Lineage Selection and Enhanced Tissue Integration of Functional and Cryopreservable Human Embryonic Stem Cell-Derived Neurons

Dissertation

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CONTENTS

ABBREVIATIONS

°C	Degree Celsius
ApoER2	ApoE receptor type 2
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
bp	Base pair
BrdU	Brom-desoxyuridine
cAMP	Cyclic adenosine monophosphate
CC	Corpus callosum
CAM	Cell adhesion molecule
Cdk5	Cyclin-dependent kinase 5
cDNA	Complementary DNA
Chat	Choline acetyltransferase
CNS	Central nervous system
Cx	Connexin
CXCR4	Chemokine (C-X-C motif) receptor 4
Dab1	Disabled-1
DAPI	4’,6-diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleotidtriphosphate
DsRED2	Red fluorescent protein 2
EB	Embryoid body
EGF	Epidermal growth factor
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescence protein
ES cells	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor

Flk/KDR	Vascular endothelial growth factor receptor 2
Flt1	Fms-related tyrosine kinase 1
Flt-4	Fms-related tyrosine kinase 4
g	Gram
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GC	Granular cell
GCL	Granular cell layer
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
h	Hour
HC	Hippocampus
HB-EGF	Heparin-binding EGF
hES cell	Human embryonic stem cell
Hz	Hertz
ICM	Inner cell mass
iPS cell	Induced pluripotent stem cell
kg	Kilogram
KO-SR	Medium containing KO-DMEM and serum replacement
LIS1	Lissencephalic 1
Lt-hESNSC	Long-term self-renewing hES cell derived neural stem cells
M	Molarity
MAP	Microtubule associated protein
MEF	Mouse embryonic fibroblasts
mg	Milligram
min	Minute
mM	Millimolar
mOsm	Milliosmolar
ms	Millisecond
MZ	Marginal zone
Neo	Neomycin
nM	Nanomolar
NSC	Neural stem cell
OB	Olfactory bulb
P	Postnatal day

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PNS	Peripheral nervous system
PO	Poly-l-ornithine
PSA-NCAM	Polysialylated neural cell adhesion molecule
PSC	Postsynaptic current
PTB	Phosphotyrosine-binding
RMS	Rostral migratory stream
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
sec	Second
SDF-1	Stromal cell-derived factor-1
SHH	Sonic hedgehog
SGZ	Subgranular zone
SOX2	Sex determining region Y box 2
SR	Serum replacement
ST	Striatum
SVZ	Subventricular zone
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
TX	Transplantation
VEGF	Vascular endothelial growth factor
VLDLR	Very low-density lipoprotein receptor
VZ	Ventricular zone
µm	Micrometer
µM	Micromolar

1. INTRODUCTION

Stem cells are characterised by two unique features that distinguish them from other types of cells. First, they have the ability of substantial self-renewal. The second is that they have the potential to differentiate into various cell types. During early mammalian development pluripotent stem cells give rise to all three germ layers. The proliferation and differentiation of these pluripotent stem cells are regulated by environmental factors and intrinsic signals.

Human embryonic stem (hES) cells are pluripotent stem cells. By studying their biology important insight into early processes of human development can be gained which might be of major importance for future therapeutic applications. A prerequisite for such applications is to determine the conditions for the differentiation of hES cells into specific somatic cell types or even cellular subtypes.

Of particular interest is the generation of cells of the central nervous system (CNS) as the CNS is characterised by unsurpassed complexity and unlike many other tissues of the human body, has a limited capacity for self-repair in response to injury.

In the last few years increasing knowledge about hES cells, including the production of neural stem cells (NSCs) from hES cells has build the foundation for using hES cells for various applications. First of all, they provide the opportunity to study processes underlying the development of the human CNS *in vitro*. Secondly, they are candidates for future therapeutic applications in neurodegenerative diseases. For the latter, it is necessary to determine the factors that regulate NSC self-renewal for *in vitro* expansion, to understand how to differentiate NSCs into specific neural subtypes and how to produce sufficient numbers of ideally pure populations of such subtypes. Furthermore, it is necessary to identify the signals controlling progenitor cell migration. This may allow directing grafted cells to particular targets within the CNS.

1.1. Stem cells and their neurogenic potential

Stem cells are undifferentiated cells that are able to self-renew and give rise to at least one, but often many specified cell types (Weissman, 2000a). They have varying repertoires. A pluripotent stem cell can give rise to every cell of the organism except the trophoblast of the placenta (Gage, 2000). A multipotent stem cell can only give rise to cells of specific tissues (van der Kooy & Weiss, 2000). To maintain a stable stem cell population and at the same time produce restricted daughter cells, the division of stem cells is often asymmetric, meaning one daughter cell differentiates into a more mature progeny, while the other daughter cell remains a stem cell (Knoblich, 2001). Although stem cells should be able to

divide without limits, they do not have to divide rapidly. In fact later in life, many stem cells divide at a relatively low rate (Donovan & Gearhart, 2001).

Embryonic stem (ES) cells, which can be derived from the inner cell mass of blastocysts (Smith, 2001) are pluripotent, which means that they can differentiate into cells derived from any of the three germ layers. Somatic stem cells, which can be found throughout the body during and after embryonic development are multioptent, meaning they can only differentiate into tissue specific cell types. A wide variety of adult mammalian tissues including CNS, bone marrow, skeletal muscle, intestine, pancreas, liver and epidermis harbour somatic stem cells (Hall & Watt, 1989; Potten & Loeffler, 1990; Weissman, 2000b). They are needed to maintain tissue homeostasis and repair lesions after an injury. NSCs are the somatic stem cells of the nervous system. They appear in the embryonic mammalian CNS (Cattaneo & McKay, 1990; Reynolds & Weiss, 1992; Kilpatrick & Bartlett, 1993; Temple, 2001) and the peripheral nervous system (PNS) (Stemple & Anderson, 1992). Adult NSCs have been found mainly in two adult neurogenic regions: the hippocampus and the subventricular zone (SVZ), but also in some non-neurogenic regions including the spinal cord (McKay, 1997; Rao, 1999; Gage, 2000).

1.1.1. Generation of pluripotent stem cells

There are different ways to generate pluripotent stem cells. For instance, pluripotent ES cells can be obtained from the inner cell mass of blastocysts (Figure 1.1) (Smith, 2001).

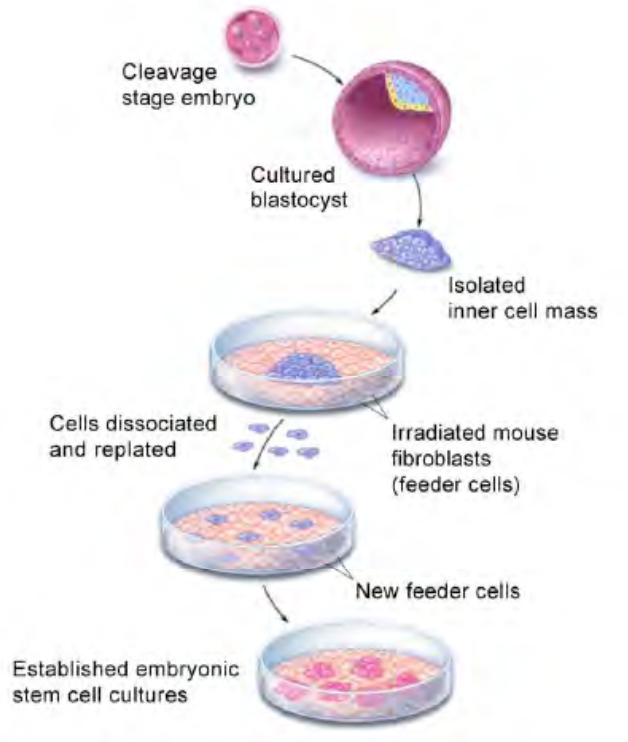

Figure 1.1: Generation of embryonic stem cell cultures. The inner cell mass of a pre-implantation embryo is isolated and cultivated on fetal fibroblasts. Cell lines can be established by dissociation and propagation of the obtained colonies. Adopted from Winslow & Duckwall (Winslow & Duckwall, 2001).

For this purpose, the inner cell mass is isolated and plated onto fibroblasts to maintain the undifferentiated state of the isolated ES cell. The developing ES cell colonies can then be further cultivated. ES cells have until now been generated from various species, including humans (Thomson *et al.*, 1998). More recently, ES-like cells have also been isolated from later stage blastocysts and also from the morula stage (Stojkovic *et al.*, 2004; Strelchenko *et al.*, 2004). For the generation of ES cells through therapeutic cloning, the nucleus of a somatic stem cell is transferred into an enucleated oocyte (Wilmut *et al.*, 1997). By isolating the inner cell mass of the developing blastocyst, ES cells with the same nuclear genome as the donor cell can be obtained.

Recent studies also showed the generation of induced pluripotent stem (iPS) cells, which is a type of pluripotent stem cell artificially derived from a non-pluripotent cell such as neonatal or adult human fibroblasts, by transcription factor-based reprogramming (Figure 1.2) (Takahashi & Yamanaka, 2006; Okita *et al.*, 2007; Wernig *et al.*, 2007).

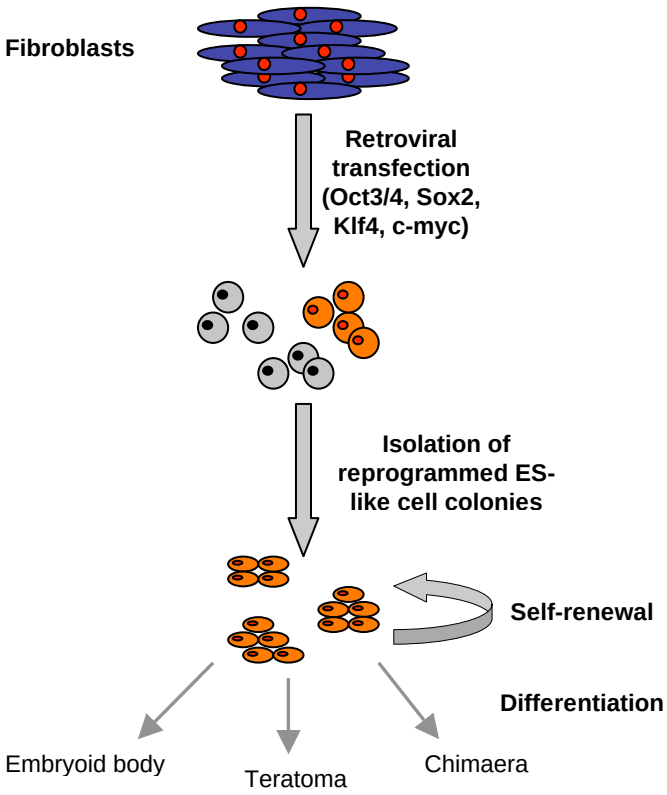

Figure 1.2: Generation of iPS cells. Specialised cells from fetal tissue can be rejuvenated and reprogrammed into cells resembling ES cells by introducing master transcription regulators into the cells, followed by a selection process to isolate reprogrammed cells. These cells resemble ES cells in that they display key properties of self-renewal and the ability to mature into many different cell types. The latter were observed in embryoid bodies, in teratomas and in chimeras (Surani & McLaren, 2006).

The reprogramming is typically achieved via virus-mediated gene transfer of master transcription regulators such as Oct-3/4, Sox2, Klf4, c-myc, Nanog and Lin28 (Nakagawa *et al.*, 2008). After 3-4 weeks, small numbers of transfected cells start to become

morphologically and biochemical similar to pluripotent stem cells. They can be isolated by virtue of their morphology, doubling time or a reporter gene expression. Ongoing studies are comparing iPS cells to ES cells, for identifying similarities and differences between these pluripotent stem cells. The expression of certain stem cell genes, DNA methylation patterns, doubling time, teratoma and chimera formation, and their differentiation potential are currently under investigation (Maherali *et al.*, 2007; Wernig *et al.*, 2007).

Pluripotent stem cells generated by therapeutic cloning or by transcription factor-based reprogramming may offer major advantages for cell replacement strategies. On the one hand, patient-specific pluripotent stem cell lines could be generated to prevent rejection of transplanted cells. On the other hand, human pluripotent stem cell lines with the genotype of specific human diseases could be generated. These cells could then be differentiated *in vitro* into relevant populations, such as dopamine neurons from Parkinson's patients. This would enable the study of disease pathogenesis *in vitro* and the development of new treatment strategies.

