmental shift in *TrkB* mRNA expression from its full-length to its truncated form occurs at the end of neurogenesis (Klein *et al.*, 1990; Allendoerfer *et al.*, 1994). Signaling through the TrkB receptor is coupled to intracellular Ca^{2+} elevations via several intracellular signaling pathways (Behar *et al.*, 1997; Polleux *et al.*, 2002) and BDNF also directly activates TRPCs, thus allowing influx of extracellular Ca^{2+} (Li et al. 2005; Amaral and Pozzo-Miller 2007a, b). Signaling through TrkB is important for the proper migration of newborn cortical neurons as well as tangentially migrating interneurons (Behar *et al.*, 1997, 1998; Polleux *et al.*, 2002; Barnabé-Heider and Miller, 2003; Medina *et al.*, 2004; Alcántara *et al.*, 2006) and BDNF is involved in regulating laminar fate determination (Ohmiya *et al.*, 2002; Alcántara *et al.*, 2006; Fukumitsu *et al.*, 2006). BDNF also regulates neuroblast migration along the RMS from the SVZ to the olfactory bulb (Bagley and Belluscio, 2010).

Neurotrophic factors have been shown to interact with glutamate during activity-dependent plasticity and to have a role in protecting cells from excitotoxic cell death (reviewed by Mattson, 2008). BDNF increases the release of both glutamate and GABA by cortical neurons (Matsumoto *et al.*, 2001; Nagano *et al.*, 2003) and regulates the expression of AMPA and $GABA_A$ receptors (Narisawa-Saito *et al.*, 1999; Mizoguchi *et al.*, 2003a, b; Nagano *et al.*, 2003). Glutamate and GABA, in turn, regulate *Bdnf* mRNA expression (Zafra *et al.*, 1990, 1991; Berninger *et al.*, 1995; Hayashi *et al.*, 1999). In the case of glutamate this occurs through activation of iGluRs (Hayashi *et al.*, 1999; Zafra *et al.*, 1990, 1991). The modulatory actions of BDNF and the neurotransmitters glutamate and GABA on each other are thus important during development.

2.6 Hypoxia and pH regulation in neural progenitor cell differentiation

Oxygen is fundamental for most life on earth. Oxygen deprivation can lead to pathological conditions while various pathological conditions, such as ischemia and stroke, can cause reduced oxygen supply to the tissues (hypoxia). Cell culture is usually performed in standardized conditions with an oxygen level of 21%, the oxygen level in the atmosphere. In most parts of both the developing and adult brain the oxygen levels are, however, much lower, ranging between 1-9%, levels which are considered physiologically normal (normoxic) in the brain (Horie *et al.*, 2008; Santilli *et al.*, 2010). Culture of neural progenitors in mildly hypoxic (2-10%), although physiologically normal, conditions has been shown to promote survival, proliferation, and differentiation of rodent and human neural progenitor cells (Studer *et al.*, 2000; Horie *et al.*, 2008; Santilli *et al.*, 2010). Mildly hypoxic conditions increase glutamatergic, serotonergic, and dopaminergic but decrease GABAergic

differentiation (Studer *et al.,* 2000; Horie *et al.,* 2008). Hypoxia also increases glutamate receptor (mGluR5) expression in neural progenitors (Zhao *et al.,* 2012). Even total oxygen deprivation (anoxia) increased cell survival and proliferation of neural progenitor cells *in vitro* as long as enough glucose was available (Bürgers *et al.,* 2008). Increased SVZ neurogenesis has been shown in mice subjected to temporary hypoxia (Fagel *et al.,* 2006) and both focal and global ischemia increase cell proliferation, neural progenitor cell differentiation, and migration of newborn neurons in the brain (Takagi *et al.,* 1999; Yagita *et al.,* 2001; Kee *et al.,* 2001; Tonchev *et al.,* 2003; Fagel *et al.,* 2006; Nochi *et al.,* 2012). (Reviewed by Zhu *et al.,* 2005; Simon and Keith, 2008; Kokaia and Lindvall, 2003)

In addition to hypoxia, decreased extracellular pH and increased concentrations of neurotransmitters, including glutamate and GABA, are consequences of pathological conditions, contributing to brain damage through excessive activation of iGluRs and acid-sensing ion channels (Vannucci *et al.,* 1999; Huang and McNamara, 2004; Xiong *et al.,* 2004; Brazel *et al.,* 2005; Santilli *et al.,* 2010). Extracellular and intracellular pH regulation is important during development as it influences various cellular processes, including cell proliferation and glutamate receptor function (Pouysségur *et al.,* 1985; Saybasili, 1998). Excessive glutamatergic neurotransmission as a consequence of disturbed glutamatergic signaling causes excitotoxic cell death (Portera-Cailliau *et al.,* 1997; reviewed by Mattson, 2008; Lau and Tymianski, 2010) but a reduced extracellular pH may, in fact, have cell-protective effects by reducing glutamate receptor activity (Saybasili, 1998; reviewed by Dingledine *et al.,* 1999; Yamakura and Shimoji, 1999; Niciu *et al.,* 2012). Neurogenesis after ischemic insults also involves glutamatergic mechanisms (Arvidsson *et al.,* 2001; Brazel *et al.,* 2005). Extracellular oxygen and pH levels are important regulators of neurogenesis, both in normal and pathological conditions, and these actions are mediated, at least in part, through the actions of glutamate receptors.

2.7 Clinical implications

Neurogenesis in response to traumatic brain injury, stroke or neurodegenerative disease involves the generation of functional neurons from progenitor cells residing within the brain. The CNS contains stem and progenitor cells capable of generating new neuronal cells but adult neurogenesis is restricted and unable to replace all lost neurons. Transplanted neural progenitors have been shown to migrate to the diseased or injured area of the brain but cell differentiation and integration of the transplanted cells into the existing neural network is inefficient (reviewed by Martino and Pluchino, 2006; Kokaia and Lindvall, 2012; Miller and Bai, 2012). It is thus important to delineate cellular processes steering cell proliferation, differentiation, migration, and functionality to enhance cell-based therapies to treat insults or diseases within the CNS.

3 AIMS OF THE STUDY

The main goal of this thesis was to increase the understanding of mechanisms regulating differentiation and migration of neural progenitor cells; the role of neurotransmitters and their receptors, specifically glutamate and glutamate receptors, the actions of the neurotrophic factor BDNF, and the reactivity of the cells to changes associated with ischemic conditions.

The specific aims of the thesis were as follows:

I. To identify and characterize glutamate receptor expression in differentiating neural progenitor cells.

II. To specify the role of glutamate and glutamate receptors in neural progenitor cell differentiation and migration.

III. To elucidate the effects of BDNF on neural differentiation, neurotransmitter receptor expression, and cell motility.

IV. To study the effects of hypoxia and acidosis on differentiating neural progenitor cells.

4 MATERIALS AND METHODS

4.1 Neural progenitor cell preparation (I-IV)

Neural progenitor cells were isolated from the dorsal wall of the lateral ventricles of embryonic day 14 (E14) FVB mice, as indicated in Figure 7. Pregnant females were briefly anesthetized with CO_2 and killed by cervical dislocation. The embryos were dissected from the uterus, placed in ice-cold PBS, and heads separated at the cervical spine. The brain was removed from the skull and the brain tissue dissected under microscope on ice in ice-cold PBS and dissociated in 5 ml Hank's balanced salt solution (Gibco, Life Technologies) containing 2 mM glucose (Sigma), 1.33 mg/ml trypsin (Gibco, Life Technologies), 0.7 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid (Sigma) for 30 min at 37ºC. After 15 min and at the end of incubation the tissue was triturated manually to dissociate cells from the tissue. Cells were centrifuged for 5 min at 1500 rotations per minute (rpm) and resuspended in 4 ml 0.5x Hank's balanced salt solution containing 0.9 M sucrose (Sigma). After centrifugation for 10 min at 2000 rpm the cells were resuspended in 2 ml Earle's balanced salt solution (EBSS, Gibco, Life Technologies) and centrifuged through 12 ml of EBSS solution containing 4% bovine serum albumin (BSA, Sigma) and 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Sigma) for 7 min at 1500 rpm by pipetting the cells on top of the EBSS-BSA-HEPES solution. The dissociated cells were plated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 media containing B27 supplement (both from Gibco, Life Technologies), 2 mM L-glutamine, 15 mM HEPES, 100 U/ml penicillin and 100 U/ml streptomycin (all from Sigma, pH 7.4), in the presence of 10 ng/ml bFGF and 20 ng/ml EGF (both from PeproTech), in a 5% CO_2-humidified incubator at 37ºC.

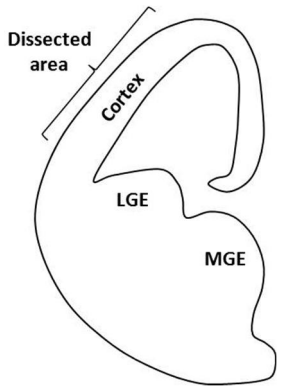

Figure 7. Neural progenitors were isolated from the dorsal wall of the lateral ventricles of E14 mice.

4.2 Cell culture and neural differentiation (I-IV)

Cells were grown as free-floating aggregates termed neurospheres. Early passage neurospheres were frozen for later use. The culture media was refreshed twice a week by changing 50% of the media and growth factors were added three times per week. Cells were passaged by manual trituration at approximately 7-10 day intervals. Neurospheres were cultured for up to 10-15 passages. For neuronal differentiation neurospheres (200-250 µm)

were plated on poly-DL-ornithine (Sigma) coated culture dishes or cover glasses in the absence of bFGF and EGF. At this stage HEPES was excluded from the culture media in works **II** and **III**. Growth factor withdrawal induced spontaneous neural differentiation. For blocking studies (**II**) cells were differentiated in the presence of 10 µM 2-methyl-6-(phenylethynyl)-pyridine (MPEP, Abcam Biochemicals) or 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma). To study the effects of BDNF (**III**) on neural cell differentiation BDNF (PeproTech) was added to growth media at a concentration of 10 ng/ml. When differentiating cells for prolonged periods of time (5 days) the culture media was changed on day two or three of differentiation.

4.3 Gene expression analysis (I)

4.3.1 RNA isolation and cDNA synthesis

Total RNA from cells differentiated for 1 and 5 days was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and quantified using NanoDrop ND8000 spectrophotometer (Thermo Scientific). Complementary deoxyribo-nucleic acid (cDNA) was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit and random hexamer primers according to the manufacturer's instructions (Roche Applied Science).

4.3.2 Quantitative real-time polymerase chain reaction

The resulting cDNA was amplified with the LightCycler® 480 SYBR Green I Master Kit and LightCycler® 480 system (Roche Applied Science). Primers, designed using the National Center for Biotechnology Information Primer-BLAST primer designing tool, were used at a final concentration of 1 µM. Melting peak analysis and analysis of polymerase chain reaction (PCR) fragments on agarose gel was performed to verify product size and specificity. The PCR data was normalized to β-actin and 18S ribosomal RNA using geNorm software.

4.4 Immunocytochemistry (I-IV)

Differentiating cells were fixed for 20 min at room temperature using 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS, pH 7.4). Cells were permeabilized and blocked for unspecific staining with PBS containing 0.1% Triton X-100 (except when staining for O4), 10% normal serum and 1% BSA (all from Sigma) for 60 min at room temperature. Cells were incubated with primary antibodies in PBS containing 0.1% Triton X-100 (except when staining for O4), 1% normal serum and 1% BSA overnight at 4ºC. Secondary antibodies were applied for 1h at room temperature in the dark in 1% BSA-PBS. All antibodies used (Table 2) were tested extensively using neural progenitor cells differentiated for various lengths of time and with several different concentrations of each antibody to examine staining patterns and

evaluate specificity of the antibodies. In addition to morphological criteria the specificity of the antibodies was determined by performing parallel staining using different antibodies recognizing the same protein and using Western blot. For secondary antibody controls primary antibodies were omitted, resulting in the disappearance of all staining. Cells were mounted using Vectashield mounting media containing 4´,6-diaminodino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories). Alternatively, in publication **III**, cells imaged using confocal microscopes were mounted using ProLong® Gold Antifade Reagent (Molecular Probes, Life Technologies). Cells were viewed and photographed using an Olympus AX70 Provis microscope (Olympus) (**I-IV**) with x10 (numerical aperture 0.30) or x20 (numerical aperture 0.50) object magnification, equipped with fluorescence optics and a charge-coupled device camera (PCO AG), or, for high-resolution imaging at x63 (numerical aperture 1.40), a Leica TCS SP2 AOBS laser scanning confocal microscope (Leica Microsystems) (**III**).