However, there are still concerns with respect to potential therapeutic applications of such pluripotent stem cells. While the use of hES cells generated through therapeutic cloning bears various ethical problems, the generation of iPS cells have major biological drawbacks. The current need of viral transfection of potentially oncogenic factors include the risk of insertion mutagenesis. This can lead to the creation of cells, which might undergo uncontrolled proliferation and tumorigenesis. Even though iPS cells can be generated from mouse and human fibroblasts without the oncogenic c-myc retrovirus, the reprogramming efficiencies are thereby decreasing strongly (Marson *et al.*, 2008; Nakagawa *et al.*, 2008). Because of that, the method of cre-mediated excision of the integrated c-myc carrying viral genome from the iPS cells might be a useful tool for the eventual application of iPS cells in human therapies (Hanna *et al.*, 2007). Efforts are also focusing on identifying alternatives to the viral delivery system such as using small molecules (Huangfu *et al.*, 2008), protein transduction of reprogramming factors (Bosnali *et al.*, 2008) or a plasmid-mediated system (Okita *et al.*, 2008) to generate iPS cells.

1.1.2. Strategies for the differentiation of pluripotent stem cells

For tissue regeneration and other biomedical applications pluripotent stem cells need to be differentiated into tissue specific cell types. A large variety of different cell types have already been generated from mouse and human ES cells including neural cells (Okabe *et al.*, 1996; Reubinoff *et al.*, 2001; Zhang *et al.*, 2001), cardiomyocytes (Xu *et al.*, 2002; Laflamme *et al.*, 2007), insulin-producing cells (Soria *et al.*, 2005; D'Amour *et al.*, 2006; Schroeder *et al.*, 2006), and several others. Key prerequisites for the biomedical application of ES cell-derived

somatic cell types are purity and cell type specification, as residual pluripotent ES cells may cause the formation of teratomas after transplantation (Thomson *et al.*, 1998; Amit *et al.*, 2000; Reubinoff *et al.*, 2001). Generally, two different strategies exist for the generation of highly enriched somatic cell populations: directed differentiation and lineage selection (Figure 1.3).

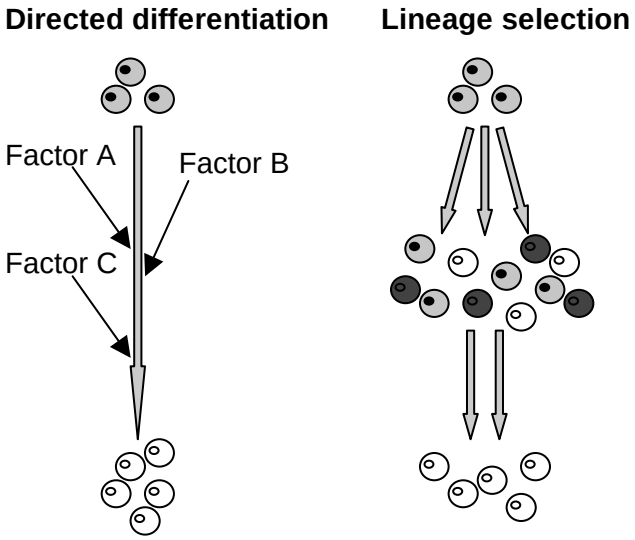

Figure 1.3: Strategies for the generation of pluripotent stem cell derived enriched somatic cell types. Directed differentiation is based on the sequential treatment with specific growth factors in defined media, guiding all cells towards the desired phenotype. For lineage selection, all cells are differentiated spontaneously into several phenotypes. The desired phenotype can then be isolated by cell type-specific selectable markers. Adopted from Wernig *et al.* (modified) (Wernig *et al.*, 2003).

The directed differentiation *in vitro* is based on the application of specific media compositions and extrinsic factors in a defined manner and sequence (Schuldiner *et al.*, 2000). The aim of this strategy is to induce the entire cell population to differentiate into the desired cell type. Early methods to direct the differentiation of ES cells to neural fates used retinoic acid treatment (Bain *et al.*, 1995), sequential culture in serum and serum-free media (Okabe *et al.*, 1996) or co-culture with specific stromal cell lines such as PA6 (Kawasaki *et al.*, 2000). Recently, several protocols were established to differentiate ES cells into NSCs (see also 1.1.3) (Conti *et al.*, 2005; Koch *et al.*, 2006). Although generating cells with a neural phenotype from ES cells is well established, it should be noted that there are many neuronal and glial subtypes with different physiological functions. Therefore, obtaining a purified subtype for basic research or for tissue repair is considerably more challenging. To achieve this, directed differentiation into NSCs has been combined with lineage selection. Lineage selection is based on the selection of a desired phenotype from a pool of heterogeneously differentiated cell types. Cells can be either selected on the basis of the expression of a specific surface antigen by immunological methods like immunopanning, magnetic or fluorescence activated cell sorting (FACS) (Roy *et al.*, 1999; Malatesta *et al.*, 2000; Roy *et*

al., 2000a; Carpenter *et al.*, 2001; Schmandt *et al.*, 2005), or by genetically modifying the ES cells or the ES cell derived NSCs with a selectable marker only expressed in the desired cell population. For selection, an antibiotic resistance gene or a fluorescence reporter gene under the control of a cell type-specific promoter is commonly used. The mixed cell population is either treated with a specific antibiotic, allowing only those cells to survive which express the marker, or subjected to FACS, which again leads to the enrichment of those cells expressing specifically a fluorescent protein in the desired cell type (Klug *et al.*, 1996; Roy *et al.*, 1999; Rietze *et al.*, 2001; Pasumarthi & Field, 2002; Wernig *et al.*, 2002; Glaser *et al.*, 2005).

1.1.3. Long-term self-renewing neuroepithelial stem cells from hES cells

In the laboratory where the presented work was performed Koch and co-workers established a population of stably proliferating neural stem cells from hES cells (lt-hESNSC) (Koch *et al*., 2006; Koch *et al.*, in revision). These cells can be expanded as adherent homogeneous monolayer across more than 100 passages, have a stable neurogenic differentiation pattern and are amenable to genetic modification (Koch *et al.*, 2006; Ladewig *et al.*, 2008), thereby overcoming many of the shortcomings of previously available cell populations. Immunhistochemical data and transcription factor expression data showed that under standard conditions these cells adopt a highly restricted regional identity comparable to an anterior and ventral hindbrain localization, an area with close spatial relationship to ventral mesencephalic progenitors. It was possible to demonstrate that even after extensive passaging these cells can be recruited into adjacent regional identities such as ventral midbrain dopaminergic neurons or spinal cord motoneurons. Furthermore, first direct evidence was provided for synaptic integration of lt-hESNSC-derived neurons into brain tissue after transplantation (Koch *et al.*, in revision).
The lt-hESNSC culture system provided an important basic for the studies presented in this work.

1.1.4. Potential therapeutic use of stem cells in CNS disorders

The CNS has a very limited regenerative capacity. Thus it is of major interest to investigate the ability of human NSCs engrafted into the brain to survive, migrate and integrate in a functional and meaningful manner.
Studies have shown that stem cells derived from the embryonic or fetal human brain can be successfully grafted into the developing rodent CNS. Once transplanted, these cells survive, migrate and integrate into the host tissue, giving rise to cells from the three fundamental

neuronal lineages i.e. neurons, astrocytes and oligodendrocytes (Brustle *et al.*, 1998; Flax *et al.*, 1998; Uchida *et al.*, 2000; Englund *et al.*, 2002b; Peng *et al.*, 2002; Honda *et al.*, 2007).
However, transplantation studies in the adult CNS are more challenging. As the tissue is fully established, developmental cues are limited and space is more constricted (Svendsen & Caldwell, 2000) leading to restricted migration and integration of the transplanted cells. Engraftment of fetal or ES cell derived neural progenitors in the adult CNS could show that transplanted cells survive but form a graft core meaning that the majority of the transplanted cells remain mainly situated at the grafted site (Guzman *et al.*, 2008). Restricted migration of the transplanted cells could be observed 10 to 15 weeks following engraftment (Fricker *et al.*, 1999; Aleksandrova *et al.*, 2002; Englund *et al.*, 2002a; Tabar *et al.*, 2005; Roy *et al.*, 2006; Guzman *et al.*, 2008). It was suggested that physical or molecular barriers caused by glial scarring at the transplantation site are the reason for the restricted outgrowth of transplanted cells (Reier *et al.*, 1983; Rudge & Silver, 1990). Such effect might be solved by micro-transplants, which minimize scarring at the grafted site (Nikkhah *et al.*, 1995; Davies *et al.*, 1997).
Nevertheless, cell replacement therapies for diseases of the adult brain have attracted attention since the first reports of successful transplantation of embryonic dopaminergic cells to patients with Parkinson's disease (Lindvall & Hagell, 2001). Parkinson's disease is characterized by a loss of dopamine-producing midbrain neurons with cell bodies in the substantia nigra. These neurons project to the striatum and are essential for motor function. Parkinson's patients suffer from various symptoms including resting tremor, difficulty in walking, and loss of facial expression. The disease is typically progressive due to ongoing loss of neurons. The first transplantation studies with fetal tissue in animal models of Parkinson's disease have shown that grafted dopaminergic cells are able to release dopamine at near normal levels and that the animals show significant behavioural recovery (Annett *et al.*, 1994; Herman & Abrous, 1994; Lindvall *et al.*, 1994). Positive effects have also been observed in clinical trails with human patients (Olanow *et al.*, 1996; Lindvall, 1999). Major improvements, however, were only seen in patients aged 60 years or younger (Freed *et al.*, 2001). Moreover, some patients receiving transplants developed dyskinesias, movement disorders associated with excessive dopamine levels in the brain. Further success of these transplantation approaches has been constrained by limited availability of fetal tissue, limited migration of grafted cells, and poor differentiation and survival of the grafted neurons (Richardson *et al.*, 2004). In addition to these problems, fresh fetal tissue cannot be standardized and raises ethical questions that have been debated intensely (Bjorklund & Lindvall, 2000).

Many of these issues can be better addressed by working towards an *in vitro* culture system. The knowledge about hES cells, including techniques of producing stable well-characterised NSCs from hES cells has provided prospects to generate large numbers of donor cells for neural repair. Many studies already show that neural progenitors derived from ES cells can give rise to dopaminergic neurons. This is mainly achieved by the combined use of FGF8 and Shh, which effectively induce dopaminergic neurons from ES cell-derived neural progenitors (Lee *et al.*, 2000; Yan *et al.*, 2005). Addition of ascorbic acid, brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), dibutyryl cyclic-AMP, and transforming growth factor-beta 3 (TGF-β3) yields cultures containing 30% to 50% neurons expressing beta-III tubulin, of which 65% to 80% express tyrosine hydroxylase required for dopamine synthesis. These neurons release dopamine upon depolarization, and form at least rudimentary synapses *in vitro* with transmitter re-uptake abilities (Kim et al., 2007; Joannides *et al.*, 2007). Following transplantation these cells survive, maintain their dopaminergic phenotype and functionally engraft in the brain (Sanchez-Pernaute *et al.*, 2005; Yang *et al.*, 2008). Using cultured ES cell-derived neural precursors as a source for transplantation therapies may, on the one hand, obviate some of the technical limitations associated with the use of fresh fetal tissue (Ostenfeld & Svendsen, 2003), but may also on the other hand, bear the risk of teratoma formation. Currently, the only way to ensure that teratomas do not form is to differentiate the ES cells in advance, enrich for the desired cell type and screen for the presence of undifferentiated cells. In addition, hES cell-derived neural precursor transplants have been found to give rise to proliferating neural clusters rather than individually incorporating neurons (Roy *et al.*, 2006) indicating that even committed progenitors can proliferate excessively after transplantation. This problem might be solved by using more restricted precursor cells or by the purification of desired postmitotic subtypes of neurons or glia.

Compared to cell replacement therapy for Parkinson's disease, in which one specific type of neurons has to be replaced by a direct local cell transplantation, cell therapy for stroke or spinal cord injury is a major challenge as transplanted NSCs need to replace a range of neuronal types, remyelinate axons and repair complex neural circuitries. In addition, it is required that transplanted cells reach the lesion site by following a gradient of inflammatory cues such as cytokines and chemokines (Ransohoff, 2002). As a preliminary step towards this goal, it was shown that human NSCs transplanted into the brains of rodents after stroke survived, migrated, and differentiated into various types of neurons (Aoki *et al.*, 1993; Ben-Hur *et al.*, 2003; Imitola *et al.*, 2004; Kelly *et al.*, 2004). Other degenerative diseases of the adult CNS such as Alzheimer's disease and amyotrophic lateral sclerosis would also require the migration of transplanted cells towards specific sites within the CNS. Many

neurodegenerative diseases are associated with a non-permissive environment, which can inhibit regenerative processes. These circumstances create an even bigger challenge for cell replacement therapy.

Thus, the major difficulties yet to be solved are how to direct and control the differentiation of specific phenotypes required for replacement and repair in each disease, how to purify lineage specific subtypes and how to improve cell migration and integration into the affected site of the CNS.

1.2. Cell migration in the vertebrate CNS

Neurons of the vertebrate CNS migrate from their places of birth to their location of function. During the last few years, significant progress has been made in the understanding of the molecular mechanisms, which control neuronal migration, either during development or within the adult CNS. Hence, the developing and adult CNS provides insight into the complex mechanisms involved in neuronal migration, which is a vital component for learning how to enhance the migration and integration of grafted NSCs for future neuronal cell replacement strategies.

1.2.1. Migration in early CNS development

In vertebrates the early embryo is divided into three germ layers: ectoderm, mesoderm and endoderm. The CNS, including the brain, the spinal cord and the retina of the eye, arises from the ectoderm. During development the ectoderm thickens depending on inductive signals from the underlying mesoderm to form the neural plate. During neurulation the neural plate folds and forms the neural tube (Kandel, 2000) (Figure 1.4). At that stage the neural tube consists of a single layer of rapidly dividing NSCs. Extensive proliferation within the germinal neuroepithelium leads to the generation of two different cell populations forming the ventricular zone (VZ) i.e. radial glial cells through symmetric cell division and neuroblasts through asymmetric cell division. During further development asymmetric cell division replaces the initial symmetric cell division of the radial glial cells and neural precursor cells arise (Temple, 2001). Radial glial cells span through the neuroepithelium while keeping attached to the pial surface and the luminal side of the neural tube. They serve as guiding tracks for neuroblasts, which migrate along the radial glial cells to the pial side where they settle as post mitotic neurons (Rakic, 1972). The neuroepithelial stem cells also give rise to another population of adult NSCs generating the SVZ. The regional identity of the cells at different positions along the dorsal-ventral axis of the tube is specified by the antagonistic signalling of the primary bone morphogenetic protein (BMP) and sonic hedgehog (Shh). The anterior-posterior axis is specified by various morphogens including fibroblast growth factors (FGFs), Wnt proteins and retinoic acid (RA) (Liu & Niswander, 2005). The extent of cell proliferation is not uniform and therefore results in the formation of the three brain vesicles: the prosencephalon, the mesencephalon and the rhombencephalon. The prosencephalon later gives rise to various structures of the brain including the cerebral cortex, the hippocampus, the thalamus and the olfactory bulb.

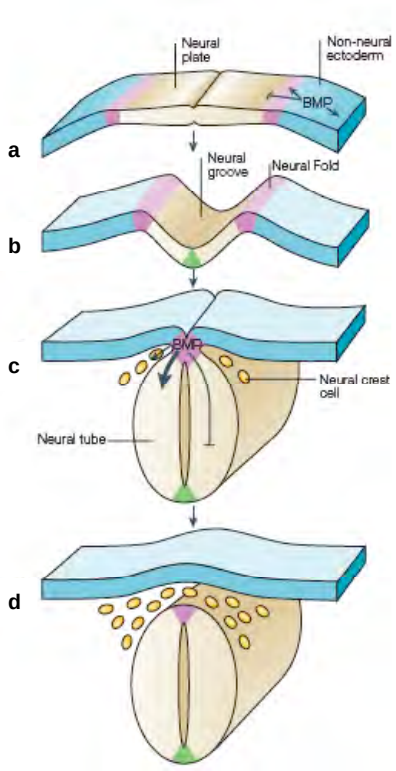

Figure 1.4: Formation of the neural tube. (**a**) The CNS arises from a specialised epithelium: the neural plate. (**b**) During neurulation the neurale plate folds, (**c**) the lateral edges of the neural plate fuse and (**d**) segregate from the non-neural epithelium to form the neural tube. Neural crest cells derived from the dorsal neural tube migrate out to form the PNS, melanocytes and cartilage in the head. Adopted from Liu & Niswander (Liu & Niswander, 2005).

1.2.2. Mechanisms of neuronal migration in the CNS

Following the patterning of the neural tube, cell migration plays an essential role in the formation of the CNS. Once the cells are specified, they migrate away from the ventricular zone to their final destination. The most complex migration can be found in the forebrain where cellular movement creates an organized structure of cell layers consisting of different neuronal subtypes which later give rise to the cerebral cortex. Two different types of migration have been identified in the forebrain: radial migration and tangential migration (Marin & Rubenstein, 2003).

Radial migration of neural precursor cells from the VZ towards the pial surface involves at least two different types of movement: soma translocation and glia-guided migration (Nadarajah, 2003) (Figure 1.5). Soma translocation is prevalent during early stages of cortical development and appears to be largely independent of radial glial cells (Morest, 1970; Nadarajah & Parnavelas, 2002). In glia-guided migration, neurons migrate along a radial glial scaffold which extends from the inner to the outer surface of the neural tube (Rakic, 1972) and receive guidance as they migrate. In soma translocation, however, the route is determined before the somata leave the VZ (Hatten, 1990; Rakic *et al.*, 1994). Neural precursor cells migrate radially from the VZ towards the pial surface, building up the cortical

cell layers in an inside to outside manner. As a result, each newly generated neuron migrates past the previously formed neuron (Rakic *et al.*, 1974; Nadarajah & Parnavelas, 2002). Subsets of neurons also migrate parallel to the surface of the brain in a tangential orientation (Hatten, 1999; Nadarajah & Parnavelas, 2002; Marin & Rubenstein, 2003) (Figure 1.5). During development, neurons migrate tangentially from the subpallium towards the cerebral cortex and the hippocampus (Corbin *et al.*, 2001). These neurons invade the cortex along the marginal zone or through the cerebral wall in a manner that is presumed to be independent of radial glial cells and give rise primarily to GABAergic interneurons (Stuhmer *et al.*, 2002). Some neurons can switch dynamically between tangential and radial migration as frequently observed in cortical interneurons, cerebellar granule neurons (Komuro & Rakic, 1995; Polleux *et al.*, 2002) and in spinal cord dorsal column neurons (Gray *et al.*, 1990).

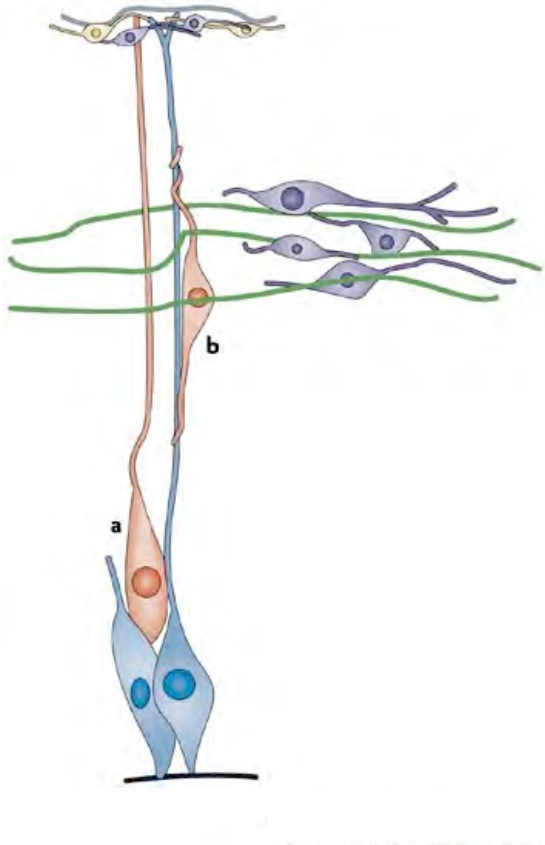

Figure 1.5: Radial and tangential migration of neurons in the developing cortex. Radially migrating neurons either use **(a)** soma translocation with a long leading process or **(b)** migrate in close proximity to a radial glial process (blue). Tangentially migrating neurons (purple) invade the cortex along the marginal zone or through the cerebral wall in a manner that is presumed to be independent of radial glial cells. Adopted from Ghashghaei *et al.* (Ghashghaei *et al.*, 2007).

Aside from the neuronal migration within the developing CNS neuronal migration also takes place in the adult CNS as neurons are constantly generated throughout adulthood from NSCs. These NSCs occupy specific niches in the dentate gyrus of the hippocampus (Eriksson *et al.*, 1998; Roy *et al.*, 2000b) and in the lateral ventricle SVZ and subcortical white matter (Lois & Alvarez-Buylla, 1993; Palmer *et al.*, 1997; Doetsch *et al.*, 1999; Temple, 2001; Doetsch, 2003). The NSC niche in the rodent CNS comprises several types of cells. Firstly, a layer of ependymal cells, which line the ventricle regions and shield the NSC niche from the ventricle. Secondly, NSCs, which are located adjacent to the ependymal cells and

extend with a single cilium structure through the boundary of ependymal cells to contact the ventricle region. NSCs divide and give rise to transit-amplifying cells, which in turn give rise to migratory neuroblasts (Figure 1.6). Both, in the SVZ and in the SGZ, blood vessels and the specialised basal lamina derived from endothelial cells represent essential components of the NSC niche. They are supposed to generate a variety of signals that control stem cell self-renewal and lineage commitment.

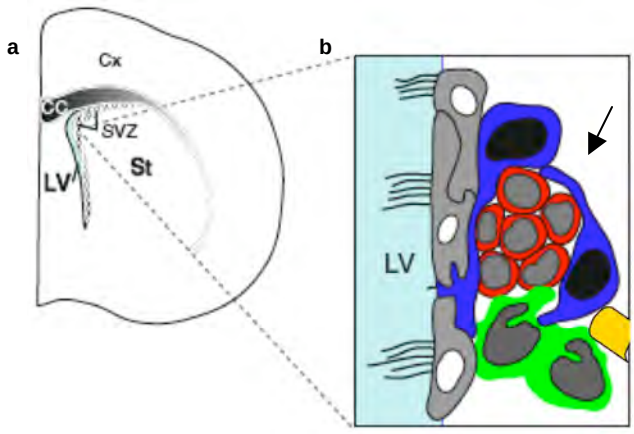

Figure 1.6: Schematic illustration of the SVZ NSC niche in the rodent brain. (a) The SVZ is located next to the lateral ventricle (LV) in the brain. **(b)** A detail of the lateral wall of the lateral ventricle. Lineage committed (post mitotic) migratory neuroblasts (red), NSCs (blue) and transit amplifying cells (green), the basal lamina (arrow), ciliated ependymal cells (grey) and brain microvessels (yellow) are in physical contact with each other to form the NSC niche. Cortex (Cx), corpus callosum (CC), striatum (St). Adopted from Martino & Pluchino (Martino & Pluchino, 2006).

Neuroblasts born in the SGZ migrate a short distance into the overlying dentate gyrus (Gage, 2000) whereas neuroblasts born in the SVZ migrate a long distance towards the olfactory bulb, where they differentiate into interneurons (Alvarez-Buylla *et al.*, 2001). Apart from radial and tangential migration, neuroblasts migrating long distances use a third mode of migration called chain migration (Lois & Alvarez-Buylla, 1994). Migrating cells which form chain-like structures appear to be either attached to one another, or attached to axons of neighbouring neurons (Lois *et al.*, 1996). These observations have lead to the conclusion that chain migration might be independent of glial cells. It is thought that these neurons are more likely to use corticofugal fibres, marginal zone neurons or the pial membrane as migratory guides. In the rostral migratory stream, however, tubes of astrocytic cells appear to be surrounded by chains of migrating neurons (Doetsch & Alvarez-Buylla, 1996), indicating that glial cells may play an active role in the regulation of chain migration (Mason *et al.*, 2001).

Neuronal migration, either in the developing or adult CNS involves four steps as follows. The first step is the initiation when the cell is activated from the stationary state to a mobile form. During this process the cell extends a leading neurite preceded by a growth cone that extends and contacts as it explores the microenvironment. During such manoeuvres the

nucleus and cell soma remain largely immobile. It is not until the leading neurite is consolidated and bipolar morphology acquired that soma translocation actually occurs (Lambert de Rouvroit & Goffinet, 2001). In a second step the nucleus moves towards a leading process called nucleokinesis which critically depends on the cytoskeleton (Morris *et al.*, 1998). Nucleokinesis begins with the stabilization of the leading process. The centrosome moves into the neurite quickly followed by the translocation of the nucleus towards the centrosome (Schaar & McConnell, 2005; Tsai & Gleeson, 2005). Once motile, the cell must maintain the migratory state and respond to guidance cues to find its final position. The third step is the retraction of the tailing process, which currently is poorly understood. Finally, unlike other cells, neurons form defined structures when reaching their final destination which is the fourth step of neuronal migration (Lambert de Rouvroit & Goffinet, 2001).

1.2.3. Factors regulating neuronal migration in the CNS

All steps involved in neuronal migration depend on extracellular cues such as various extracellular matrix components, growth factors and neurotransmitters either generated from cells of the NSC niche or from the surrounding tissue (Figure 1.7). These extracellular cues act through intracellular signalling cascades which in turn regulate the cytoskeletal machinery leading to cell locomotion mechanisms (Sobeih & Corfas, 2002). Studying the role of the extracellular cues on neuronal migration in the embryonic and adult CNS can teach us important lessons about the complex mechanisms involved in neuronal migration following NSC transplantation.