Table 2. Antibodies used in the original publications.

Antibody	Host	Dilution	Used in work	Manufacturer
Primary antibodies				
anti-Calbindin D-28K	Rabbit	1:100	III, IV	Millipore
anti-CNPase	Mouse	1:500	II	Abcam
anti-GFAP	Rabbit	1:500	II	Sigma
anti-GLAST	Guinea pig	1:500	I, III	Millipore
anti-EAAT1	Rabbit	1:500	II, IV	Abcam
anti-GluR1	Rabbit	1:100	I	Millipore
anti-GluR2	Mouse	1:100	I, II	Millipore
anti-GluR3	Mouse	1:100	I	Millipore
anti-GluR4	Rabbit	1:100	I	Millipore
anti-MAP-2	Mouse	1:500	II, III, IV	Millipore
anti-MAP-2	Rabbit	1:500	I, II, IV	Millipore
anti-mGluR5	Rabbit	1:50	II	Millipore
anti-NeuN	Mouse	1:50	III, IV	Millipore
anti-O4	Mouse	1:1000	II	Millipore
anti-S100β	Rabbit	1:500	II	Abcam
anti-TrkB	Rabbit	1:100	III	Santa Cruz
anti-Tuj1	Mouse	1:500	I	Covance
Secondary antibodies				
AlexaFluor 488 anti-rb IgG	Goat	1:2000	I-IV	Molecular Probes
AlexaFluor 488 anti-ms IgG	Donkey	1:2000	II	Molecular Probes
AlexaFluor 488 anti-ms IgM	Donkey	1:2000	II	Molecular Probes
AlexaFluor 568 anti-rb IgG	Goat	1:5000	II	Molecular Probes
AlexaFluor 568 anti-ms IgG	Goat	1:5000	I-IV	Molecular Probes
AlexaFluor 568 anti-gp IgG	Goat	1:750	I, III	Molecular Probes

Gp, guinea pig; ms, mouse; rb, rabbit

4.5 Ca^{2+} imaging (I-III)

For the Ca^{2+} imaging experiments 20-30 neurospheres were plated on poly-DL-ornithine coated 25 mm cover glasses and differentiated for 1 or 5 days. The cells were then incubated with 4 μM fura-2 acetoxymethyl ester (fura-2-AM, Molecular Probes, Life Technologies) dissolved in dimethyl sulfoxide (Sigma) at 37ºC for 20 min in HEPES-buffered media (HBM, pH 7.4) consisting of 137 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose and 1 mM probenecid (all from Sigma) before imaging. After fura-2-AM loading of cells the cover glass was attached to a temperated perfusion chamber on the microscope (Nikon TMS inverted microscope, x20 objective) and perfused at 2 ml/min at 37ºC. Using 340 nm and 380 nm light excitation using a filter changer under the control of the InCytIM-2 fluorescence imaging program (Intracellular Imaging Inc.) and a dichroic mirror (DM430, Nikon) up to 100 cells could be imaged simultaneously. Cells derived from only one neurosphere were imaged in each experiment. Light emission was measured through a 510 nm barrier filter with an integrating charge-coupled device camera (COHU Inc.). A ratioed (340 nm/380 nm) image was acquired each second. Agonists, modulators and blockers were applied to the cells in HBM at the following concentrations: AMPA, 30 μM; CNQX, 10 μM; cyclothiazide (CTZ), 10 μM (all from Sigma); (S)-3,5-dihydroxyphenylglycine (DHPG), 10 μM (Abcam Biochemicals); GABA, 100 μM (Sigma); KA, 30 μM (**I**), 50 μM (**II**) (Tocris Bioscience); NMDA, 50 μM, in Mg^{2+}-free HBM together with glycine, 10 μM; philanthotoxin (PhTx), 5 μM (both from Sigma); pyr-2, 10 μM, (Calbiochem, Merck Millipore); pyr-3, 10 μM; SKF-96365, 10 μM (both from Tocris Bioscience).

4.6 Time-lapse imaging (II, III)

Time-lapse imaging of cellular movement was performed using a self-contained cell culture instrument combining phase contrast microscopy, automation, and environmental control (Cell-IQ® system, Chip-Man Technologies Ltd). The instrument contains an integrated incubator (±0.2ºC), two incubation gas flow controllers, precision movement stages (x, y axes: ±1 μm; z axis: ±0.4 μm), and an automated optics module fully controlled through machine vision based firmware and analysis software. The imaging system enables continuous monitoring of adherent cells in two plates in an integrated plate holder. Machine vision enables analysis of a continuous time-lapse image series of living cells for observing morphologic changes and cell movement without the use of labels and dyes.

4.7 Measurement of membrane potential (II, IV)

Changes in the resting membrane potential of individual neural progenitor cells were measured using the potentiometric bisoxonol dye bis-(1,3-dibutylbarbituric acid)-trimethine oxonol ($DiBAC_4(3)$, Molecular Probes Invitrogen, Life Technologies), an anionic probe that exhibits enhanced fluorescence intensity when the cell membrane is depolarized and

decreased fluorescence intensity due to efflux of the probe upon hyperpolarization of the cell membrane, as described earlier (Louhivuori *et al.*, 2010). Briefly, differentiating cells grown on 25 mm cover glasses were washed 3 times with HBM (pH 7.4), placed in the measuring chamber and transferred to the heat controlled chamber-holder on the microscope (Nikon TMS inverted microscope, x20 objective). For the experiment, 500 nM $DiBAC_4(3)$ was added to the perfusion solution (HBM, pH 7.4) and allowed to equilibrate across the cell membrane for 15 min (1 ml/min) before the data acquisition process was started. The cells were excited with 490 nm wavelength lights for 80 ms (rate of data capture 25/min) and the emitted fluorescence captured at 530 nm. For calibration of the response, cells were then treated with depolarizing (20, 40 or 140 mM) K^+ solutions containing 500 nM $DiBAC_4(3)$.

4.8 Measurement of intracellular pH (IV)

Intracellular pH of neural progenitor cells was measured using the cell membrane-permeable probe 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and the dual-wavelength InCytIM-2 fluorescence imaging program. For the experiments, cells differentiated for 5 days on 25 mm covers glasses were loaded with 4 µM BCECF-AM in HBM for 30 min at 37ºC. After the dye loading step the cells were washed 3 times with HBM, placed in the measuring chamber and transferred to the heat controlled chamber holder on the microscope (Nikon TMS inverted microscope, x20 objective). During the experiment, the cells were perfused (1 ml/min) with HBM (pH 7.4). The cells were excited alternately with 490 nm and 440 nm wavelength lights for 80 ms (rate of data capture 34/min). The BCECF-AM fluorescence emission ratios (490 nm/440 nm) of the 510 nm fluorescence were turned into pH values by using a standard curve (calibration curve obtained by using BCECF-AM and nigericin as described earlier, Nordström *et al.*, 1995). Intracellular pH was studied by exposing the cells to low pH extracellular medium or by using the ammonium prepulse technique. In this technique, non-charged ammonia, NH_3, (in equilibrium with ammonium ion, NH_4^+) enters cells and is converted to ammonium (NH_4^+) after which an increase in cytoplasmic pH can be observed. After washout, the reverse reaction takes place. Ammonium (NH_4^+) dissociates into ammonia (NH_3) and protons (H^+). Ammonia (NH_3) rapidly leaves the cells and the protons that are released simultaneously cause a rapid acidification in the cytosol.

4.9 Image analysis (II, III)

Quantification of immunofluorescence intensity was performed using the ImageJ software. The area of interest was framed using identical regions of interest starting from the neurosphere edge from parallel neurosphere cultures stained with the same staining conditions and imaged using the same exposure times. A background reading was obtained from areas devoid of cells. This reading was reduced from fluorescence intensity values. Time-lapse image analysis was also performed using ImageJ freeware and motility was analyzed using MTJ track software.

4.10 Data analysis (I-IV)

The data collected during Ca^{2+} imaging and measurement of membrane potential and intracellular pH was analyzed with the InCyt 4.5 software (Intracellular Imaging Inc.) and further processed with Origin 6.0 software (OriginLabCorp.). In the different original publications the area of cell migration was divided into different cellular layers for analysis as follows:

I (cells differentiated for 1 day and 5 days):

- inner (i) layer: 21 µm at day 1 and 42 µm at day 5, starting from the innermost single cells that could be distinguished next to the neurosphere (corresponds to layers 1 + 2 in **II** and **III**)
- middle (m) layer: consisted of cells within the next 42 µm at day 1 and 84 µm at day 5 (corresponds to layers 3 + 4 in **II** and **III**)
- outer (o) layer: consisting of all cells outside the middle layer (corresponds to layers 5 + 6 in **II** and **III**)

II/III (cells differentiated for 1 day and 5 days):

- layer 1: consisted of the innermost single cells that could be distinguished next to the neurosphere
- layers 2-5: corresponded to 21 µm layers at day 1 and 42 µm layers at day 5 of differentiation
- layer 6: consisted of all accessible cells outside layer 5

IV (cells differentiated for 5 days):

- inner layer: cells that had migrated less than 100 µm (corresponds to: inner layer + first ²/₃ part of middle layer in **I**; layers 1-3 + first ½ part of layer 4 in **II/III**)
- outer layer: cells that had migrated more than 100 µm (corresponds to: last ¹/₃ part of middle layer + outer layer in **I**; last ½ part of layer 4 + layers 5-6 in **II/III**)

4.11 Statistical analysis (I-IV)

The data is presented as means ± SEM (**I-IV**) or fold change ± SEM (**I**) and was evaluated statistically using Students *t*-test. A value of $p < 0.05$ was considered significant (**I-IV**). In studies **II** and **III** significances are indicated as follows: ns (non-significant), $p > 0.05$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

5 RESULTS

5.1 Immunocytochemical characterization of neurosphere-derived cells (I-IV)

Neurospheres were plated on poly-DL-ornithine coated glass slides and differentiated for defined periods of time in the absence of mitogens, thus inducing spontaneous neural differentiation of cells migrating out of the neurospheres. Immonocytochemical analysis of differentiating cells revealed positive staining for markers of radial glia, neurons, astrocytes, and pre-oligodendrocytes, see Figure 8. The radial glial marker GLAST (Hartfuss *et al.*, 2001) was expressed from the beginning of cell migration by cells emerging from the neurospheres and forming a compact layer of cells (**I-IV**), and as seen in Figure 8. GLAST expression was concentrated to the inner migration layers but was strongest in the tips of the individual radial glial processes, as determined by measurement of intensity of GLAST immunofluorescent staining (**II**: Figure 3). Neuronal staining for calbindin (**III**: Figure 4), musashi-1 (not shown), and doublecortin (not shown), markers of neural progenitors and early neurons (Palmer *et al.*, 1995; Sakakibara *et al.*, 1996; des Portes *et al.*, 1998; Anderson *et al.*, 2001); Tuj1 (**I**: Figure 2), an early marker of cells committed to neuronal fate (Menezes and Luskin, 1994); and microtubule associated protein (MAP) -2 (**I-IV**), see also Figure 8, a marker for neuronal cells (Dehmelt and Halpain, 2005); was seen from the first day of differentiation in neuron-like cells with small cell bodies and thin processes. The expression of neuronal markers was initially weak but increased with time in differentiating culture. Expression of neuron-specific nuclear (NeuN) protein (**III**: Figure 5) (Mullen *et al.*, 1992) was not seen at 1 day of differentiation. After 5 days of differentiation NeuN-positive nuclei could be seen in some, but not all, MAP-2-postitive cells, particularly in the outer migration layers (**III**: Figure 5). Neuron-like cells were initially seen migrating on and between the radial glial processes but accumulated outside the radial glial layer after prolonged differentiation. Neuronal marker expression was also highest in cells outside the radial glial cell layer (**III**: Figure 5). GFAP-expression was seen in astrocytic processes (Imura *et al.*, 2003) extending from the core of the neurosphere (**II**: Figure 4), see also Figure 8, and cells weakly positive for the late astrocyte marker S100β (Raponi *et al.*, 2007) were identified in the differentiating cultures (**II**: Figure 4). Expression of O4, identifying pre-oligodendrocytes (Bansal *et al.*, 1992), could be seen in a few cells in cultures differentiated for 5 days (**II**: Figure 4), see also Figure 8, but was not identified at 1 day of differentiation. No expression of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase), a marker for mature oligodendrocytes (Pfeiffer *et al.*, 1993), was found in the cultures at these time points (not shown). Taken together, these results show that neural progenitor cells derived from the dorsal wall of the lateral ventricles of E14 mice and grown as neurospheres differentiate into all the major cell types of the CNS (Davis and Temple, 1994; Vescovi *et al.*, 1999; D'Amour and Gage, 2003; reviewed by Kriegstein and Alvarez-Buylla, 2009) and gain a more mature neuronal phenotype upon prolonged time in differentiating culture and as they migrate further away from the neurosphere, as was also shown previously (Kärkkäinen *et al.*, 2009).

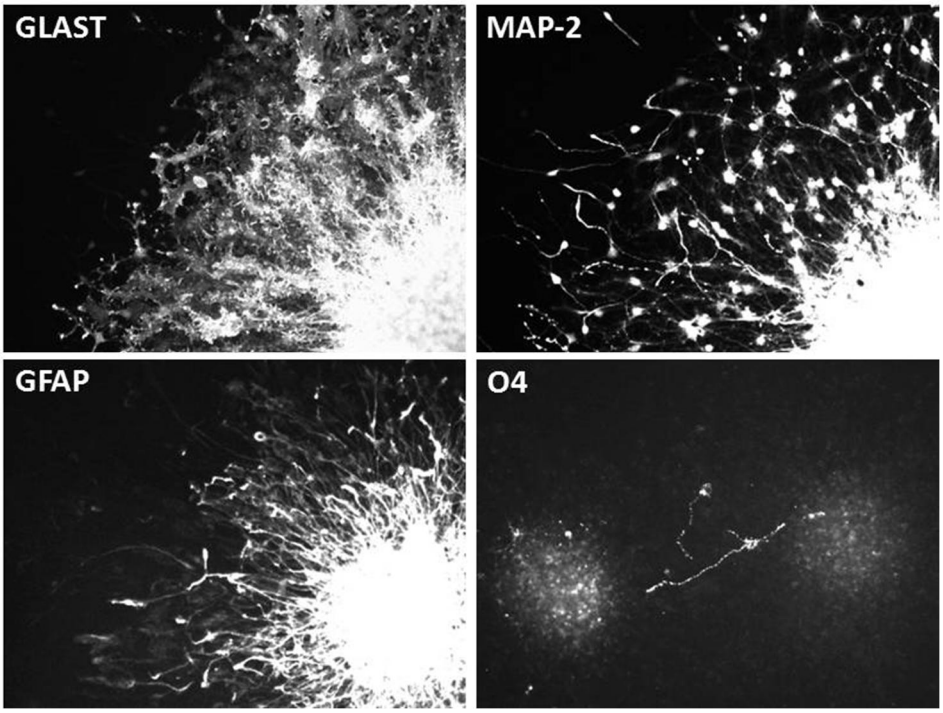

Figure 8. Immunocytochemical staining of neural progenitor cells differentiated for 5 days. Cells migrate out from the neurosphere and differentiate into and express molecular markers of radial glia (GLAST), neuronal cells (MAP-2), astrocytes (GFAP), and pre-oligodendrocytes (O4).

5.2 Differentiating neural progenitor cells express functional glutamate and GABA receptors (I-III)

Differentiating cells migrating out from neurospheres express mGluR5, as shown by immunocytochemistry (**II**: Figure 1). The expression of mGluR5 was strong in cultures differentiated for 1 day but decreased with time in differentiating culture. Expression of mGluR5 was concentrated to the inner migration layers and correlated strongly with the expression of GLAST. The differentiating cells exhibited a functional response, as determined using Ca^{2+} imaging and, in the case of AMPA/KA receptors, measurement of membrane depolarization, by showing a strong elevation in their intracellular free Ca^{2+} concentration when stimulated with the group I mGluR agonist DHPG (**II**: Figure 1, **III**: Figure 7), the response of which is mediated by mGluR5 in embryonic and postnatal neural progenitors (Castrén *et al.*, 2005; Kärkkäinen *et al*., 2009). A low concentration (10 μM) of the agonist was used during Ca^{2+} imaging in order to monitor Ca^{2+} entry through Ca^{2+}-permeable channels (Kim *et al*., 2003; Berg *et al.*, 2007). The response to stimulation with DHPG was highest in the inner migration layers where all cells showed a Ca^{2+} response at 1 day of differentiation (**II**: Figure 1, **III**: Figure 7). The cells exhibiting a Ca^{2+} response to DHPG correlated well with radial glial cells expressing mGluR5 and GLAST. After 5 days of differentiation the cells responding to stimulation with DHPG had shifted slightly towards the outer cell layers (**II**: Figure 1, **III**: Figure 7). At this time about 80% of the cells in the innermost migration layers exhibited a response to DHPG and the response declined towards the outer cell layers. SKF-96365 (10 μM), a non-selective blocker of many transient receptor

potential channels (Zhu *et al.*, 1998; Clapham *et al.*, 2005), pyr-2 (10 μM), which blocks most TRPC channels and store-operated Orai channels, and pyr-3 (10 μM), a selective TRPC3 channel blocker (Kiyonaka *et al.*, 2009), partly inhibited the Ca^{2+} response to DHPG (**II**: Figure 1). The mGluR5-mediated Ca^{2+} elevation in response to stimulation with DHPG thus seems to partly be mediated by transient receptor potential channels.

Using quantitative real-time PCR and immunocytochemistry differentiating neural progenitor cells was shown to express mRNA transcripts for AMPA receptor subunits *Gria1-4* and GluA1-3 protein (**I**: Figure 1 and Figure 2, **II**: Figure 2). No expression of GluA4 protein was identified in the differentiating cells although several antibodies were tested. AMPA receptor subunit mRNAs showed increased expression levels with increased time in differentiating culture. Both radial glial and neuron-like cells expressed GluA1-3 protein after 1 day of differentiation (not shown). After 5 days of differentiation GluA1 and GluA2 co-localized more strongly with neuron-like cells while GluA3 was identified in both glial and neuronal cells (**I**: Figure 2, **II**: Figure 2). Stimulation of migrating cells with AMPA (30 μM), a specific AMPA receptor agonist, caused a transient Ca^{2+} influx in the cells, as shown using Ca^{2+} imaging (**I**: Figure 3). The Ca^{2+} response to AMPA was prolonged in the presence of cyclothiazide (CTZ, 10 μM), a positive allosteric modulator of AMPA receptors that blocks AMPA receptor desensitization (Yamada and Tang, 1993). KA is a potent agonist of AMPA receptors, which causes a sustained response, does not cause AMPA receptor desensitization, and only exhibits a transient effect on KA receptors (Kiskin *et al.*, 1986; Paternain *et al.*, 1995). Stimulation of differentiating cells with KA (30 μM or 50 μM) caused a Ca^{2+} elevation in the inner migration layers (inner layer in **I**, layers 1 + 2 in **II**/**III**) in 20-50% of the cells at 1 day of differentiation and in 60-80% of the cells at 5 days of differentiation (**I**: Figure 3, **II**: Figure 2, **III**: Figure 7). The Ca^{2+} response to KA increased towards the outer migration layers so that all or almost all of the cells in the outer migration layers responded to stimulation with KA, both after 1 day and 5 days of differentiation. The distribution of cells exhibiting Ca^{2+} responses to stimulation with KA correlated well with the distribution of GluA1-3 and neuronal marker expression. The voltachromic dye $DiBAC_4(3)$ was used to measure the resting membrane potential of the migrating cells and to confirm the ionotropic nature of the response to KA. At 1 day of differentiation the cells in the inner migration layers showed a more depolarized resting membrane potential (~-52 mV) compared to the cells in the outer migration layers (~-86 mV) (**II**: see text). This was also true for cells differentiated for 5 days but the difference in the resting membrane potential was much smaller at this time point, being ~-75 mV in the inner layers and ~-79 mV in the outer layers (**II**: see text), values which are similar to results obtained in study **IV** (see below, section 5.7). When the cells were stimulated with KA a depolarizing response was seen in most of the cells in both the inner and the outer migration layers with the cells in the outer layers depolarizing more robustly, most probably as a result of decreased Ca^{2+}-permeability of the AMPA receptors in the outer cell layers, both at 1 day and 5 days of differentiation (**II**: Figure 2).

Functional NMDA and GABA receptors appeared in the cell cultures at later stages of differentiation compared to mGluR5 and AMPA/KA receptors. Very few cells showing Ca^{2+}

responses to stimulation with NMDA (50 μM in Mg^{2+}-free conditions in the presence of 10 μM glycine, conditions in which NMDA responses are optimal) could be detected after 1 day of differentiation (**III**: Figure 7). After 5 days of differentiation around 20% of the cells in layers 2-6 (**II**/**III**) showed Ca^{2+} responses when stimulated with NMDA. At 5 days of differentiation ≤ 30% of cells in the different migration layers responded to stimulation with GABA (100 μM) (**III**: Figure 8).

5.3 Up-regulation of *Gria1* mRNA and expression of Ca^{2+}-permeable and Ca^{2+}-impermeable AMPA receptors by differentiating neural progenitor cells (I, II)

Quantitative real-time PCR showed that the mRNA levels for the different *Gria1-4* splice variants increased upon differentiation of neurosphere-derived cells (**I**: Figure 1). An increase in *Gria1-4* mRNA expression, which was significant ($p < 0.05$) for *Gria2* splice variant *3* (*Gria2/3*), could be seen already after 1 day of differentiation when compared to proliferating neurospheres (unpublished results). Messenger RNA of the *Gria1* splice variants *1* (*Gria1/1*) and *2* (*Gria1/2*) were markedly and significantly upregulated as the differentiation progressed from day 1 to day 5. The *Gria4* splice variant *1* (*Gria4/1*) mRNA was also significantly upregulated, but not to the same extent as mRNA of the *Gria1* isoforms. As mentioned above, GluA1-3, but not GluA4, protein was detected the differentiating cultures (**I**: Figure 2).

Ca^{2+} imaging confirmed the presence of functional AMPA receptors in differentiating cells with cells responding to stimulation with both AMPA and KA (see above). Philanthotoxin (PhTx, 5 μM or 10 μM), an antagonist of Ca^{2+}-permeable GluA2 unedited AMPA receptors (Washburn and Dingledine, 1996), considerably reduced the Ca^{2+} influx in response to KA (**I**: Figure 3, **II**: Figure 2). The PhTx-mediated inhibition was larger in cells differentiated for 1 day (~60%) compared with cells differentiated for 5 days (~40%), indicating the presence of a larger amount of unedited GluA2 containing Ca^{2+}-permeable AMPA receptors at 1 day of differentiation and an increased expression of edited GluA2 containing Ca^{2+}-impermeable AMPA receptors in cells differentiated for 5 days (**I**: Figure 3). These results suggest a functionally more mature neuronal phenotype for the cells differentiated for a longer period of time, as was expected based on previous results obtained by our laboratory (Kärkkäinen *et al.*, 2009). The remaining Ca^{2+} response to KA in the presence of PhTx was blocked by CNQX (10 μM), a blocker of all AMPA/KA receptors (Honoré *et al.*, 1988). This confirmed that the Ca^{2+} response to KA was indeed mediated by AMPA/KA receptors.