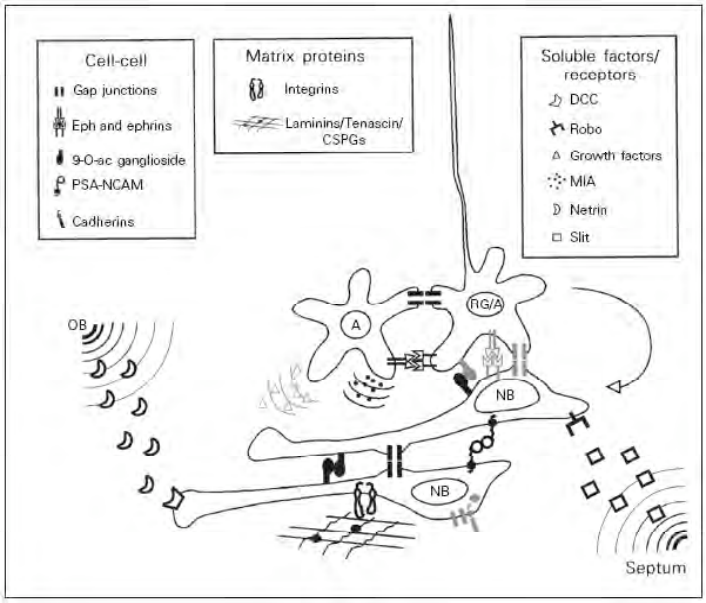

Figure 1.7: Summary scheme of putative molecules governing neuroblast migration from the NSC niche in the SVZ towards the olfactory bulb. Influences of diffusible factors, cell-cell and cell-ECM interactions on migration are illustrated. Neuroblasts (NB), astrocytes (A) and radial glia (RG). Adopted from Menezes *et al.* (Menezes *et al.*, 2002).

It has recently been suggested that EGF and TGFα play significant roles in radial migration in the cortex. The EGFR and its ligands, heparin-binding EGF (HB-EGF) and TGFα, are expressed in the germinal layers of the telencephalon. Mice lacking the EGFR show accumulation of neuronal precursors in the telencephalic proliferative zone, suggesting a defect in their migration (Threadgill *et al.,* 1995). In addition, a study using a replication defective retrovirus, provided evidence that the ability of neural cells to migrate depends on their level of EGFR expression, with cells overexpressing EGFR displaying increased radial migration in the cortex and olfactory bulb (Caric *et al.*, 2001). Another growth factor that has been shown to be crucial for neuronal migration is FGF2. FGF2 deficient mice have an abnormal organization of the cerebral cortex, caused by a failure of neural progenitors to migrate to their target layer (Dono *et al.*, 1998).

Neurotrophins, including BDNF and neurotrophin-4 (NT-4), which are known to act as neural survival factors, have also been shown to affect neuronal migration by regulating the expression of reelin (Brunstrom *et al.*, 1997; Sobeih & Corfas, 2002, Ringstedt, 1998) which is the best studied extracellular matrix (ECM) protein involved in neuronal migration (Frotscher, 1997; D'Arcangelo & Curran, 1998). Reelin is thought to function by allowing migrating neuronal cells to detach from the radial glia, as they reach the cortical surface and thereby making it possible for younger neurons to pass the older ones (Dulabon *et al.*, 2000). That would explain the inverted layering in the cerebral cortex seen in reelin mutatants (see 1.2.4) (Rice & Curran, 2001). Laminins and HSPGs are other components of the ECM that are known to influence neuronal migration (Liesi *et al.*, 1995; Hu, 2001).

Integrins and cell adhesion molecules (CAMs) are cell surface molecules that have been reported to be involved in the regulation of neuronal migration. Integrin α6β1 which is found to be expressed in neuroblasts, functions by linking the cell membrane with the ECM. Blocking of integrin α6β1 was shown to inhibit chain migration (Rice & Curran, 2001). Integrin ligands such as laminins have been found to be involved in the migration of cerebellar granule cells (Liesi *et al.*, 1995). CAMs are important for cell-cell recognition by affecting the adhesiveness of neurons to other neurons and to the ECM. Studies showed that mice lacking N-CAM (neural-CAM) exhibit a decreased migration of neural precursors along the rostral migratory stream resulting in a reduction in size of the olfactory bulb (Chazal *et al.*, 2000).

Furthermore, cell junctions play a role in neuronal migration. Recent studies showed that gap junction subunits connexin 26 (Cx26) and connexin 43 (Cx43) are expressed at the contact points between radial fibers and migrating neurons, and acute down regulation of the Cx26 or Cx43 impairs migration of neurons to the cortical plate. The gap junctions provide dynamic adhesive contacts that interact with the internal cytoskeleton to enable leading process

stabilisation along radial glial fibers as well as the subsequent translocation of the nucleus. They are necessary for glia-guided neuronal migration (Elias *et al.*, 2007).

Soluble factors create chemical gradients that are critical for neural migration. These factors are generated from the surrounding tissue and include attractive and repulsive molecules such as netrins, semaphorins, ephrins, slit, and various neurotrophic factors (Figure 1.7) (Marin *et al.*, 2003; Marin & Rubenstein, 2003). Slit is expressed in the septum and acts in combination with astrocyte-derived migration-inducing activity (MIA) as a repellent for SVZ cells to migrate towards the olfactory bulb (Mason *et al.*, 2001). Ephrins are expressed throughout the rostral migratory stream and are associated with astrocytes functioning in axonal pathfinding (Conover *et al.*, 2000). Netrin is expressed among others by cells of the olfactory bulb and acts as an attractant by influencing the orientation of the leading processes of neurons expressing the netrin receptor DCC (deleted in colorectal cancer) (Alcantara *et al.*, 2000). Chemokines are another family of soluble factors with over 40 proteins involved in migration in the CNS. They typically signal through specific transmembrane G-protein-coupled receptors and were originally identified as factors modulating the migration of leukocytes. Studies in the developing cerebellum showed the expression of the chemokine SDF-1 in cerebellar pia and its receptor CXCR4 in granule cell precursors. Deletion of either of these genes results in premature invasion of the cerebellar anlage by granule cells (Ma *et al.*, 1998; Zou *et al.*, 1998).

Apart from studying the role of extracellular cues on neuronal migration *in vivo* their role on neuronal migration have also been studied *in vitro*. To this end, slice cultures were used to study endogenous cell migration with the possibility to supply chemoattractants, receptor blocking antibodies or other reagents, which might be involved in neuronal migration (Krull & Kulesa, 1998; Tucker, 2001). A more reductionist way of studying migration with respect to chemoattractants is the use of microchemotaxis chambers (Boyden chambers) (Richards & McCullough, 1984). In this method, a membrane separates the upper and the lower part of a chamber. Possible chemoattractants are placed in the wells of the lower compartment and cells are plated in the upper well. If the studied agent acts as a chemoattractant the cells from the upper well will migrate through the pores of the membrane towards the lower well. By applying such methods it could be shown that PDGF and VEGF are acting as a chemoattractant for FGF2 stimulated neural progenitors *in vitro* (Forsberg-Nilsson *et al.*, 1998; Zhang *et al.*, 2003).

1.2.4. Neuronal migration defects in the CNS

By analysing genetic mammalian brain malformations, several genes could be identified which are involved in the regulation of neural migration (Table 1.1), including Doublecortin (DCX), Filamin A and Reelin.

Table 1.1: Overview of genes responsible for human congenital disorders featuring neuronal migration defects. Adopted from Ayala *et al.* (Ayala *et al.*, 2007).

Genes	Human Disorders	Gene Loci	Syndromes	Reference
Reelin	Lissencephaly with cerebellar hypoplasia	7q22	Mental retardation, hypotonia, epilepsy, and myopia	Hong et al., 2000
Lis1	Isolated lissecencephaly sequence and its severe form Miller-Dieker Syndrome	17p13.3	Mental retardation, epilepsy, and premature death; Miller-Dieker Syndrome also has craniofacial abnormalities	Ozmen et al., 2000; Reiner et al., 1993
14-3-3 ε	Miller-Dieker Syndrome	17p13.3	Craniofacial abnormalities	Toyo-oka et al., 2003
DCX	Isolated lissecencephaly sequence in males and Subcortical band heterotopia in females	Xq22.3-23	Mental retardation, and epilepsy; less severe in female due to X mosaic inactivation	Lambert de Rouvroit and Goffinet, 2001
Filamin A	Periventricular heterotopia	Xq28	Epilepsy and vascular signs	Fox et al., 1998
Fukutin	Fukuyama-type congenital muscular dystrophy	9q31	Mental retardation, epilepsy and muscular dystrophy	Gressens, 2005
POMGnT1 (protein O-mannose β-1,2-N-acetylglucosaminyltransferase)	Muscle-eye-brain disease	1p32-34	Mental retardation, severe myopia, glaucoma and muscular dystrophy	Yoshida et al., 2001
Disc-1	Schizophrenia	1q42.1	Schizophrenia	Kamiya et al., 2005; Millar et al., 2000

The DCX gene was identified by positional cloning and is located on the X-chromosome. It encodes a 361 amino-acid protein that associates with and stabilizes microtubules, but shows no similarity to any classical microtubule associated protein (MAP) (Francis *et al.*, 1999; Gleeson *et al.*, 1999; Taylor *et al.*, 2000). DCX is specifically and abundantly expressed in neuronal progenitors of the developing and adult CNS (Chelly, 1998; des Portes *et al.*, 1998; Gleeson *et al.*, 1998; Francis *et al.*, 1999; LoTurco, 2004; Rao & Shetty, 2004; Couillard-Despres *et al.*, 2005; Bernreuther *et al.*, 2006). An evolutionary conserved domain is repeated in the N-terminal part of the protein and called the DC repeat. Each repeat alone is able to bind tubulin but neither is sufficient to mediate microtubule stabilization. Like classical MAPs, the interaction of DCX with the microtubules can be regulated by a number of different kinases (Schaar *et al.*, 2004; Tanaka *et al.*, 2004). The pathologies associated with DCX mutations are characterised by a disorganisation of the layers in the cerebral cortex also known as the “double-cortex” (Feng & Walsh, 2001), in which neurons accumulate inappropriately within the white matter beneath the normal cortical layers (Figure 1.8 c) or scattered across the cortical layers. Mutations in the DCX gene cause the “double-cortex” (subcortical laminar heterotopias) malformation in heterozygous carrier

women and the X-linked lissencephaly in males (Figure 1.8) (des Portes *et al.*, 1998; Gleeson *et al.*, 1999). Knockdown of the DCX expression by RNAi in adult rats confirmed the role of DCX in neuronal migration as affected cells accumulated in the white matter or scattered across the cortical layers compared to their normal position in the cortex of control animals (Bai *et al.*, 2003). Overexpression of the wild-type DCX in neurons results in an increase in their migration rates of up to 30%. Further, this study indicated, that DCX in addition to its role in microtubule stabilisation, is also involved in dynein-driven nuclear translocation as DCX was also found in the neuronal soma and to associate with microtubules, which surround the nucleus (Tanaka *et al.*, 2004).

Biochemical and genetic evidence thus point to a role of DCX in crucial microtubule-based events in neuronal migration. However, which step of the migration process is affected in DCX mutants' remains to be clarified.

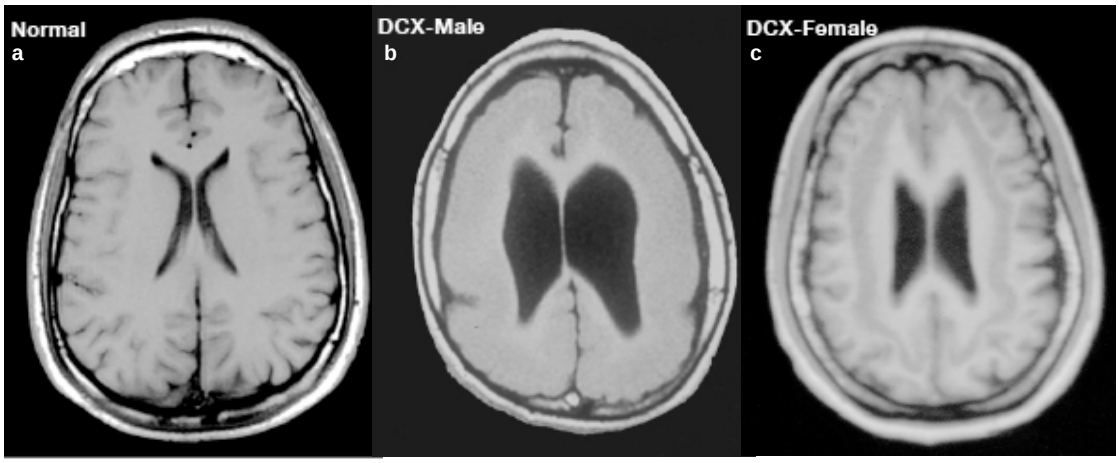

Figure 1.8: Pathologies associated with DCX mutations. MRI image of the cerebral cortex from **(a)** a human being, **(b)** a male patient and **(c)** a female patient with DCX mutation. Adopted from Feng *et al.* (Feng & Walsh, 2001).