5.4 Effects of glutamate receptor antagonists on neural progenitor cell differentiation and motility (II)

Neural progenitor cells are cultured in the presence of glutamate receptor agonists as the growth media contains glutamate and aspartate. Glutamate has been shown to modulate neurogenesis (reviewed by Schlett, 2006). To study glutamate receptor function in neural

progenitor cell differentiation and migration antagonists of metabotropic and ionotropic glutamate receptors were added to the culture media of differentiating cells. The presence of MPEP (10 μM), a selective blocker of mGluR5 (Gasparini *et al.*, 1999), in the culture media dramatically affected radial glial cells by considerably impairing the extension of radial glial processes, resulting in a flattening and shortening of the glial processes (**II**: Figure 3 and Figure 5). The effect of MPEP on the radial glial cells could be seen already after 1 day of differentiation but was more pronounced after 5 days of differentiation. Initially MPEP also transiently increased the number of rapidly moving neuron-like cells outside the radial glial cell layer but with prolonged time in culture the numbers of neuron-like cells declined and no difference in the number of rapidly moving cells in MPEP-treated cultures compared to control cultures could be seen after 30h in time-lapse culture (**II**: Figure 5). Block of mGluR5 through the action of MPEP did, however, promote the motility of individual neuron-like cells by advancing the progress of the cells and reducing the number of turns the cells made (**II**: Figure 6 and Figure 7). Treatment with MPEP reduced the Ca^{2+} response to DHPG in the outer cellular migration layers at both 1 day and 5 days of differentiation. It also reduced the Ca^{2+} response to KA in the outer cell layers at 5 days of differentiation (**II**: Figure 9).

Addition of CNQX (10 μM) to the growth media of differentiating cells, thus blocking AMPA/KA receptor activation, in turn enhanced the extension of radial glial processes during the first days of differentiation (**II**: Figure 3 and Figure 5). After 5 days of differentiation the radial glial layer appeared somewhat flattened in cultures treated with CNQX but no major differences in the morphology of the cells compared to cells in control cultures could be seen at this time point. CNQX did not significantly affect the number of neuron-like cells outside the radial glial cell layer but affected the motility of these cells by reducing their progression and increasing the number of turns the cells made (**II**: Figure 5, Figure 6, and Figure 7). The cells that moved freely outside the radial glial cell layer typically extended two neurites, a leading process and a trailing process, one on each side of the cell body. In the cultures treated with CNQX the cell body often moved back and forth along the neurites in a wobbly manner with little movement of the processes (**II**: Figure 8). Conversely, in cells in control cultures the somal progression correlated closely with movement of the leading process. Treatment of differentiating cells with CNQX increased the Ca^{2+} response to DHPG in the outer cell layers at both 1 day and 5 days of differentiation and also in the inner cell layers at 5 days of differentiation (**II**: Figure 9). CNQX treatment did not significantly affect the Ca^{2+} response to KA.

5.5 Effects of BDNF on neuronal marker, neurotransmitter receptor, and TrkB receptor expression and distribution (III)

Cells differentiated for 1 day showed weak immunocytochemical staining for the early neuronal marker calbindin (**III**: Figure 4). Calbindin-positive cells were found in all migration layers of differentiating neural progenitors but the presence of BDNF (10 ng/ml) in the growth media of differentiating cells significantly increased the number of calbindin-positive cells in the outer migration layers. In control cultures at 1 day of differentiation

calbindin-positive neuron-like cells could mainly be seen migrating on or among the radial glial cells but a few cells had already moved outside the radial glial cell layer and showed weakly positive staining for MAP-2. Cells differentiated in the presence of BDNF and that had migrated outside the radial glial cell layer had clearly MAP-2-positive processes. No NeuN-positive cells were identified at day 1 of differentiation in either control or BDNF-treated cultures (not shown). Cells in cultures differentiated for 5 days showed positive staining for both MAP-2 and NeuN (**III**: Figure 5). Cells differentiated for 5 days in the presence of BDNF had increasingly migrated outside the radial glial cell layer and exhibited an increased number of NeuN-positive cells in the outermost migration layers. BDNF did not affect the intensity of GLAST staining or the morphology of radial glial cells in the different cell layers (**III**: Figure 1 and Figure 4).

Ca^{2+} imaging of cells differentiated for 1 or 5 days showed no effect of BDNF on the Ca^{2+} response to stimulation with DHPG (**III**: Figure 7). This supports the results obtained through immunocytochemical analysis of GLAST-stained radial glia that BDNF does not affect the extension of radial glial processes. BDNF also had little or no effect on the distribution of cells responding to stimulation with KA. At 5 days of differentiation BDNF did, however, clearly affect the distribution, but not the number, of NMDA-responsive cells. In the cultures differentiated in presence of BDNF there was a marked increase in cells responding to NMDA in the outer migration layers. At 5 days of differentiation BDNF also affected the distribution of cells responding to GABA, shifting the GABA-responsive cells towards the outer migration layers (**III**: Figure 8). There was a clear correlation of the cells responding to stimulation with both NMDA and GABA.

The BDNF high-affinity receptor TrkB was found, using immunocytochemistry, to be expressed by neurosphere-derived cells already 1 day after initiation of differentiation (**III**: Figure 6). The TrkB receptor expression was localized to distinct perinuclear areas, typically on one side of the soma and from where a neurite was extending. Some TrkB expression was also seen in neurites. After 5 days of differentiation the TrkB expression in the neurites had increased and overlapped partly with the expression of MAP-2. Addition of BDNF to the growth media of differentiating cultures increased the number of TrkB expressing cells in the outermost migration layers but not the total number of TrkB expressing cells. Taken together, these results show that BDNF does not affect the development of the radial glial network but promotes the migration and maturation of neuronal cells.

5.6 Migration of neurosphere-derived cells and effect of BDNF on cell motility (III)

A suitable substrate for cell attachment and growth factor removal induces the neurospheres to attach to the substrate and cells to start migrating out of the neurospheres. At first thick radial glial processes extended from the neurospheres but soon after neuron-like cells emerged, initially migrating on and among the radial glial cells (**III**: Figure 1). The neuronal cells had long processes that either remained attached to the neurosphere or detached from

the neurosphere, thus allowing cells to move independently outside the radial glial cell layer. As described above, the presence of BDNF in the differentiating cultures did not affect the development of the radial glial cell layer. BDNF did, however, increase the number of freely moving neuron-like cells outside the radial glial layer. The freely moving cells moved in a saltatory manner with surges of motion and occasional periods of stalling (**III**: Figure 2). As the cells moved in surges they extended a long leading process as well as a shorter trailing process, which were partly retracted when the cells became stationary. The cells extended processes in various directions while pausing, seemingly exploring the surroundings. Analysis of cell motility patterns revealed that BDNF increased the average speed of motion of freely moving cells (**III**: Figure 3). BDNF did not affect the speed of movement during surges or the maximal speed but decreased the duration of stationary periods and increased the duration of surges, thus increasing the total distance cells moved during a defined period of time.

5.7 Neurosphere-derived radial glial and neuronal cells show differences in intracellular pH and react differently when exposed to hypoxia and acidosis (IV)

The intracellular pH (pH_i) of differentiating cells can be measured using the pH indicator dye BCECF-AM. In cells differentiated for 5 days a resting pH_i of pH 7.00 ± 0.01 was measured in cells in the inner migration layer (**IV**) that primarily corresponded to GLAST-positive radial glial cells. The cells in the outer migration layer (**IV**), chiefly corresponding to MAP-2- and NeuN-positive neuron-like cells, exhibited a lower resting pH_i, pH 6.79 ± 0.01 (**IV**: Figure 1). Using $DiBAC_4(3)$ to measure the membrane potential of the migrating cells it was found that the inner cell population showed a more depolarized resting membrane potential (~-66 mV) compared to the outer cells (~-81 mV) (**IV**: see text). These results correlated well with results obtained elsewhere (see above, section 5.2). When subjecting the cells to acute hypoxia the cells in the inner migration layer responded with a decrease in pH_i by 0.15 units but recovered quickly when the hypoxic treatment ended (**IV**: Figure 2). The cells in the outer migration layer barely showed any change in pH_i when subjected to acute hypoxia. In contrast, all cells hyperpolarized to an equal extent when exposed to acute hypoxia. When exposing differentiating cells to transient acidosis (pH 6.0 and pH 4.0) the cells in the outer migration layer, with a lower resting pH_i, showed a marked decrease in pH_i that returned back to normal when the extracellular pH was switched back to that of physiological levels (pH 7.4) (**IV**: Figure 4 and Table 1). The cells in the inner migration layer, with a higher resting pH_i, showed a less robust response to extracellular acidification and alkalinized in response to depolarizing stimuli with 30 mM extracellular K^+ (**IV**: Figure 5). The cells in the outer migration layer did not alkalinize when depolarized with K^+. Subjecting the migrating neural progenitors to 20 mM ammonium chloride (ammonium chloride pre-pulse) induced an immediate increase in the pH_i of all cells (**IV**: Figure 6 and Table 2). In the continued presence of ammonium chloride the pH_i started to decline in the cells in the outer migration layer. When ammonium chloride was removed there was a further rapid decline in pH_i of the outer cell population to levels below the initial pH_i levels after which the pH_i slowly started to recover. The cells in the inner migration layer, which also

showed an increased pH_i in the presence of ammonium chloride, did not, however, exhibit a decline in pH_i during ongoing ammonium chloride treatment. When ammonium chloride was removed the pH_i of these inner layer cells rapidly returned to normal. When barium chloride (100 μM) was present in the imaging solution during the ammonium pre-pulse experiment the two cell populations could not be distinguished from each other on the basis of changes in pH_i (**IV**: Figure 7).

Neural progenitor cells were subjected to long-term acidosis by differentiating the cells in media with pH 6.8, pH 6.5 or pH 6.2 for a total of 72h. Cells grown in pH 6.8 and pH 6.5 survived well, exhibiting no major morphological or migratory changes (**IV**: Figure 9), see also Figure 9 where the cells have been immunocytochemically stained for MAP-2. Only a slight reduction in MAP-2-positive cells could be seen after differentiation for 24h in pH 6.5 or after 72h in pH 6.8. Differentiation of neural progenitors at pH 6.2 did, however, considerably hamper migration of both radial glial and neuronal cells. This effect could be seen already after 24h in culture and became more apparent with prolonged time (72h) in culture, see Figure 9.

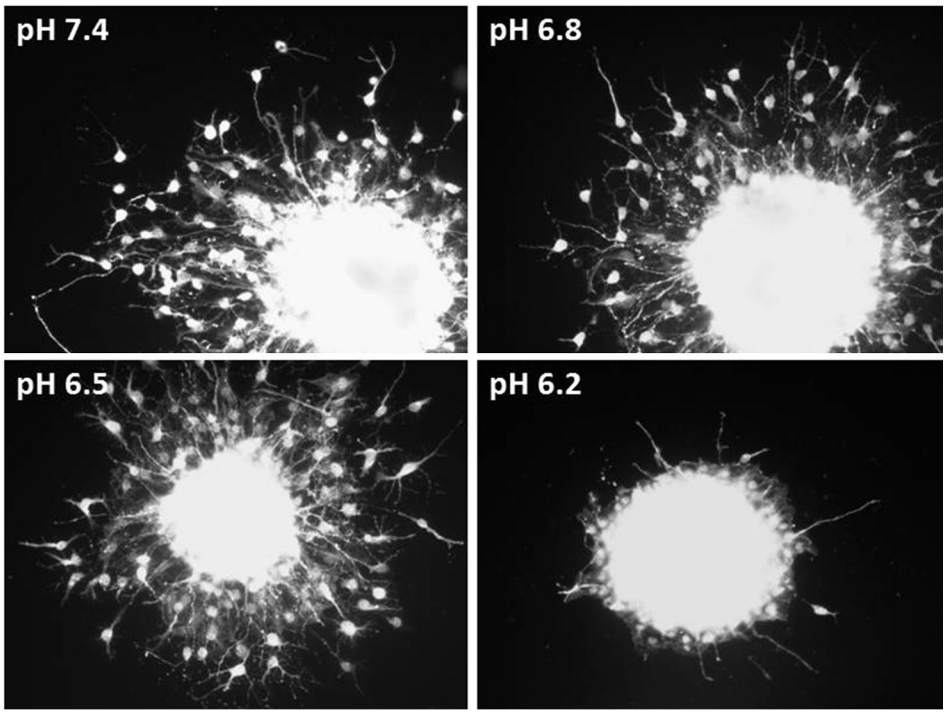

Figure 9. Effect of pH in culture conditions on differentiating neural progenitor cells. Cells cultured in normal (pH 7.4) or acidic conditions (pH 6.8, pH 6.5, and pH 6.2) for 72h immunocytochemically stained for MAP-2.

6 DISCUSSION

The CNS in its entirety derives from multipotent neural progenitor cells of the developing brain. Development of the nervous system is a tightly regulated process that coordinates cell proliferation, survival, migration, differentiation, synaptogenesis, and cell death. Adult neurogenesis in response to brain injury involves increased proliferation of neural progenitor cells residing within the neurogenic zones of the brain and migration of newborn neurons to the site where brain damage has occurred. Neural progenitors have enormous potential during development but neurogenesis in the adult brain is markedly restricted due to the more restricted potential of the progenitor cells in the adult. While thousands of cells are estimated to be born in the adult brain each day it is thought that most of the newborn cells die (reviewed by Gage, 2000), possibly as a consequence of their inability to integrate into the existing neural circuits. In addition, mechanisms leading to cell death are activated in damaged brain tissue and regenerative axon extension impaired due to glial scarring. (Reviewed by Moskowitz and Lo, 2003; Wieloch and Nikolich, 2006). Adult neural stem cells are thus unable to repair damage in the brain following cell loss or are only able to do so to a small extent. By promoting endogenous neural progenitor cell proliferation, differentiation, and integration into the neural network it might be possible to enhance recovery from nervous system disease or trauma. (Reviewed by Gage, 2000; Hagg, 2005; Christie and Turnley, 2013). Neurotrophic factors are molecules that could possibly enhance endogenous neurogenesis (Gao and Chen, 2009; Im *et al.,* 2010; Zhu *et al.,* 2011). The growing knowledge about cell manipulation *in vitro* also drives the development of cell-based therapies to treat insult or disease within the CNS. While it was shown in a trial with Parkinson's patients that tissue transplants can aid in functional recovery from neurodegenerative disease (Spencer *et al.,* 1992) cell transplantation does not yet provide a means of cure from neurodegenerative disease. It is also clear from numerous studies that much research is still needed to elucidate the mechanisms behind recovery from neurodegenerative disease or nervous system injury after cell transplantation. Mechanisms of functional recovery after cell transplantation are not purely based on the integration of new neurons into the neural network but also on anti-inflammatory actions mediated by the transplanted cells (Pluchino *et al.,* 2005; Einstein *et al.,* 2007). It is also clear that the functional integration of transplanted cells into the existing neural network is poor. (Reviewed by Martino and Pluchino, 2006; Kokaia and Lindvall, 2012; Miller and Bai, 2012). It is therefore of importance to characterize the behavior of neural progenitor cells and their derivatives to understand the process of cell differentiation and maturation in more detail, to steer stem cell differentiation in a wanted direction, and to promote cell survival and neural network integration. It has been suggested that glutamate-mediated signaling play important roles in neuronal development by promoting and/or inhibiting the proliferation, differentiation, and migration of neural progenitor cells (reviewed by Schlett, 2006). This study has focused on delineating some of the fundamental factors influencing neural progenitor cell differentiation and migration to enhance the understanding of the behavior of

these cells and to aid in studies aimed at developing therapeutic agents or cell transplants for therapeutic purposes.

6.1 The methods – a critical view

The neurosphere model is a widely used cell model for studying neural progenitor cell proliferation and differentiation as well as neural development *in vitro*. Much of the currently available information regarding these features derives from studies utilizing this cell model. In this study neural progenitor cells from embryonic mice were used. Mouse embryos are easily available and the preparation of cortical progenitors from embryonic mice straightforward. The neurotransmitter responsiveness of cells derived from embryonic neural progenitors has also been shown to correspond to those of cells derived from progenitor cells of postnatal mice (Castrén *et al.,* 2005; Kärkkäinen *et al.,* 2009). The mouse is a good model to study human development and disease as the mouse shows considerable similarity to human anatomy and physiology, including the structure and development of the cortex (reviewed by Ayala *et al.,* 2007; Bystron *et al.,* 2008). Observations made in rodent models of corticogenesis are thus applicable to humans. Indeed, most of the data presented in this thesis derives from work using rodents but is extended and supported by data obtained from studies using other species, including non-human primates and humans. The use of mice also allows for modification of the mouse genome, something that is not possible in humans. Although no genetically engineered mice were used in this study parallel projects utilizing mice with various genetic mutations are currently ongoing in the laboratory.

Isolation of embryonic neural progenitor cells occurs by dissection of the dorsal wall of the lateral ventricles followed by enzymatic dissociation and subsequent rounds of centrifugation through various solutions to enrich for neural progenitors. After plating the neural progenitor cells grow in suspension culture in the presence of mitogens, forming neurospheres. Any contaminating cells adhere to the bottom of the culture vessel and are removed upon replating of the cells in a new culture vessel. As any other cell culture system the neurosphere culture system has it pros and cons (reviewed by Jensen and Parmar, 2006). The proliferation of the neural progenitor cells is affected by cell plating density (Tropepe *et al.,* 1999). In addition, the composition of the growth media and the concentration of intrinsic and extrinsic factors, such as growth factors, affect cell proliferation and differentiation (Irvin *et al.,* 2003). The method of passaging (enzymatic vs. manual) may affect the properties of the cells, as do the passage number as the cells tend to lose their neurogenic potential, becoming more gliogenic, upon long-time culture. The reason for this is not known. We made use of the fact that neurospheres can be frozen, thus allowing us to freeze neurospheres at early passages to be thawed when the existing cultures grew older. The sensitivity of the culture system to variation underscores the importance of standardized cell culture routines and may lead to difficulties in comparing research results obtained by different laboratories. The neurospheres are also heterogenous in nature, consisting of cells at various stages of differentiation (reviewed by Jensen and Parmar, 2006). Thus, one should not consider the neurosphere system as a model for studying neural stem cells but as a model to study the various

progenitor cells arising within the neurospheres. The neurosphere system does, however, comprise a valuable model to study neural development *in vitro*. Importantly, it has been demonstrated that cortical progenitor cells expanded as neurospheres develop in a similar manner to as they do *in vivo* (Shen *et al.,* 2006). The intrinsic properties of the neurospheres as well as the fact that they are grown in defined conditions provides a model system that allows for the study of forebrain development and adult neurogenesis, and factors affecting these processes, in cell culture.

Real-time PCR was used to study the change in gene expression of AMPA receptor subunits in differentiating cultures. Real-time PCR is a quantitative, efficient, and highly sensitive method for studying changes in gene expression although care has to be taken in choosing the suitable gene or genes for normalization. We decided to perform normalization using the geNorm software, which evaluates the stability of expression of the normalization genes (Vandesompele *et al.,* 2002), thus allowing us to exclude the weakest gene out of three, leaving us to normalize the obtained data to the remaining two genes. The analysis was based on data obtained from whole cultures differentiated for 1 and 5 days. This setting is not the most optimal as these cultures includes not only the differentiating cells but also the neurospheres. However, as the expression of AMPA receptor subunits is lower in proliferating neural progenitors than in differentiating cells (unpublished results) and increase upon cell differentiation we concluded that the increase in AMPA receptor gene expression observed in this study was a result of increased AMPA gene expression by differentiating cells. By collecting RNA from only differentiated cells it would probably be possible to obtain even greater differences in the gene expression levels in cells differentiated for various periods of time. It would therefore be of interest to perform single cell PCR to see if this is indeed the case.

Immunocytochemical analysis can give us a view of the state of a cell by characterizing the expression of specific proteins. Today there are a lot of commercially available antibodies, which are more or less well characterized. The challenge when it comes to immunocyto-chemical characterization of differentiating cells is to define the specificity of the resulting staining. The cells might or might not express a certain marker or the expression may be very weak. The use of any antibody thus requires rigorous evaluation to determine its specificity (Fritschy, 2008). We thus employed various methods for determining antibody specificity, i.e. morphological analysis, double staining with antibodies recognizing different epitopes of the same protein, blocking peptides, Western blot analysis etc., as well as careful titration of the antibody concentrations used, to ensure the specificity of the antibodies used in this work.

Intracellular Ca^{2+} is essential in that it regulates many cellular functions (Zheng and Poo, 2007). Fura-2-AM (Grynkiewicz *et al.,* 1985) is a sensitive ratiometric and membrane-permeable fluorescent indicator dye for measuring intracellular Ca^{2+}. The peak excitation wavelength of the dye changes upon binding to Ca^{2+} and as a consequence it is possible to measure the amount of free intracellular Ca^{2+} by performing ratioed measurements at 340 nm and 380 nm wavelengths. Some problems associated with the use of fura-2-AM are compartmentation, incomplete de-esterification of the dye (intracellular esterases cleave AM from the fura-2 dye and traps the dye inside the cell), leakage of the dye into the extracellular

medium, and photobleaching of the dye when exposed to excitation light. Photobleaching can be minimized by reducing excitation light intensity (Becker and Fay, 1987) and optimal loading conditions should be determined to reduce compartmentation (Malgaroli *et al.*, 1987). Possible leakage of dye into the extracellular medium that could yield fluorescence background is not a problem in a setting where a perfusion system like the one we have used is utilized as any leaked dye is washed away with the perfusate. Incomplete de-esterification is, in turn, avoided by loading the cells for a long-enough time to allow for the esterases to cleave the AM product away from the dye, thus minimizing fluorescent artifacts caused by the also fluorescent fura-2-AM.

Changes in intracellular pH affect various metabolic reactions as well as the function of cellular organelles. The membrane-permeable dye BCECF-AM allows accurate measurement of changes in intracellular pH (Adams and Levin, 2012). The ratioed measurements are made by alternatively exciting the cells with 440 nm and 490 nm lights, which is detected at 510 nm, and the emission ratios are turned into pH values by using a standard curve. As with fura-2-AM the AM group of BCECF-AM is cleaved intracellularly by esterases, allowing the free BCECF to fluoresce according to the intracellular pH. Photobleaching, leakage, and acid load caused by BCECF-AM hydrolysis are problems associated with the use of BCECF-AM. Problems associated with photobleaching and dye leakage can be dealt with as described above. In the cytosol BCECF ionizes, releasing protons into the intracellular environment. This can result in an acid load, which may lead to a physiological response that may interfere with the measurements. To avoid this problem the loading concentration of the dye should be kept as low as possible but still high enough to obtain an adequate fluorescence signal (Aharonovitz *et al.*, 1996).

The membrane potential of the differentiating cells were measured using $DiBAC_4(3)$, a lipophilic potential-sensitive probe that enters the cells and binds to intracellular membranes and proteins. Depolarized cells accumulate the negatively charged dye, exhibiting enhanced fluorescence intensity when the cell membrane is depolarized. Hyperpolarization causes efflux of the dye and decreased fluorescence intensity. The cells are excited with 490 nm wavelength lights and the emitted fluorescence captured at 530 nm. Problems associated with the use of $DiBAC_4(3)$ is temperature fluctuations and, as with any fluorescent dye, photobleaching (Adamas and Levin, 2012).