Studies in mice have identified additional mutant genes, which cause defects in the cortical structure. Among the most well known are Filamin A and Reelin (Marin & Rubenstein, 2003).

Filamin A is an actin cross-linking protein that cooperates with members of the Arp2/3 complex to form orthogonal actin networks. Filamin A is able to interact with a diverse group of transmembrane proteins and serves as a scaffold for signalling proteins of the Rho family of GTPases, which are also involved in neurite extension and in neuronal migration (Luo, 2000; Dhavan & Tsai, 2001; Stossel *et al.*, 2001). Mutations in the Filamin A gene cause periventricular heterotopias where neurons completely fail to migrate out of the germinal layers and instead form clusters that line the lateral ventricles of the brain (Figure 1.9 b) (Fox *et al.*, 1998).

When neurons reach their final destination and stop migrating, they continue to autonomously assemble into defined architectures. Observations in reeler mice show that the normal function of the reelin signalling pathway is essential for proper radial organization and layering of post migratory neurons at all levels of the CNS (Lambert de Rouvroit & Goffinet, 1998). Reelin is a large glycoprotein (about 400 kDa) secreted by several neurons (D'Arcangelo *et al.*, 1995) and binds to the VLDLR (very low-density lipoprotein receptor) and ApoER2 (ApoE receptor type 2). Both have a short cytoplasmatic tail that interacts with the tyrosine kinase adapter Dab1 (Disabled 1), which is expressed in the cytoplasm of migrating cortical neurons as a 555 amino-acid protein comprising an N-terminal phosphotyrosin-binding (PTB) domain responsible for interacting with the lipoprotein receptors. Mutations of Reelin and Dab1 both generate a reeler phenotype (Howell *et al.*, 1997; Sheldon *et al.*, 1997; Ware *et al.*, 1997), which is characterised by an abnormal lamination of the cerebral and cerebellar cortices including inversion of the normal “inside-out” pattern found in mammals (D'Arcangelo *et al.*, 1995; Hirotsune *et al.*, 1995) (Figure 1.9 d). A crucial importance of Dab1 phosphorylation in neuronal positioning is demonstrated by the generation of Dab1 knock-in animals in which the fifth tyrosine residues have been replaced by phenylalanine. These mice display a similar phenotype to Dab1 null mutants (Howell *et al.*, 2000).

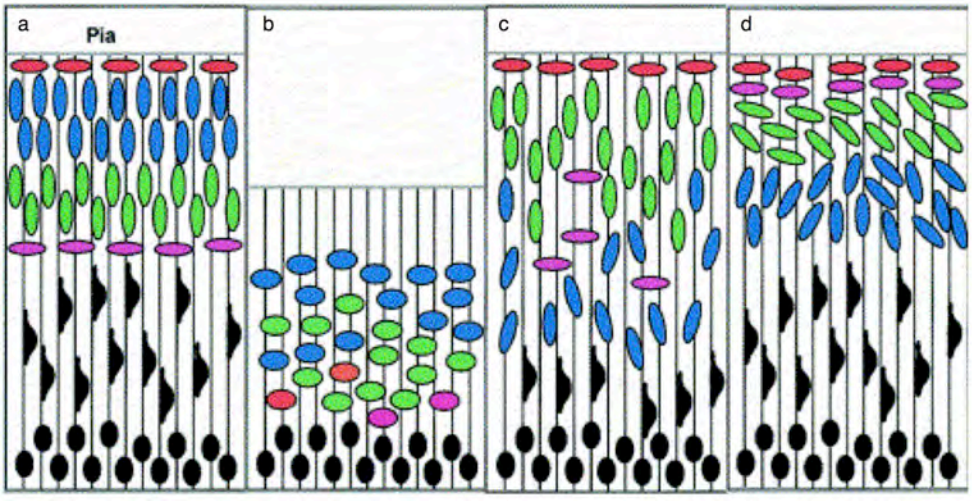

Figure 1.9: Schematic illustration of normal and different abnormalities of cortical development. (a) In the normal cortex, cells proliferate in the VZ and migrate along radial fibers. Early neurons settle horizontally in the marginal zone (MZ) (red) and subplate (pink). Early born neurons (green) and younger neurons (blue) demonstrate the so-called “inside-outside” gradient. **(b)** Mutations in the Filamin A cause periventricular heterotopias in which neurons cannot migrate from the VZ and settle locally. **(c)** In case of a DCX-mutation as occurs in type 1 lissencephaly, initial migration is satisfactory but many neurons settle at a subcortical level. **(d)** In the absence of Reelin signalling, cortical neurons (green) settle obliquely in the cortex. The next generation (blue) cannot cross the first one and the gradient is directed from outside to inside. Adopted from Lambert de Rouvroit & Goffinet (Lambert de Rouvroit & Goffinet, 2001).

1.3. Objectives of this study

The main goal of this project was to establish a new lineage selection protocol in order to generate pure cultures of immature neurons from human ES cells. To that end a reporter construct carrying the human Doublecortin (DCX) promoter and a fluorescent marker gene had to be introduced into long term proliferating human ES cell-derived neural stem cells (lt-hESNSC). As a prerequisite a detailed analysis of the endogenous DCX expression during the time course of neural differentiation and terminal maturation had to be performed. After finding appropriate transfection methods for lt-hESNSC the faithful expression of the fluorescent marker in differentiating lt-hESNSC had to be validated by comparison to the endogenous DCX expression level. Time period of differentiation as well as procedures for the fluorescent activated cell sorting (FACS) of DCX-EGFP-positive neurons had to be developed in order to achieve maximum post-FACS survival rates. Following immunocytochemical characterization electrophysiological properties of selected neurons including single cell patch clamp analyses had to be performed to analyse the influence of the selection procedure on the functional characteristics of the cells. In order to facilitate conservation of selected neurons the aim was to develop cryopreservation procedures enabling long-term storage of human neurons.

A second major aim of this study was to characterise the migratory properties of DCX-EGFP-selected neurons *in vitro* and *in vivo*. *In vivo*, expression of DCX is closely associated with a migratory phenotype. Consequently, the migratory capacity of DCX-EGFP selected neurons had to be explored *in vitro* (e.g. transwell filter assays), on hippocampal slice cultures as well as after transplantation into different regions of the adult rodent brain. A particular aspect was to compare the properties of a selected pure neuronal population with a mixed cell population containing neurons as well as neural stem/progenitor cells. These studies should provide insight into potential paracrine effects between neural stem/progenitor cells and early neurons in the graft.

Thus, the focus of this study was to develop a technology to generate large numbers of immature human ES cell derived neurons, to use these cells to study migration and integration of transplanted neural populations and to find ways to enhance the integration potential of human ES cell derived neurons for neural cell replacement.

2. MATERIALS

Cell culture plastic ware including dishes, centrifugation tubes, cell strainer, cell scraper and incubation tubes were obtained from BioRad Laboratories (Munich, Germany), Corning Coster (New York, USA), Eppendorf (Hamburg, Germany), Falcon/Becton Dickinson (Heidelberg, Germany), Greiner (Nürtingen, Germany), Millipore (Billerica, USA) and Nunc (Wiesbaden, Germany). Glass materials were obtained from Schott (Mainz, Germany).

2.1. Technical equipment

Appliance	Name	Manufacturer	Registered office
Autoclave	D-150	Systec	Wettenberg, Germany
Balance	BL610	Sartorius	Göttingen, Germany
Balance	LA310S	Sartorius	Göttingen, Germany
Block heater	Thermomixer compact	Eppendorf	Hamburg, Germany
Capillary	Capillary GB150T	Science Products	Hofheim, Germany
Cell culture centrifuge	Megafuge 1.0R	Kendro	Hanau, Germany
Counting chamber	Fuchs-Rosenthal	Faust	Halle, Germany
Cryostat	Cryostat HM 560	Microm Laborgeräte	Walldorf, Germany
Digital camera	C 5050 Zoom	Olympus Optical	Hamburg, Germany
Digital camera	Canon Power Shot G5	Canon	Krefeld, Germany
FACS® sorter	FACS® DiVa	BD Biosciences	San Jose, USA
Fluorescence lamp	HAL100	Carl Zeiss	Hamburg, Germany
Fluorescence lamp	Fluo-Lamp U-RFL-T	Olympus	Jena, Germany
Freezer -80°C	HERAfreeze	Kendro	Hanau, Germany
Freezing container	Namgene^{TM}Cryo 1°C	Nalge Nunc	New York, USA
Glass-Microelectrode Puller	PE-21	Tritech Research	Los Angeles, USA
Gel chamber	Agagel	Biometra	Göttingen, Germany

Appliance	Name	Manufacturer	Registered office
Incubator	HERAcell	Kendro	Hanau, Germany
Inverse light microscope	Axiovert 25	Carl Zeiss	Jena, Germany
Liquid nitrogen store	MVE 611	Chart Industries	Burnsville, USA
Micro Drill	High Speed Micro Drill	Fine Science Tools	Heidelberg, Germany
Microliterpipet	Microliterpipet 1710N	Hamilton	Bonaduz, Switzerland
Microscope	Axiovert 40 CFL	Carl Zeiss	Jena, Germany
Microscope	Axiovert 200M	Carl Zeiss	Jena, Germany
Microscope	Axioskop 2	Carl Zeiss	Jena, Germany
Microscope	ApoTom	Carl Zeiss	Jena, Germany
Microscope	IX 81 Confocal	Olympus	Hamburg, Germany
Microscope	Imager Z 1	Carl Zeiss	Jena, Germany
Microscope camera	Axiocam MRM	Carl Zeiss	Jena, Germany
Microscope camera	ProgRes C14	Jena Optic	Jena, Germany
Microscope laser	Laser Mells Griot	Laser Mells Griot Lasergroup	Carlsbad, Germany
Microscope slides	Superfrost plus	Menzel-Gläser	Braunschweig, Germany
Nucleofector	Nucleofector™	Amaxa	Cologne, Germany
Plastic coverslips	Thermanox®plastic coverslips	Nunc	Wiesbaden, Germany
PCR cycler	T3000 Termocycler	Biometra	Göttingen, Germany
pH-meter	CG840	Schott	Mainz, Germany
Pipetteboy	Accu-Jet	Brand	Wertheim, Germany
Polyester membrane	Transwell-Clear	Corning	Bodenheim, Germany
Power supply	Standard Power Pack P25	Biometra	Göttingen, Germany
Refrigerators/Freezers 4°C and -20°C	G 2013 Comfort	Liebherr	Lindau, Germany

Appliance	Name	Manufacturer	Registered office
Shaker	Bühler Schüttler WS 10	Johanna Otto	Hechingen, Germany
Sterile laminar flow hood	HERAsafe	Kendro	Hanau, Germany
Stereo microscope	STEMI 2000-C	Carl Zeiss	Göttingen, Germany
Stereotactic Frame	Stereotactic Frame	Stoelting	Illinois, USA
Sterilizer	Compact Hot Bead Sterilizer	Fine Science Tolls	Heidelberg, Germany
Thermocycler	T3 Thermocycler	Biometra	Göttingen, Germany
Table centrifuge	Centrifuge 5415R	Eppendorf	Hamburg, Germany
Transplantation tool	Micromanipulator	Self-made	Bonn, Germany
Transplantation tools	Transplantation Tool Set	Fine Science Tools	Heidelberg, Germany
Transwell	Millicell, 8 µm	Millipore	Billerica, USA
UV-Vis Spectrophotometer	BioPhotometer	Eppendorf	Hamburg, Germany
Vacuum pump	Vacuubrand	Brand	Wertheim, Germany
Vibroslicer	VSLM1	Campden Instruments	Sileby, GB
Water bath	1008	GFL	Burgwedel, Germany

2.2. Chemicals and reagents

Chemicals	Manufacturer	Registered office
2-Mercaptoethanol	Invitrogen	Karlsruhe, Germany
Adenosine 3’, 5’-Cyclic monophosphate	Sigma	Deisenhofen, Germany
Affi-Gel®Blue-Gel	BioRad	Munich, Germany
Agar	Sigma	Deisenhofen, Germany
Agarose	PEQLAB	Erlangen, Germany
Ampicillin	Sigma	Deisenhofen, Germany