Time-lapse imaging using the Cell-IQ® system enables the acquisition of continuous image series of living adherent cells for observing morphologic changes and cell movement without the use of labels and dyes and without disturbing the environment of the cells (Narkilahti *et al.*, 2007; Toimela *et al.*, 2008). Continuous monitoring of cells is more informative than conventional endpoint microscopy. A challenge in the analysis of living cells arise due to the fact that cells move and may also changes their morphological appearance. A major issue is thus to determine the morphological features of the cell type of interest and to quantify these features.

6.2 Glutamate-mediated neural progenitor cell differentiation and migration

Neurotransmitter-mediated signaling plays important roles in neuronal development and the majority of differentiating cells migrating out from SVZ-derived neurospheres respond to various neurotransmitter receptor ligands (Castrén *et al*., 2005; Kärkkäinen *et al*., 2009). It is known that glutamate is involved in promoting and/or inhibiting the proliferation and differentiation of neural progenitor cells (reviewed by Schlett, 2006). In this thesis, it is shown that differentiating cells derived from neural progenitors of the embryonic mouse brain show a distinct spatial and temporal distribution of functional neurotransmitter receptors. Cell systems *in vitro* usually lack directional properties. In these studies the neurosphere model has been used as it provides a definite point of origin for the differentiating cells, thus allowing the study of cell migration out of the neurosphere as a function of time and distance.

6.2.1 Differentiation potential of neurosphere-derived cells

Neural progenitor cells isolated from the lateral ventricles of embryonic mice proliferate as free-floating neurospheres in the presence of the growth factors bFGF and EGF (Reynolds and Weiss, 1992; Doetsch *et al.,* 1999; Gritti *et al.,* 1999). Upon growth factor removal the neurospheres adhere to a suitable growth substrate and cells start to migrate out of the neurosphere and differentiate. Neurospheres contain multipotent stem cells as well as more restricted progenitor cells (Brazel *et al.,* 2005; reviewed by Jensen and Parmar, 2006). In the studies conducted for this thesis it was shown that cells migrating out of the neurospheres are initially GLAST-positive radial glial cells with thick processes. Soon after, neuron-like cells with a small cell body and thin neurites and showing positive staining for markers of immature neurons (calbindin, doublecortin, and musashi-1) appear, migrating on and among the radial glial cell processes and eventually moving outside the radial glial cell layer. A similar behavior of differentiating human fetal neurosphere-derived cells has been observed (Caldwell *et al.,* 2001). As the cells migrate further away from the neurosphere they mature and become positive for markers of mature neurons (MAP-2, NeuN). Astrocytes and pre-oligodendrocytes were also identified in the cultures but to a lesser extent, thus confirming that these cells are indeed multipotent. That no mature oligodendrocytes could be identified in the cultures, as shown by the lack of immunoreactivity towards the myelin protein CNPase, is most likely explained by the time in differentiating culture conditions. Pre-oligodendrocytes were only identified after 5 days in culture and an even longer time of differentiation would be required for the appearance of mature oligodendrocytes. In a culture system utilizing embryonic stem cell-derived neurospheres less than 10% of cells were CNPase-positive after 10 days of neurosphere differentiation (Covey *et al.,* 2012), supporting this hypothesis.

6.2.2 Distinct spatial and temporal distribution of glutamate receptor subtypes

Our studies showed that when analyzed using Ca^{2+} imaging the glutamate receptor-mediated Ca^{2+} response was initially mainly metabotropic in nature, responding to the group I mGluR agonist DHPG, and correlated with the distribution of GLAST- and mGluR5-expressing radial glial cells in the inner migration layers. Neural progenitor cells in the developing and adult brain of both rodents and humans express mGluR5 (Di Giorgi-Gerevini *et al.,* 2005; Castiglione *et al.,* 2008; Zhao *et al.,* 2011; Nochi *et al.,* 2012; Zhao *et al.,* 2012). While the proliferation and differentiation of neural progenitors from the SVZ of adult mice seemed to be affected by the presence of an mGluR1 antagonist and potentially express mGluR1b, but not mGluR1a, protein (Castiglione *et al.,* 2008) mGluR1 expression has not been identified in the neurogenic zones of the embryonic brain (Di Giorgi-Gerevini *et al.,* 2004). Pharmacological studies have confirmed that the response to group I mGluR agonists in differentiating embryonic and postnatal murine neural progenitor cell cultures is mediated by mGluR5 (Castrén *et al.,* 2005; Kärkkäinen *et al*., 2009). It is thus deduced that the metabotropic Ca^{2+} response to DHPG seen in our studies is mediated by mGluR5. The results summarized above may reflect a developmental expression pattern of the group I mGluRs, with mGluR5 being present early during embryonic development and mGluR1 expression emerging in the neurogenic zones at a later time point.

The mGluR5-mediated Ca^{2+} response described in our studies was sensitive to antagonists of transient potential receptor channels (SKF-96365, pyr-2, pyr-3), which is in line with previous data showing that mGluR5 couples to TRPC channels (Gee *et al.,* 2003; Kim *et al*., 2003; Berg e*t al*., 2007; Ben-Mabrouk *et al.,* 2012). The TRPC channels are non-selective Ca^{2+}-permeable channels activated by PLC-dependent mechanisms that are important in neuronal development, including neural progenitor cell proliferation (reviewed by Tai *et al.,* 2009).

We found that the expression of mGluR5 and the Ca^{2+} response to DHPG was high in the beginning of the differentiation period and decreased towards the outer migration layers and with time in culture. The expression patterns of iGluRs were shown to be the opposite. This has also been shown before (Kärkkäinen *et al.,* 2009; Louhivuori *et al.,* 2011). AMPA/KA receptors are expressed during early nervous system development (LoTurco *et al.,* 1995; Scherer and Gallo, 1998; Haydar *et al.,* 2000; Maric *et al.,* 2000; Brazel *et al.,* 2005). In the culture system used in the studies described in this thesis an ionotropic Ca^{2+} response through the activation of AMPA/KA receptors could be seen from the first day of differentiation. This response increased towards the outer migration layers and with increased time in differentiating culture conditions so that almost all of the cells in the outer layers responded to stimulation with KA. These results correlate well with the gene and protein expression data obtained. We found *Gria1-4* mRNA transcripts to be upregulated upon prolonged differentiation, in particular the expression of the *Gria1* subunit mRNA. Similar results have been obtained previously in cultures of differentiating human neural progenitors where *Gria1* mRNA was upregulated to a larger extent than *Gria2-4* mRNA upon neural differentiation (Whitney *et al.,* 2008). These results indicate a distinct role for GluA1 in neural progenitor cell differentiation. While protein products of all of the four AMPA receptor subunits are

present in the adult brain (Monaghan *et al.,* 1984; Petralia and Wenthold, 1992; Arai *et al.,* 1997) we only identified GluA1-3 protein in our differentiating cultures. This is in accordance with results showing GluA1-3 expression by cells in the developing cortical plate (Métin *et al.,* 2000). Functional NMDA receptors were only barely detectable at 1 day of differentiation but their expression increased upon differentiation. This is in line with previous results showing that functional NMDA receptors emerge later in development than AMPA/KA receptors (LoTurco *et al.,* 1991, 1995; Sah *et al.,* 1997; Sadikot *et al.,* 1998; Behar *et al.,* 1999; Maric *et al.,* 2000).

AMPA/KA receptors are present and have important functions during early neural development (Scherer and Gallo, 1998; Maric *et al.,* 2000; Nguyen *et al.,* 2001; Suzuki *et al.,* 2006; Whitney *et al.,* 2008). We found that AMPA/KA receptors are functional in neural progenitor-derived cells *in vitro* from the first day of differentiation, as indicated by the presence of KA-responsive channels in all migration layers throughout the differentiation period. The AMPA receptors are initially mostly Ca^{2+}-permeable and become more Ca^{2+}-impermeable as the cells mature, as shown by the decreasing extent to which PhTx blocked the KA-induced Ca^{2+} response. Although Ca^{2+}-permeable GluA2-lacking AMPA receptors are expressed by several classes of interneurons and have important functions in neuronal plasticity (reviewed by Liu and Zukin, 2007) the Q/R-editing of GluA2 is a physiologically essential feature of glutamatergic neurons as the presence of Ca^{2+}-permeable GluA2-containing AMPA receptors in glutamatergic neurons of postnatal mice have been linked to seizures and premature death of the affected animals (Higuchi *et al.,* 2000). The results obtained in our studies are in agreement with previous studies on human fetal neural progenitors, which initially express unedited GluA2-containing Ca^{2+}-permeable AMPA receptors but show increased amounts of the *Gria2* pre-mRNA editing enzyme ADAR2 as well as *Gria2* pre-mRNA editing as the cells differentiated into neurons and astrocytes (Whitney *et al.,* 2008). Our results suggest a functional maturation of the differentiating cells as they migrate away from the neurosphere and after prolonged time in culture. These results, obtained using Ca^{2+} imaging, are supported by analysis of immunocytochemically stained cells showing increased expression of markers of mature neuronal cells, such as MAP-2 and NeuN, in cells in the outermost migration layers in cells differentiated for longer time periods.

6.2.3 Effects of glutamate receptor antagonists

Both metabotropic (Di Giorgi-Gerevinin *et al.,* 2005; Castiglione *et al.,* 2008; Gandhi *et al.,* 2008; Zhao *et al.,* 2011; Nochi *et al.,* 2012; Zhao *et al.,* 2012) and ionotropic (LoTurco *et al.,* 1995; Haydar *et al.,* 2000; Bai *et al.,* 2003; Hirasawa *et al.,* 2003; Deisseroth *et al.,* 2004; Brazel *et al.,* 2005; Suzuki *et al.,* 2006; Whitney *et al.,* 2008) glutamate receptors have been implicated in neural progenitor cell survival, proliferation, and differentiation. Glutamate is found in high levels in the brain already during early development (Behar *et al.,* 1999; Haydar *et al.,* 2000) and present in the growth media in which the neural progenitors are propagated and differentiated. By blocking the activation of particular glutamate receptor

subtypes it was found that these glutamate receptor subtypes are of importance in the development of radial glia and in neuronal cell movement. Antagonizing mGluR5 function using MPEP resulted in impaired extension of radial glial processes while AMPA/KA receptor block using CNQX had a reverse effect on the radial glial cells. The effects of blocking mGluR5 could be distinguished already after 1 day of differentiation and became increasingly visible with time. Blocking AMPA/KA receptors initially increased the length of the radial glial processes but after 5 days of differentiation the effect was less pronounced. mGluR5, together with mGluR3, is involved in the regulation of filopodial movement in astrocytes (Lavaille *et al.,* 2011). The same mechanism may be at play in radial glial process extension. Expression of AMPA/KA receptors, which are involved in neurotransmission, is ubiquitous in neuronal cells but AMPA/KA receptors are also expressed by glial cells (Steinhäuser and Gallo, 1996; Chew *et al.,* 1997; Scherer and Gallo, 1998). The role of AMPA/KA receptor expression in glial cells is not yet fully understood but has been implicated in neuronal plasticity and pathogenesis (Steinhäuser and Gallo, 1996). Interference with AMPA/KA receptors on glial cells may thus affect these functions.

Although the protein expression and functional response of mGluR5 was weak in the outer cell layers containing mainly neuronal cells we found that mGluR5 had a distinct role in regulating neuronal motility. Neurosphere-derived neuronal cells moved in patterns of alternating bursts of motion and stationary periods. During the stationary periods cells extended thin processes in many directions. This kind of saltatory movement has previously been identified for radially migrating (locomoting) neurons in organotypic slice cultures (Nadarajah *et al*., 2001). Cells migrating through the process of somal translocation were, in turn, shown to move in a constant steady pace (Nadarajah *et al*., 2001). By blocking mGluR5-mediated glutamate signaling neuronal cell movement was increased. This occurred through enhanced cell progression and shortened stationary phases. Immobile phases are times during which cells explore their surroundings (Gomez and Spitzer, 1999). As the cells treated with the mGluR5 antagonist MPEP had less time to explore their environment during the shortened stationary periods they also changed direction less frequently. A similar effect after TRPC1 knockdown in immortalized GnRH neurons has recently been shown, with the receptor knockdown promoting cell migration (Storch *et al.,* 2012). TRPC channels are activated by mGluRs (Gee *et al.,* 2003; Kim *et al*., 2003; Berg e*t al*., 2007; Ben-Mabrouk *et al.,* 2012) and function, among other things, as sensors of environmental cues (reviewed by Tai *et al.,* 2009). These channels regulate Ca^{2+}-mediated neurite extension as well as growth cone morphology and turning by mediating cellular responses to various chemoattractants and -repellants (Greka *et al.,* 2003; Bezzerides *et al.,* 2004; Li *et al.,* 2005; Shim *et al.,* 2005; Wang and Poo, 2005). The mGluR5-mediated effects on neuronal cells observed here may result from minute local changes in intracellular Ca^{2+} in neuronal cells, possibly through the actions of TRPC channels, and might sensitize cells to environmental cues by regulating cell movement.

Antagonizing AMPA/KA receptor function had a reverse effect on neuronal cell motility compared to block of mGluR5. While mGluR5 inactivation resulted in increased neuron-like cell movement, possibly through TRPC-mediated mechanisms, AMPA/KA receptor

inactivation caused a marked decrease in cell movement and changed the pattern the cells moved in, causing the cells to progress less and turn more frequently, probably as a result of the prolonged periods of immobility. Glutamate has been implicated in neuronal migration by signaling through ionotropic glutamate receptors (Behar *et al.*, 1999; Manent *et al.*, 2005, 2006; reviewed by Heng *et al.*, 2007; Manent and Represa, 2007). While NMDA receptor activation is of importance in neuronal migration and neurite extension/retraction (Behar *et al.*, 1999; Manent *et al.*, 2005, 2006; reviewed by Heng *et al.*, 2007; Manent and Represa, 2007) differentiating embryonic neural progenitor cells express NMDA receptors only at very low levels and only during the later period of differentiation, as shown in study **III**. AMPA/KA receptors are, however, present from the beginning of the differentiation period. Presence of the AMPA/KA receptor antagonist CNQX in the differentiation media did not effect somal movement but disturbed neurite motility, leading the cell body to move back and forth along the leading and trailing processes that made little progress. This is in line with data showing that activation of AMPA receptors in neurons derived from human neural progenitors or a human neuroblastoma cell line increase neurite formation and that antagonizing AMPA receptor function reversed this effect (Voss *et al.*, 2007; Whitney *et al.*, 2008). In contrast, AMPA receptor activation caused neurite retraction in tangentially migrating neurons in the developing rat cortex (Poluch *et al.*, 2001) and inhibited actin dynamics and the motility of axonal filopodia in embryonic hippocampal neurons (Chang and De Camilli, 2001). Blocking of AMPA/KA receptors using CNQX has, however, been shown to decrease migration of interneurons to the hippocampal primordium in embryonic corticohippocampal organotypic slice cultures due to disturbed cellular motility (Manent *et al.*, 2006; reviewed by Manent *et al.*, 2011). Interneurons migrating tangentially in the IZ express GluA2-lacking Ca^{2+}-permeable AMPA receptors (Métin et al., 2000). Cells in our cultures express GluA2 and are initially Ca^{2+}-permeable but become more Ca^{2+}-impermeable with time. The varying effects on neurite extension and cell movement mediated through AMPA/KA receptors described above might be explained by the difference in cell types studied, which most likely express different AMPA receptor subtypes and thus exhibit differences in Ca^{2+}-permeability of the AMPA receptors. This can be likened to the fact that neuronal migration is differentially affected by neurotransmitter signaling depending on cell type in question. The neurons of the hippocampus provide one example of this as migration of glutamatergic hippocampal neurons is mostly mediated through NMDA and $GABA_A$ receptors while AMPA receptors are involved in the migration of hippocampal interneurons (reviewed by Manent *et al.*, 2011). The neural progenitor cells used in this study primarily differentiate into glutamatergic neurons (Gorski *et al.*, 2002) but one cannot completely rule out the presence of a small amount of contaminating MGE-derived interneuron progenitors in the cultures, which, if present, might contribute to the data obtained.

Ca^{2+} signaling in the cell is a complex process. It is well known that intracellular Ca^{2+} signaling, both spontaneous and receptor-mediated, is coupled to cell differentiation, cell movement, neurite outgrowth, and growth cone dynamics (Gomez and Spitzer, 1999; Ciccolini *et al.*, 2003; Bolsover, 2005; reviewed by Gomez and Zheng, 2006; Zheng and Poo, 2007). Neurotransmitter receptors that regulate intracellular Ca^{2+} concentrations, such as mGluR5 and AMPA/KA receptors, expressed on cells at particular times during development

will thus strongly influence and regulate cellular functions such as neurite extension and cell migration. The glutamate receptor-mediated effects on cell movement and morphology observed here may thus be linked to intracellular Ca^{2+} regulation but the exact molecular mechanisms remain, however, to be elucidated.

6.3 Effects of BDNF on neurosphere-derived cells

The neurotrophic factor BDNF is an important regulator of neuronal development, including cell migration and differentiation (reviewed by Huang and Reichardt, 2001; Dicou, 2009). Our results show that while neurosphere-derived cells expressing mGluR5 and AMPA/KA receptors did not respond to the presence of BDNF in the differentiation media, neuronal cells that expressed NMDA receptors did. More specifically, BDNF shifted the location of NMDA-responsive cells towards the outer migration layers while the number of NMDA-responsive cells in the cultures remained the same. The distribution of the NMDA-responsive cell population correlated with cells expressing the high-affinity BDNF receptor TrkB, which has been shown to be expressed during early cortical neurogenesis (Klein *et al.*, 1990; Allendoerfer *et al.*, 1994; reviewed by Huang and Reichardt, 2001), and the expression of which was found at similar levels in similar cultures in a previous study (Louhivuori *et al.*, 2011). TrkB expression has been shown to increase as SVZ-derived neural progenitors (Gascon *et al.*, 2005) and cells of the adult SGZ mature towards a more neuronal phenotype (Donovan *et al.*, 2008), reflecting the increased expression of TrkB seen in these cultures after 5 days of differentiation compared to 1 day of differentiation. BDNF increases the number and survival of newborn neurons deriving from the SVZ (Kirschenbaum and Goldman, 1995; Zigova *et al.*, 1998; Bath *et al.*, 2008). Our results, showing increasing numbers of cells positive for neuronal markers in the outer migration layers in the presence of BDNF supports the role of BDNF in neuronal migration. BDNF also increased the number of TrkB-positive neuronal cells in the outer migration layers but the total number of TrkB-positive cells remained roughly the same as in cultures differentiated in the absence of BDNF. Even though the progenitor cells used in this study primarily differentiate into glutamatergic neurons (Gorski *et al.*, 2002) the NMDA receptor expressing BDNF-responsive cell population also expressed functional GABA receptors at low levels at 5 days of differentiation. Expression of functional GABA receptors in the proliferative zones of embryonic brain and in neural progenitors has been shown previously and GABA is known to regulate progenitor cell proliferation and neuronal migration (LoTurco *et al.*, 1995; Ma and Barker, 1995; Behar *et al.*, 1996, 1998, 1999, 2000, 2001; Haydar *et al.*, 2000; Lopez-Bendito *et al.*, 2003; Nguyen *et al.*, 2003; Cuzon *et al.*, 2006; Inada *et al.*, 2011). While BDNF has been shown to regulate expression levels of both glutamate and GABA receptors (Narisawa-Saito *et al.*, 1999; Mizoguchi *et al.*, 2003a, b; Nagano *et al.*, 2003) in this study BDNF seemed to mainly regulate the migration of a certain subpopulation of cells that migrated freely outside the neurosphere. BDNF did not seem to affect glutamate or GABA receptor expression levels and did not affect radial glial process extension. A subpopulation of embryonic cortical neurons has been shown to migrate in response to BDNF acting as a chemoattractant (Behar *et al.*, 1997) and embryonic cortical interneurons migrate increasingly

towards the cortex in response to BDNF (Polleux *et al.*, 2002). While BDNF was shown to increase the rate of tangential migration of neuroblasts that moved in a saltatory manner in the RMS (Bagley and Belluscio, 2010) BDNF did not increase the maximum speed of cell movement in the study conducted here. BDNF did, however, increase the average speed of cell movement by shortening stationary periods and increasing the time the cells moved actively. A similar effect where neurotrophin-4 promoted the motility of interneurons migrating tangentially has been shown (Polleux *et al.*, 2002). Various guidance cues affecting cellular movement act through Ca^{2+}-dependent mechanisms (reviewed by Gomez and Zheng, 2006; Zheng and Poo, 2007). BDNF can act through Ca^{2+}-dependent mechanisms (Behar *et al.*, 1997) and the motogenic actions of BDNF seen here may be mediated through such mechanisms. This is supported by the fact that the cell population responding to BDNF was a specific subpopulation of cells expressing functional and highly Ca^{2+}-permeable NMDA receptors. Since BDNF increased the number of calbindin-positive cells (calbindin is a Ca^{2+}-binding protein expressed predominantly by GABAergic but also by glutamatergic cells; Kondo *et al.*, 1990; Anderson *et al.*, 2001) in the outer cell layers and affected cells responding to stimulation with both NMDA and GABA the effect of BDNF on cell motility might also, at least partly, be mediated through a GABA receptor-coupled mechanism. That BDNF increases the number of neuronal cells as well as neurite formation in neuronal cells derived from striatal neural progenitors, cells which are mainly calbindin-expressing GABAergic neurons (Ahmed *et al.*, 1995), supports this hypothesis. Further work is needed to clarify the exact mechanisms behind the BDNF-mediated mechanisms seen here.

6.4 Intracellular pH and the effects of hypoxia/acidosis on differentiating neural progenitor cells

Maintenance of oxygen and pH levels is crucial for proper brain homeostasis as hypoxia and disturbed pH levels cause brain damage (Kaku *et al.*, 1993). Hypoxia/ischemia and alkalinizing conditions have been associated with increased activation of NMDA receptors and voltage-gated Ca^{2+} channels, increasing intracellular Ca^{2+} levels and neuronal excitability and leading to excitotoxic cell damage (Saybasili, 1998). It is, however, known that hypoxic conditions, to which decreased pH levels are associated, promote stem cell proliferation and differentiation as well as migration of newly generated neurons towards injured brain areas (Yagita *et al.*, 2001; Fagel *et al.*, 2006; Horie *et al.*, 2008; Santilli *et al.*, 2010). A lowered pH may, in fact, have neuroprotective effects by decreasing NMDA receptor activation and blocking voltage-gated Ca^{2+} channels, thus protecting cells from excitotoxic injury (Giffard *et al.*, 1990; Tang *et al.*, 1990; Kaku *et al.*, 1993; Saybasili, 1998). As ischemic conditions can promote neurogenesis after ischemic insult (reviewed by Kokaia and Lindvall, 2003) identification of factors and mechanisms involved in promoting ischemia-induced neurogenesis may be of future therapeutic use.