Chemicals	Manufacturer	Registered office
Apo-Transferrin	Millipore	Schwalbach, Germany
Ascorbic acid	Sigma	Deisenhofen, Germany
B27 supplement	Invitrogen	Karlsruhe, Germany
BDNF	R&D Systems	Wiesbaden, Germany
Bromphenol blue	Sigma	Deisenhofen, Germany
BSA	Sigma	Deisenhofen, Germany
Collagenase type IV	Invitrogen	Karlsruhe, Germany
Cyclosporine	Novartis	Nürnberg, Germany
Cytocon^{TM}Buffer II	Evotec Technologies	Hamburg, Germany
DMEM, high glucose	Invitrogen	Karlsruhe, Germany
DMEM/F12 (1:1)	Invitrogen	Karlsruhe, Germany
DNA ladder (100bp)	Fermentas	Leon-Rot, Germany
DNA loading buffer (6 x)	Fermentas	Leon-Rot, Germany
DNase (cell culture)	CellSystems	St. Katharinen, Germany
dNTPs	PEQLAB	Erlangen, Germany
DMSO	Sigma	Deisenhofen, Germany
EDTA	Sigma	Deisenhofen, Germany
EGF	R&D Systems	Wiesbaden, Germany
Endostatin	Sigma	Deisenhofen, Germany
Ethanol	Merk	Darmstadt, Germany
Ethidium bromide	Sigma	Deisenhofen, Germany
FCS	Invitrogen	Karlsruhe, Germany
FGF2	R&D Systems	Wiesbaden, Germany

Chemicals	Manufacturer	Registered office
Forskolin	Sigma	Deisenhofen, Germany
Freezing medium, serum free	Sigma	Deisenhofen, Germany
G418	PAA Laboratories	Cölbe, Germany
Gelatine	Invitrogen	Karlsruhe, Germany, Germany
Glucose	Sigma	Deisenhofen, Germany
Glycerol	Sigma	Deisenhofen, Germany
Heparin sodium salt	Sigma	Deisenhofen, Germany
Horse serum	Invitrogen	Karlsruhe, Germany
Insulin	Sigma	Deisenhofen, Germany
Isopropanol	Sigma	Deisenhofen, Germany
Ketanest	Parke Davis	Karlsruhe, Germany
L-Glutamine	Invitrogen	Karslruhe, Germany
Laminin	Sigma	Deisenhofen, Germany
Matrigel, not growth factor reduced	BD Bioscience	Heidelberg, Germany
MgCl2	Invitrogen	Karslruhe, Germany
Mowiol 4-88	Merck	Darmstadt, Germany
Myo-inositol	Sigma	Deisenhofen, Germany
N2 supplement	Invitrogen	Karslruhe, Germany
NaHCO3	Sigma	Deisenhofen, Germany
Neurobasal medium	Invitrogen	Karslruhe, Germany
Non-essential amino acids	Invitrogen	Karlsruhe, Germany
Nucleofector^{TM}Solution T8352	Amaxa	Cologne, Germany
Paraformaldehyde	Sigma	Deisenhofen, Germany

Chemicals	Manufacturer	Registered office
PBS	Invitrogen	Karlsruhe, Germany
PDGF-AA	R&D Systems	Wiesbaden, Germany
Poly-l-ornithine	Sigma	Deisenhofen, Germany
Polyvinyl-alcohol 72000	Merck	Darmstadt, Germany
Progesterone	Sigma	Deisenhofen, Germany
Putrescine	Sigma	Deisenhofen, Germany
Propidium iodide	Sigma	Deisenhofen, Germany
Reverse transcriptase	Roch	Mannheim, Germany
Rompun	Parke Davis	Karlsruhe, Germany
Serum replacement	Invitrogen	Karlsruhe, Germany
Sodium azide	Sigma	Deisenhofen, Germany
Sodium pyruvate	Invitrogen	Karlsruhe, Germany
Sodium selenite	Sigma	Deisenhofen, Germany
Staurosporine	Sigma	Deisenhofen, Germany
Taq DNA polymerase	Invitrogen	Karsruhe, Germany
Tris	Merck	Darmstadt, Germany
Triton-X-100	Sigma	Deisenhofen, Germany
Trypan blue	Invitrogen	Karlsruhe, Germany
Trypsin-EDTA	Invitrogen	Karlsruhe, Germany
Trypsin-inhibitor	Invitrogen	Karlsruhe, Germany
Vectashield®mounting medium	Axxora	Loerrach, Germany
VEGF 165	R&D Systems	Wiesbaden, Germany
Z-VAD-fmk	R&D Systems	Wiesbaden, Germany

2.3. Cell lines and animal stocks

Primary embryonic fibroblasts	mouse strain CD1
Human ES cell line H9.2	Haifa, Israel (Amit *et al.*, 2000)
Fox Chase Scid/Beige mice	Charles River, Sulzfeld, Germany
Sprague-Dawley rats	Charles River, Sulzfeld, Germany

2.4. Plasmids

The phu-DCX3509-EGFP (Karl *et al.*, 2005) plasmid encodes the reporter gene EGFP under the control of the human DCX-promoter cloned into the SmaI-linearised promoterless pEGFP-N1 vector (BD/Clontech, Heidelberg, Germany). The phu-DCX3509-DsRED2 (Karl *et al.*, 2005) plasmid encodes the reporter gene pDsRED2 under the control of the human DCX-promoter cloned into the SmaI-linearised promoterless DsRED2 vector (BD/Clontech, Heidelberg, Germany). Both plasmids were kindly provided by Sebastien Couillard-Després and Ludwig Aigner.

Length: 7,6 kb; Resistance genes: Kanamycin, Neomycin

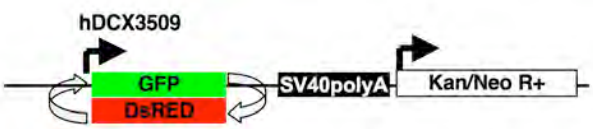

Figure. 2.1: Plasmid phu-DCX3509-EGFP and phu-DCX3509-DsRED2, human DCX promoter (hDCX), polyadenylation consensus sequence of the Simian Virus 40 (SV40polyA), Kanamycin/Neomycin resistance (Kan/Neo R+).

2.5. Cell culture reagents

2.5.1. Cell culture stock solutions

Reagent	Concentration	Solvent
Adenosine 3', 5'-Cyclic monophosphate	100 µg/ml	H_2O
Ascorbic acid	100 mM	H_2O
BDNF	10 µg/ml	Diluted acetic acid + 0.1% BSA
Endostatin	4 mg/ml	17 mM citric-phosphate buffer, pH 6.2
EGF	10 µg/ml	Diluted acetic acid + 0.1% BSA
FGF2	10 µg/ml	PBS + 0.1% BSA
G418	50 mg/ml	H_2O
GDNF	10 µg/ml	Diluted acetic acid + 0.1% BSA
Heparin	2 mg/ml	H_2O

Reagent	Concentration	Solvent
Insulin	5 mg/ml	10 mM NaOH
Myo-inositol	500 mM	H_2O
PDGF	10 µg/ml	Diluted HCl + 0.1% BSA
Polyvinyl-alcohol	50 mg/ml	H_2O
Progesterone	20 µM	Ethanol
Putrescine	0.1 M	H_2O
Sodium selenite	500 µM	H_2O
SU5402	20 mM	DMSO
Transferrin	10 mg/ml	H_2O
VEGF	20 µg/ml	PBS + 0.1% BSA
VEGF R2 (KDR) Antibody	100 µg/ml	PBS + 0.1% BSA
Z-FAD-fmk	50 mM	DMSO

2.5.2. Cell culture media

Mouse embryonic fibroblast (MEF) medium

87% DMEM

10% FCS, heat inactivated

1% Sodium pyruvate (stock: 100 mM)

1% Non-essential amino acids (stock: 10 mM)

1% L-glutamine (stock: 200 mM)

Knockout/Serum replacement (KO/SR) medium

79% KO-DMEM

20% SR

1% Non-essential amino acids (stock: 10 mM)

1 mM L-glutamine

0.1 mM β-Mercaptoethanol

4 ng/ml FGF2

Embryoid body (EB) medium

79% KO-DMEM

20% SR

1% Non-essential amino acids (stock: 10 mM)

1 mM L-glutamine

ITSFn medium

5 µg/ml	Insulin
50 µg/ml	Apo-transferrin
30 nM	Sodium-selenite
30 nM	Human fibronectin

in DMEM/F12

Neural stem cell (NSC) medium

500 ml	DMEM/F12, high glucose
5 ml	N2 supplement
1.68 mg	Glucose
20 µg/ml	Insulin

Neuronal generation (NGc) medium

50%	NSC medium
48%	Neurobasal medium
2%	B27 supplement
100 ng/ml	cAMP

Neuronal precursor freezing medium (NP-FM)

10%	DMSO
20%	Myo-inositol (stock: 500 mM)
5%	Polyvinyl alcohol (stock: 50 ng/ml)
65%	SR

2.5.3. Cell dissociation reagents

Collagenase type IV (1 mg/ml)

50 mg	Collagenase type IV
50 ml	KO/SR medium

Trypsin-EDTA (1 x)

90%	PBS
10%	10 x Trypsin-EDTA

mixed and stored at -20°C.

2.5.4. Coating materials

Poly-l-ornithin (PO)

100 x stock solution:

100 mg PO

67 ml dH_2O

sterile filtered and stored at -20°C

working dilution:

5 ml 100 x stock solution

495 ml dH_2O

sterile filtered and stored at 4°C

Gelatine (0.1%)

1 g Gelatine powder type A

1 l dH_2O

autoclaved and stored at room temperature.

Laminin (LN)

1 µg/ml Human laminin

in PBS, mixed and stored at 4°C

Matrigel

1 ml Matrigel stock solution

29 ml DMEM/F12

The matrigel stock solution is thawed overnight at 4°C. A 2 ml pipette is cooled in the -20°C freezer to avoid attachment of the matrigel. 1 ml of matrigel stock solution is pipetted quickly to 29 ml cooled DMEM/F12. The dilution is stored at 4°C.

2.5.5. FACS solutions

500 µl DNAse (stock: 1%)

100 ml Cytocon^{TM}Buffer II

2.6. Reagents for immunohistochemistry

PBS-azide (0.1%)

1 g Sodium-azide

1 l PBS

stored at room temperature

Fixation solution (4% Paraformaldehyde, PFA)

40 g PFA

1 l PBS (warmed)

mix to dissolve and adjust pH to 7.4; Cooled on ice and stored at -20°C.

Blocking solution

10% FCS

90% PBS-azide.

For the staining of intracellular markers 0.1% triton is added. The blocking solution is prepared freshly before use.

Borate buffer (0.1 M, pH 8.5)

3.8 g Sodium borate (MW 381.4)

100 ml H_2O

mix to dissolve and adjust pH to 8.5

Mowiol

6 g Glycerol

2.49 g Mowiol

in 6 ml H_2O

12 ml Tris-HCL (0.2 M, pH 8.5)

2.6.1. Primary antibodies

ms = mouse, rb = rabbit, gt = goat

Epitope and origin	Dilution	Manufacturer
A2B5 (ms) IgM	1:500	Chemicon
BrdU (ms) IgG	1:33	Becton Dickinson
GABA (rb)	1:1000	Sigma
Gad67 (rb)	1:200	Chemicon
GFP (rb)	1:2000	Abcam
GFAP (ms) IgG	1:100	ICN Biomedicals
GFAP (rb)	1:1000	DAKO
hDCX (gt) IgG	1:200	Santa Cruz
hNestin (ms) IgG	1:600	R&D Systems
hSynaptophysin (ms) IgG	1:1000	Chemicon
hNuc (ms) IgG	1:200	Chemicon
Ki67/MIB1 (ms) IgG	1:250	DAKO
MAP2ab (ms) IgG	1:500	Chemicon
NeuN (ms) IgG	1:100	Chemicon
O4 (ms) IgM	1:200	R&D Systems
PSA-NCAM (ms) IgM	1:500	Chemicon
Serotonin (rb)	1:800	Sigma
β-III-tubulin (ms) IgG	1:600	Covance
β-III-tubulin (rb)	1:2000	BAbCo
S100β (ms) IgG	1:2000	Sigma
TH (ms) IgG	1:800	Sigma

2.6.2. Secondary antibodies

Epitope and coupled dye	Dilution	Manufacturer
anti-mouse, Cy3	1:500	Dianova / Jackson ImmunoResearch
anti-mouse, FITC	1:200	Dianova / Jackson ImmunoResearch
anti-rabbit, Cy3	1:500	Dianova / Jackson ImmunoResearch
anti-rabbit, FITC	1:500	Dianova / Jackson ImmunoResearch
anti-goat, FITC	1:500	Dianova / Jackson ImmunoResearch
anti-mouse, Cy5	1:500	Dianova / Jackson ImmunoResearch
anti-rabbit, Cy5	1:500	Dianova / Jackson ImmunoResearch

2.7. Reagents for molecular biology

50 x Tris-acetate-buffer (TAE)

242 g Tris (dissolved in 500 ml H_2O)

100 ml EDTA (0.5 M, pH 8.0)

57.1 ml Glacial acetic acid

volume is adjusted to 1 litre with H_2O and stored at room temperature

1 x TAE

50 x TAE is diluted 1:50 with H_2O and stored at room temperature.

10 x Sample buffer

70% Tris, (100 mM, dissolved in H_2O, pH 8.0)

29.2% Glycerol

0.4% Bromphenol blue

0.4% Xylene cyanol

mixed and stored at 4°C.

2.7.1. Primers

Name	Primer Sequence
BDNF	Fw: 5′-gccctgtatcaacccagaaa-3′ Rv: 5′-cttcagaggccttcgttttg-3′
cKit	Fw: 5′-ctatgctctcgcacctttcc-3′ Rv: 5′-caatgaagtgcccctgaagt-3′
CXCR4	Fw: 5′-ggtggtctatgttggcgtct-3′ Rv: 5′-tggagtgtgacagcttggag-3′
EGF	Fw: 5′-cagggaagatgaccaccact-3′ Rv: 5′-cagttcccaccacttcaggt-3′
EGFR	Fw: 5′-taacaagctcacgcagttgg-3′ Rv: 5′-gttgagggcaatgaggacat-3′
FGF2	Fw: 5′-gacaggcctcctggaaactt-3′ Rv: 5′-gagggagaaaggatggaagc-3′
FGFR1	Fw: 5′-gcgagggtcagtttgaaaag-3′ Rv: 5′-gtgcagagtgtggctgtgac-3′
FGFR2	Fw: 5′-ttacgaaccatgccttcctc-3′ Rv: 5′-ttctcctcctggggaagatt-3′
FGFR3	Fw: 5′-gtgtgcaggttccgatgtta-3′ Rv: 5′-caactgaattcacggcttcc-3′
FGFR4	Fw: 5′-cctccagcgattctgtcttc-3′ Rv: 5′-catgctatcaaggtcgagca-3′

Name	Primer Sequence
FLK/KDR	Fw: 5′-ggcttgtgacccaagaatgt-3′ Rv: 5′-gcaaatccttcccatcttca-3′
FLT1	Fw: 5′-aagggaagaccccgtctcta-3′ Rv: 5′-gccagctaatgctcttccac-3′
FLT4	Fw: 5′-catcatgctgaactgctggt-3′ Rv: 5′-gagaagctgccctcttctga-3′
GAPDH	Fw: 5′-acgacccttcattgacctcaact-3′ Rv: 5′-atatttctcgtggttcacacccat-3′
NRP1	Fw: 5′-gaagcaccgagagaacaagg-3′ Rv: 5′-ctcggggtagatcctgatga-3′
PDGFA	Fw: 5′-acacgagcagtgtcaagtgc-3′ Rv: 5′-cctcacatccgtgtcctctt-3′
PDGFB	Fw: 5′-aagcacacgcatgacaagac-3′ Rv: 5′-gacggacgagggaaacaata-3′
PDGFC	Fw: 5′-ggaggcctaaaggacaggag-3′ Rv: 5′-ttaagcaaggcaacggaatc-3′
PDGFD	Fw: 5′-atggcatgggcattctgtat-3′ Rv: 5′-cgggaacacaaaagcaagat-3′
PDGFRα	Fw: 5′-ctgggtttccatccttgaga-3′ Rv: 5′-tagtaggcttcctgcgtggt-3′
PDGFRβ	Fw: 5′-gcacttttatccacccagga-3′ Rv: 5′-gtacttggctcagcctccag-3′
ß-Aktin	Fw: 5′-gaagagctacgagctgcc-3′ Rv: 5′-tgatccacatctgctgga-3′
TrkB	Fw: 5′-tcaacaaatgtggacggaga-3′ Rv: 5′-gtggtgaacacaggcatcac-3′
VEGF	Fw: 5′-cagctactgccgtccaatcg-3′ Rv: 5′-tcaccgcctcggttgt-3′

2.7.2. Kits

Name	Producer	Registered office
ApopTag® Fluorescence Direct In Situ Apoptosis Detection Kit	Chemicon	Temecula, USA
iScript™cDNA Synthesis Kit	BioRad	Munich, Germany
PureYield™ Plasmid Midiprep System	Promega GmbH	Mannheim, Germany
peqGOLD Plasmid Miniprep Kit I	Peqlab Biotechnologie GmbH	Erlangen, Germany
QIAquick PCR Purification Kit	QIAGEN GmbH	Hilden, Germany
RNeasy®Mini Kit	QIAGEN GmbH	Hilden, Germany

2.8. Software

Name	Application	Producer
AxioVs40LE 4.5.0.0	Fluorescence microscopy	Carl Zeiss
BD FACSDiva Software	FACSorter	BD Bioscience
Openlap 4.0	Fluorescence microscopy	Jena Optic
Quantity One	Electrophoresis gel documentation	Bio-Rad
Mac OS X Software	Microsoft office for Mac	Macintosh

3. METHODS

3.1. Cultivation of pluripotent hES cells

Generally, cell culture was performed under sterile conditions in a sterile hood with sterile media, glass and plastics. Cells were cultivated in an incubator at 37°C, 5% CO_2 and saturated air humidity.

3.1.1. Generation, cultivation and mitotic inactivation of murine fetal fibroblasts

All working steps were performed according to the standard operation procedures of the Institute of Reconstructive Neurobiology, University of Bonn.

3.1.2. Cultivation of hES cells

HES cells were cultivated on a layer of irradiated mouse fibroblasts (MEF). Cells were grown in serum-free KO/SR medium. Medium was changed daily and hES cells were passaged about every four days. For passaging, medium was removed and the cells were incubated in 1 mg/ml collagenase IV for one hour. Subsequently, cells were rinsed off, pipetted up and down to obtain small clusters and centrifuged in a 15 ml centrifugation tube (800 rpm, 3 min, 4°C). The cells were plated at a ratio of 1:4 on fresh MEF.
Morphologically differentiated hES cells were manually removed by scraping with a sterile injection needle (26 g 3/8, 0.45 x 10) in a horizontal sterile hood using a microscope. Subsequently, differentiated colonies were removed together with the KO/SR medium.

3.2. *In vitro* differentiation of hES cells into lt-hESNSC

In vitro differentiation of hES cells into long term proliferating hES-derived neural stem cells (lt-hESNSC) was performed as described (Zhang *et al.*, 2001; Li *et al.*, 2005; Koch *et al.*, 2006).
For the generation of embryoid bodies (EBs), hES cells were detached as described (3.1.2). The pellet was re-suspended only 5 x with a 5 ml-pipette to preserve the colonies. Aggregates were transferred into 6 cm bacterial dishes to avoid adherence. Medium was changed every other day by transferring the EBs to a 15 ml centrifugation tube. After sedimentation of the aggregates, the supernatant was replaced with fresh EB medium. Four-day-old EBs were transferred to poly-l-ornithine coated tissue culture dishes and propagated in ITSFn medium with 20 ng/ml FGF2. Within 10 days, neural tube-like structures developed in the EB outgrowth. These structures were mechanically isolated and propagated as free-

floating neurospheres in NSC medium containing 10 ng/ml FGF2 for 1-3 weeks. Neurospheres were triturated into single cells by incubating them with trypsin/EDTA for 10 min in a 15 ml centrifugation tube. Trypsin was inhibited by trypsin inhibitor. Cells were pipetted up and down to obtain single cells and centrifuged (1000 rpm, 5 min, 4°C). Single cells were plated on poly-l-ornithine/laminin coated plastic dishes. Medium was changed to NSC medium containing 10 ng/ml FGF2, 10 ng/ml EGF and 1 µl/ml B27 supplement (Figure 3.1).

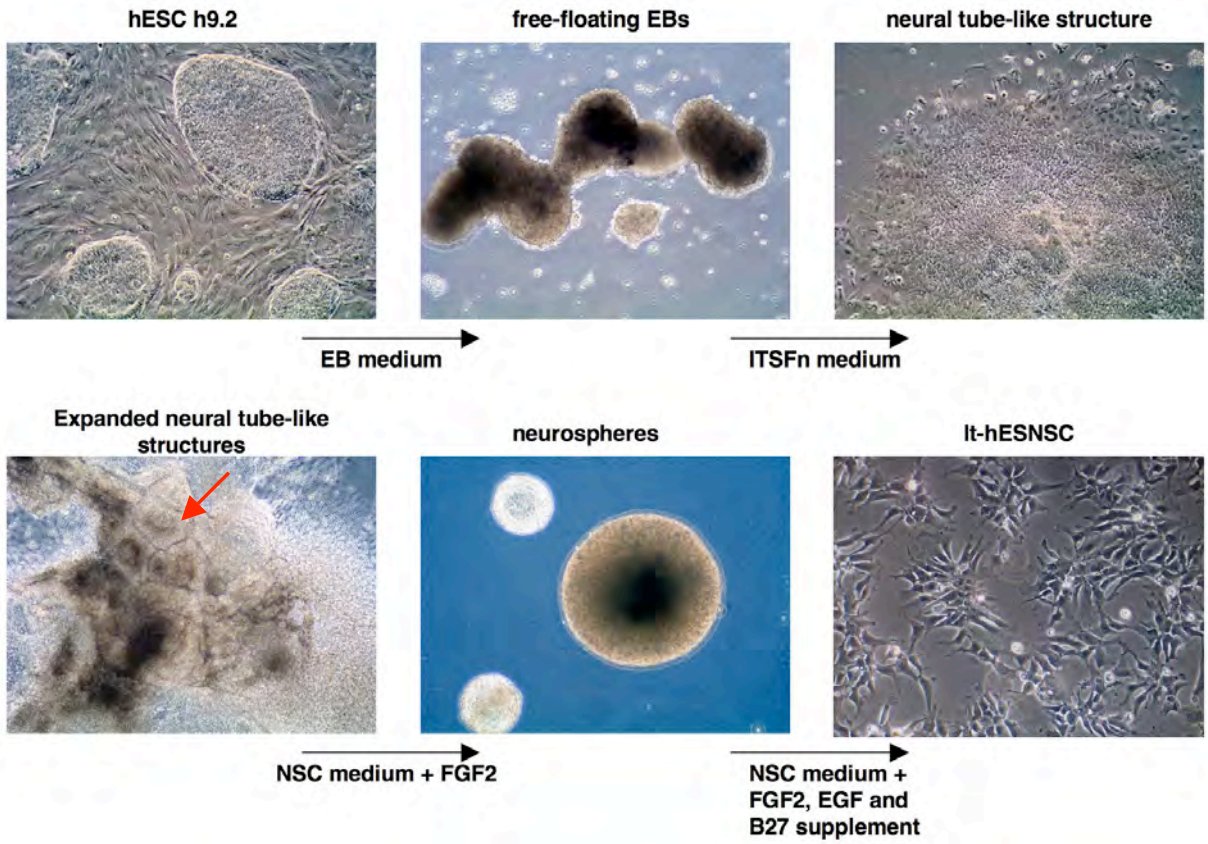

Figure. 3.1: *In vitro* differentiation of hES cells into lt-hESNSC. To induce differentiation, hES cells were aggregated to form free-floating EBs. Neural tube-like structure formation was induced in the EB outgrowth upon plating and a four-day cultivation in ITSFn medium. These structures became visible (see red arrow) in the expanded cultures. They were mechanically isolated and further propagated as neurospheres. Neurospheres were triturated into single cells, which were replated and further cultivated as stably proliferating lt-hESNSC in NSC medium.

Daily media change was performed for the first 7 days. Cells were passaged every 2-4 days at a 1:2 to 1:3 ratio by incubating the cells in the tissue culture dish with trypsin/EDTA. Trypsin was inhibited by trypsin inhibitor and cells were centrifuged in a 15 ml centrifugation tube (1000 rpm, 5 min, 4°C). Replating densities were kept above 30%. Terminal differentiation was performed by growth factor withdrawal in NGc medium. For storage trypsinized lt-hESNSC were resuspended in 1ml freezing medium (Sigma). Cells were directly transferred in a Nalgene^{TM}Cryo 1°C freezing container and placed at –80°C to achieve a 1°C/min rate of cooling. Final temperature was reached after freezing in liquid

nitrogen. Thawing was carried out by gently swirling the cryo-vial in a 37°C water bath for 1.5 min. After thawing, the cells were quickly placed into DMEM/F12 medium in a 15 ml centrifugation tube and centrifuged (800 rpm, 6 min, 4°C). The supernatant was discarded and the pellet was carefully re-suspended in 2 ml NSC medium containing 10 ng/ml FGF2, 10 ng/ml EGF and 1 µl/ml B27 supplement with a 5 ml pipette before transferring it to PO/LN coated culture dishes.