We studied the intracellular pH of differentiating embryonic neural progenitor cells and how the pH was affected by acidic and hypoxic conditions. In the brain hypoxia causes an acidification of cells (Huang and McNamara, 2004), affecting in particular astrocytes, which

have important roles in maintaining the extracellular environment in the brain (Bondarenko and Chesler, 2001). When we exposed the neurosphere-derived cells to acute hypoxia an acidification of the cells in the inner migration layers containing mainly radial glial cells could be observed, indicating that the cells in the inner migration layer function similarly as astrocytes derived from the mature brain (Bondarenko and Chesler, 2001). There was barely any change in the intracellular pH in cells in the outer cell layers containing mainly neuronal cells in response to hypoxia. A hyperpolarization of the cell membrane after exposure to hypoxia was, however, seen in all cells. Neuronal cells hyperpolarize in response to hypoxia, possibly due to activation of adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels expressed by neurons (Fujiwara *et al.*, 1987; Godfraind and Krnjević, 1993; Fujimura *et al.*, 1997; reviewed by Ballanyi, 2004). Hypoxia-induced hyperpolarization and K_{ATP} channel activation has been associated with having a neuroprotective effect during hypoxic conditions (reviewed by Ballanyi, 2004). The hyperpolarizing effect on both neuronal and glial cells might also be mediated through the activation of a certain type of two-pore potassium channels, TREK channels, expressed widely by cells in the CNS, including neural progenitors (Xi *et al.*, 2011). These channels cause K^+ efflux and hyperpolarization of the cell membrane, decreasing voltage-gated Ca^{2+} channel activity, glutamate release, NMDA receptor activation, and excitability (Franks and Honoré, 2004; reviewed by Huang and Yu, 2008). Hypoxia has been shown to upregulate TREK-1 channel expression in astrocytes while blocking TREK-1 channel activation significantly increases neuronal apoptosis (Wu *et al.*, 2012). These features implicates a neuroprotective role for also this K^+ channel during ischemic conditions

It has been shown that the pH of the brain can fall to levels close to pH 6 after global ischemia (Nemoto and Frinak, 1981), leading to severe brain damage. One of the mechanisms behind hypoxia-induced acidification causing irreversible damage to neuronal tissue might be the activation of acid-sensing ion channels that cause depolarizing currents and increased Ca^{2+}-influx (Huang and McNamara, 2004; Xiong *et al.*, 2004; Duan *et al.*, 2011). We studied the effects of long-term acidosis on neurosphere-derived cells by differentiating the cells in media with different pH. While it was clear that exposure of the cells to acidosis (pH 6.2) caused cell death within 24h the cells survived surprisingly well in less, but still markedly, acidic conditions (pH 6.8 and pH 6.5), suggesting that these neural progenitor-derived cells possess some kind of mechanism that protects them against low pH. This may be compared to the fact that neural progenitors are not harmed by the high levels of glutamate present in zones of active neurogenesis (Miranda-Contreras *et al.*, 1998, 1999, 2000; Behar *et al.*, 1999; Haydar *et al.*, 2000) or by hypoxic or even anoxic conditions (Takagi et al., 1999; Yagita et al., 2001; Bürgers *et al.*, 2008; Nochi et al., 2012), levels of glutamate and oxygen that would normally be harmful to cells in the brain. Instead these factors promote neural progenitor cell proliferation and differentiation (reviewed by Schlett, 2006; Zhu *et al.*, 2005). Clearly, neural progenitors and their derivatives react differently to certain extracellular factors than some other cells of the organism. Interestingly, we found that radial glial cells had a higher intracellular pH and resting membrane potential than neuronal cells. These two cell populations can thus be distinguished from each other by means of measuring intracellular pH and membrane potential. The intracellular pH of radial glia was also affected to a lesser

extent than that of neuronal cells, the pH of which decreased rapidly, when exposed to acute extracellular acidosis. These results suggest that the two cell types differ in their pH regulation and that the neuronal membrane is more permeable to proton influx, resulting in a lower resting intracellular pH and a higher sensitivity to changes in extracellular pH. Since no effect on intracellular pH of the neuronal cells was seen after depolarization the mechanisms behind this is probably an electro-neutral one. Differentiated cells have a more hyperpolarized membrane potential than progenitor cells (reviewed by Sundelacruz *et al.*, 2009) and the values measured here are in line with previously published results (Noctor *et al.*, 2002; Cai *et al.*, 2004; Liu *et al.*, 2005; Moe *et al.*, 2005; Liu *et al.*, 2006; Yasuda *et al.*, 2008). K^+-induced depolarization and a subsequent rise in the intracellular pH have been shown to occur in astrocytes (Brookes and Turner, 1994; Pappas and Ransom, 1994). This supports our results showing that the intracellular pH of neuronal cells was unaffected but was elevated in radial glial cells in the inner migration layer upon depolarizing stimuli, probably through the action of a voltage-dependent Na^+/HCO_3^- co-transporter known to be expressed by astro- and oligodendroglial cells (O'Connor *et al.*, 1994; Boussouf *et al.*, 1997) or a voltage-gated proton channel (reviewed by DeCoursey, 2003).

Exposing the cells to alkalinizing conditions also supported the notion that the cells in the inner and outer migration layers differ in their pH regulation. The cells in the inner layers exhibited a sustained alkalinization when exposed to ammonium chloride while the neuronal cells were only transiently alkalinized. It is well known that increased levels of ammonia in the brain are toxic. Alkaline conditions increase NMDA receptor activation, synaptic transmission, and excitotoxicity (Saybasili, 1998). Much of the ammonia in the brain is produced by neurons and removed by the actions of astrocytes (reviewed by Cooper and Plum, 1987). Here the neuronal cells in the outer cell layer were seemingly more susceptible to influx and accumulation of ammonium ions causing a transient increase in intracellular pH followed by a fall of the intracellular pH to below initial levels. This phenomenon was originally identified in squid giant axons (reviewed by Boron and De Weer, 1976) and most likely involves a barium-sensitive inward-rectifier K^+ (K_{ir}) channel permeable to ammonium ions and known to be present on neural progenitor cells (Choe *et al.*, 2000; Yasuda *et al.*, 2008). This hypothesis is supported by the fact that the two cell populations could not be distinguished from each other when the ammonium pre-pulse experiment was performed in the presence of barium. Other mechanisms for ammonium influx into neurons and astrocytes include Na^+, K^+-ATPase antiporters and Na^+-dependent K^+, Cl^- co-transporters (Kelly and Rose, 2010).

7 CONCLUSIONS

The aim of this thesis was to study factors regulating neural progenitor cell differentiation and migration to increase the knowledge about the fundamental properties of neural progenitor cells. The main findings can be summarized as follows:

- Multipotent neural progenitors deriving from the dorsal wall of the lateral ventricles of embryonic mice grow as neurospheres and differentiate into radial glia, neurons, astrocytes, and pre-oligodendrocytes. Radial glial cells are the first cells to emerge from the differentiating neurospheres, closely followed by neuron-like cells. Astrocytic processes extend from the neurosphere early during the differentiation process while pre-oligodendrocytes appear later during the differentiation process.

- The glutamatergic phenotype of the differentiating cells is initially mainly metabotropic, expressing mGluR5, and become increasingly ionotropic towards the outer migration layers and with time in culture. The differentiating cells abundantly express AMPA/KA receptors from the beginning of the differentiation period. The AMPA receptors are initially mostly Ca^{2+}-permeable but become less Ca^{2+}-permeable when differentiated for a longer time. A subset of neuronal cells expresses NMDA receptors after prolonged differentiation.

- Differentiating neural progenitor cells express *Gria1-4* mRNA and GluA1-3 protein. Expression of mRNA transcripts is upregulated as differentiation proceeds, with *Gria1* mRNA being most significantly upregulated. Neuron-like cells express mainly GluA1 and GluA2 protein while GluA3 protein is expressed by both neurons and glial cell.

- Glutamate receptors have important roles in the differentiation process and regulate radial glial process extension and neuronal cell movement. Activation of mGluR5 increases radial glial process extension and reduces motility of neuronal cells. AMPA/KA receptor activation impairs the development of the radial glial cell layer and promotes neuronal motility.

- BDNF increases the expression of neuronal markers, especially in the outer cell layers, and enhances the migration of a specific TrkB-positive NMDA- and GABA-

responsive neuronal subpopulation by prolonging periods of active movement and reducing stationary phases.

- Intracellular pH and resting membrane potential distinguishes two different cell populations migrating out of neurospheres. These two populations correspond to radial glial and neuronal cells and exhibit considerable differences when exposed to hypoxic, acidic or alkalinizing conditions. Neuronal cells show a high tolerance to prolonged acidic conditions.

- The neurosphere model is a suitable tool to study neural progenitor cell differentiation and migration and extracellular factors affecting these parameters in a spatial and temporal manner *in vitro*.

8 Future perspectives

A tightly controlled brain homeostasis is essential for the brain to perform its functions optimally. As disease or insult to the brain occurs the delicate balance of the internal environment of the brain is disturbed, affecting disease progress and recovery. Adult neurogenesis is restricted and unable to replace damaged tissue. With the advances made within the field of stem cell research during the last decades the notion of cell-based therapies or enhanced activation of endogenous neurogenesis have gained more focus. By understanding signaling pathways and mechanisms that regulate stem cell self-renewal and differentiation, both during normal and pathological conditions, the development of targeted cell grafts or identification of factors that promote endogenous neurogenesis may be possible. The careful elucidation of these mechanisms is, however, of utter importance as any negative effects would be detrimental. Newly generated neurons may restore lost neurological functions but they may also contribute to pathological features of a disease. This is possibly the case in temporal lobe epilepsy where cognitive functions are negatively affected by seizure-induced neurogenesis (Parent *et al.,* 1997).

Disturbed glutamate signaling in the diseased brain causes excitotoxic cell damage as a consequence of receptor over activation (Saybasili, 1998). It has thus been speculated that by modulating glutamate receptor function it would be possible to protect cells from further damage. In fact, glutamate receptor antagonists have been shown to reduce brain damage after ischemic stroke in animal models but no clinical trial has yet been successful, mainly due to side effects of the drugs (reviewed by Huang and McNamara, 2004; Mattson, 2008; Bruno *et al.,* 2001). In addition to being the chief excitatory neurotransmitter in the brain glutamate is also an important regulator of neural progenitor cell proliferation and differentiation into new neurons (reviewed by Schlett, 2006). This is an important thing to consider when designing drugs and treatment programs for neurological disorders. While it in some cases may be beneficial to block glutamate receptor activation during the acute phase of ischemic stroke to reduce excitotoxic cell damage glutamate receptor activation could, in turn, possibly aid in recovery from insult by enhancing cell proliferation, differentiation, and migration. Other factors linked with pathological conditions such as ischemia, like hypoxia and pH, are also important factors affecting neural progenitor cell growth and differentiation (reviewed by Kokaia and Lindvall, 2003).

The mammalian cortex with its highly organized structure is relatively conserved among species, as is its development. It has also been shown that cortical progenitor cells in culture develop according to the same schedule as they do *in vivo* (Shen *et al.,* 2006). Studying neural progenitor cell characteristics *in vitro* can give us a clue about the real situation *in vivo*. In this study it was shown that glutamate receptor function is important in neural progenitor cell differentiation and migration. Metabotropic and ionotropic glutamate receptors are expressed in distinct spatial and temporal patterns by the differentiating cells and interference with receptor function affects both radial glial and neuronal cells, but through different receptor subtypes. It was also shown that these two cell populations respond

differently to factors linked with ischemia and that BDNF affects the migration of a specific subpopulation of neuronal cells. As the specific molecular mechanisms by which these actions occur remain unclear further work to elucidate the mechanisms regulated by the factors studied here needs to be conducted. Of specific interest would be to study if the effects on cell morphology and motility seen in this work, mediated by glutamate receptors and BDNF, are due to changes in intracellular Ca^{2+} signaling affecting the cell cytoskeleton, and to elucidate the role of TRPC receptors in mGluR5-mediated neuronal motility. To clarify the possible role of GluA1 in neural progenitor cell differentiation is also of interest. The lack of agonists and antagonists selective for specific AMPA receptor subtypes complicates more specific studies of AMPA receptors and the development of subtype-selective compounds would greatly aid AMPA receptor research. The use of genetically engineered mice where specific AMPA receptor subunits have been deleted could, however, serve as models to study the role of specific receptor subunits in neural progenitor cell differentiation and migration, either in intact animals or in isolated neural progenitor cells *in vitro*. As the neurosphere-derived cells studied here exhibit differences in their intracellular pH levels and in the way they react to changes in extracellular pH and oxygen levels it would also be of important to characterize the expression of K^+- and H^+-gating ion channels and transporters on neural progenitor cells and their glial and neuronal derivatives in more detail. Understanding how glial and neuronal cells differ in their pH regulation and why they react differently to changes in pH and oxygen levels will increase the knowledge regarding the role of these factors in brain damage, neuroprotection, and neurogenesis.

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APPENDIX: Original publications