3.3. Stable nucleofection of lt-hESNSC

Lt-hESNSC were stably transfected by nucleofection (Amaxa - nucleofection program B0-33/ B0-16) with the phuDCX3509-EGFP or the phuDCX3509-DsRED2 vector (Couillard-Despres *et al.*, 2005). Three million lt-hESNSC were trypsinized and centrifuged (1000 rpm, 5 min 4°C). 3 µg DNA was added to the cell pellet. The pellet was incubated for 1 min following resuspension in 100 µl Nucleofector™Solution before cells were transferred into a nucleofection cuvette. The cuvette was inserted into the Nucleofector™ and the appropriate cell-type specific program (B0-33 or B0-16 for lt-hESNSC) was chosen before starting nucleofection. The sample was removed from the cuvette immediately after nucleofection and transferred to a PO/LN coated culture dish. The cells were subsequently selected for gentamycin resistance by adding 50 µg/ml G418 into NSC medium containing 10 ng/ml FGF2, 10 ng/ml EGF and 1 µl/ml B27 supplement. Emerging colonies were manually removed, replated and further propagated. Recapitulation of DCX expression by the EGFP or by the DsRED2 reporter was confirmed via immunocytochemistry after 6 days of cultivating the cells in NGc medium to induce differentiation.

3.4. Fluorescence activated cell sorting

FACS DiVa was used for flow cytometric cell sorting. Lt-hESNSC stable expressing EGFP or DsRED2 under the hDCX promoter, cultured in NGc medium for 8±1 days were trypsinized, gently resuspended in Cytocon™Buffer II (Evotec) containing 0.5% DNase (Invitrogen) and filtered through a 40 µm nylon mesh (Pall GmbH). The cells were sorted at a concentration of 3 million cells/ml on a fluorescence-activated cell sorter FACS DiVa (Becton Dickinson) using FACS Diva software (BD Bioscience). Cells were selected by forward-angle and sideward-angle light scatter and for EGFP or DsRED2 fluorescence's intensity, with an argon-ion laser (Coherent) operating at 488 nm. Purity of the sorted fractions was analysed by FACS reanalysis and by immunocytochemistry following replating. Prior to sorting the nozzle, sheath and sample lines were sterilized with 70% ethanol followed by washing with sterile water. A 70 µm ceramic nozzle (BD Bioscience), sheath pressure of 20 to 25 pounds per

square inch (PSI) and an acquisition rate of 2000 to 3000 events/sec was used as conditions optimized for cell sorting of immature neurons.

3.5. Preparation of primary astrocytes

Primary astrocytes were prepared according to the protocol from Polleux and Glosh (Polleux *et al.*, 2002). Mouse pups from postnatal day 3 (P3) were anesthetized and decapitated. The preparation of the brains was performed in 6 cm petri dishes, using a stereomicroscope under a sterile laminar flow hood. The brains were removed and rinsed in PBS containing 2% glucose. The specimens were incubated in 3 ml 10 x trypsin at 37°C for 3 minutes and rinsed again. Then MEF medium and 500 µl DNase (1%) were added to a total volume of 5 ml. The tissue was triturated to a cell suspension with a 1000 µl pipette. Finally, the cells were filtered through a nylon mesh (40 µm) and centrifuged for 10 min at 1000 rpm. 2.5 to 5 x 10^6 cells were plated on PO/LN coated 10 cm dishes in MEF medium. A confluent astrocyte cell layer appeared after 5 to 10 days.

3.5.1. Direct-/ in-direct shared media culture with primary astrocytes

For direct co-culture primary astrocytes were plated on PO/LN coated plastic coverslips and cultivated at least for 24 hours before plating purified human neurons on top of the primary astrocyte layer. For in-direct co-culture purified human neurons were plated on PO/LN coated 12 well culture dish. In parallel, primary astrocytes were plated on PO/LN coated transfilter membrane (4 µm pore size), which can be hooked into a 12 well culture dish. Both cell types were cultured for 24 hours before assembling.

3.6. Cryopreservation of purified human neurons

Purified neurons were centrifuged in batches of 5 million cells (1000 rpm, 5 min, 4°C). Treatment with the general caspase inhibitor z-VAD-fmk was performed in the cell solution 30 minutes before freezing in a 500 nM concentration. The cell suspension was frozen with 1.5 ml freezing medium containing 10% DMSO, 20% 500 mM myo-inositol, 5% polyvinyl alcohol stock solution and 65% Knockout™Serum-Replacement. Cells were directly transferred in a Nalgene™Cryo 1°C freezing container and placed at -80°C to achieve a –1°C/min rate of cooling. Final temperature was reached after freezing in liquid nitrogen. Thawing was carried out by gently swirling the cryo-vial in a 37°C water bath for 1.5 min. After thawing, the cells were quickly transferred into DMEM/F12 medium in a 15 ml-centrifugation tube and centrifuged (800 rpm, 6 min, 4°C). The supernatant was discarded, and the pellet was carefully re-suspended in 2 ml NGc medium with a 5 ml-pipette. Survival

rates were obtained using standard trypan blue exclusion test. Caspase-3/7 activity was measured in a luminescent assay (Caspase-Glo®3/7Assay) according to the manufacturer's instruction 20 hours after thawing and replating of purified cryopreserved immature neurons.

3.7. *In vitro* migration assays

3.7.1. Transwell migration assay

One way to study migration at the cellular level is to use microchemotaxis chambers (Boyden chambers) (Richards & McCullough, 1984). A membrane separates the upper and the lower part of the chamber. Cells are plated on the PO/LN coated membrane that separates the upper and the lower well. Chemoattractants are introduced in the well of the lower compartment e.g., by soaking agarose beads (affi-gel blue gel) with the corresponding chemoattractants. 100 µl of the pre-soaked beads are introduced in the lower well and covered with medium before placing the upper well on top. The chemoattractant will be released over time, thus creating a chemoattractive gradient. If the agent acts as a chemoattractant, the attached cells will migrate through the filter towards the gradient of the attracting factor. Small pore sizes (8 µm) require active migration rather than passive falling of the cells through the filter.

In this study agarose beads were soaked with either 30 ng/ml EGF, BDNF, SDF1, SCF, PDFG-AA, FGF2 or VEGF (see 2.5.1 for stock solutions). The chemoattractive effect of NSCs on neurons was studied by plating lt-hESNSC directly in the PO/LN coated lower well before placing the upper well with the attached neurons on top. After a culture period of 20 hours, chambers were fixed with 4% PFA for 10 min. Cells that had not migrated and were still on the upper side of the membrane were scraped off, while the migrated cells on the lower side were DAPI stained and counted (Figure 3.2).

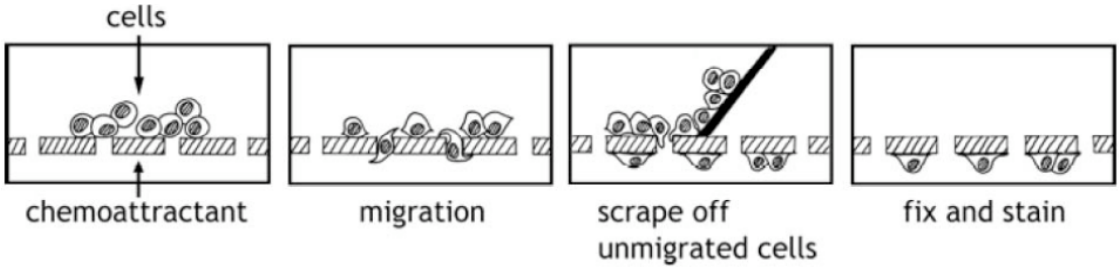

Figure 3.2: Cell migration studied by using a chemotaxis chamber. Cells are plated on the membrane of the upper well. Chemoattractants can be added to the lower well. Migration of cells from the upper well through the membrane can be measured by scraping off the remaining cells from the upper side of the membrane and counting the cells that reached the bottom side. Adopted from Erlandsson (Erlandsson, 2003).

3.7.2. Matrigel migration assay

Matrigel matrix is a soluble basement membrane extract. The major components of matrigel matrix are collagens, laminin and proteoglycans. It can provide the substrate necessary for the study of cell migration. For the matrigel migration assay the matrigel matrix was thawed at 4°C overnight and diluted at a ratio of 1:2 in cold DMEM/F12 medium. Per well of a 4-well-plate 250 µl of the matrigel matrix mixture was added and incubated at 37°C for at least 30 minutes for hardening. Cells were spotted (1 µl cell suspension containing 100 000 cells in Cytocon^{TM}Buffer II) on the gel mixture, incubated for 10 minutes at 37°C for attachment and then covered with NGc medium. Two days after plating, migration was quantified by measuring the distance the cells had migrated away from the site where they were plated.

3.8. Transplantation

In order to prepare cells for transplantation, they were trypsinized and re-suspended at the desired cell concentration in Cytocon^{TM}Buffer II containing 0.1% DNase. Cell suspension was kept on ice until transplantation was performed. Following transplantation remaining cells were replated onto PO/LN coated culture dishes for monitoring cell viability.

3.8.1. Transplantation onto rat hippocampal slice cultures

Using a vibroslicer, 400 µm horizontal sections were generated from the hippocampus of 9 – 10 day-old Wistar rats. The slices included the dentate gyrus and the entorhinal and temporal cortex (Scheffler *et al.*, 2003; Opitz *et al.*, 2007). They were transferred onto a polyester membrane and cultivated at 35°C, 5% CO_2 and saturated air humidity in an initial culture medium containing 25% normal horse serum, which was gradually replaced after 3 – 5 days by chemically defined, serum-free culture medium based on DMEM/F12, N2 supplement and B27 supplement. Medium was changed every other day and 5 – 7 days after explantation a cell suspension of 50 000 cells in a 1 µl volume was spotted onto the entorhinal cortex of the slice using an injection device.

3.8.2. Transplantation into the rodent brain

Cell transplantation into the brain of 12 week-old rats (SD) was performed as described previously (Crawley *et al.*, 2003). Briefly, SD rats were anesthetized (10 mg/kg Xylacin, 80 mg/kg Ketamine) and clamped in a stereotactic frame. A midline incision was performed and the injection position was determined (coordinates for transplants into the left striatum:

anteroposterior + 0.2mm; mediolateral –2.9mm; ventral 4.8mm, coordinates for transplants into the SVZ close to the rostral migratory stream: anteroposterior + 1.6mm; mediolateral –1.25mm; ventral 5.6mm). A hole was drilled at the target site and 1 µl of cell suspension containing 100 000 cells/µl were carefully injected over a 5 min period into either the left striatum or into the SVZ. Engrafted animals received daily injections of cyclosporine (10 mg/kg). Eight days after transplantation, the recipients were anesthetized with pentobarbital and perfused with 4% paraformaldehyde. The brains were removed and cryoprotected in 15% sucrose in PBS over night, followed by 30% sucrose in PBS for 2 days. Immunofluorescence analysis (see 3.9.2) was performed to identify engrafted human cells in 35 µm cryostat sections. All procedures were performed in accordance with institutional guidelines.

Quantification of the total number of EGFP positive cells within the corpus callosum was performed on every second out of twenty consecutive 35 µm coronal brain sections encompassing the entire transplantation area. Three animals per condition were analysed. Data were presented as mean number and standard derivation of counted EGFP positive cells per animal for each condition.

3.8.3. Transplantation into the neonatal rodent brain

Two-day-old (P2) Sprague-Dawley rats were shortly anaesthetised by hypothermia on ice for 4 minutes. Hypothermia was chosen to anaesthetise P2 rats, as anaesthetics like ketamine cannot be applied to animals at this age without a pronounced increase in the mortality rate. The animals received 2 µl of purified and cryopreserved cell suspension (50 000 cells/µl) in 2 deposits along the rostral/caudal axis of the right hemisphere by using a glass capillary. After transplantation, recipient rats were placed on a 37°C plate. Upon reaching their regular body temperature, they were placed back to the mother animal. Two weeks after transplantation, recipient rats were deeply anesthetized and perfused with 4% paraformaldehyde. The brains were removed and treated as described (see 3.9.2).

3.9. Immunocytochemistry and immunohistochemistry

3.9.1. Immunocytochemistry

Immunocytochemical analyses of the cells were performed using primary antibodies and appropriate secondary antibodies labelled with Cy3, Cy5 or FITC. Nuclei were visualised by DAPI staining (1:10000 in PBS, 4 minutes incubation). Cells were fixed in 4% paraformaldehyde for 10 minutes. For the staining of intracellular markers cells were

permeabilized with 0.1% Triton X-100 in PBS for 20 minutes. Blocking was performed with 10% FCS, in PBS for 1 hour. Samples were incubated with primary antibodies diluted in blocking solution at room temperature for 3 to 4 hours, washed twice in PBS and incubated with secondary antibody diluted in blocking solution for 45 minutes. The cells were washed in PBS, counterstained with DAPI and mounted with vectashield mounting solution.
For BrdU staining 0.5% Triton X-100 in PBS was used for permeabilizing the cells. After washing cells were incubated with 2 M HCL for 10 minutes, washed in PBS, equilibrated using 0.1 M borate buffer, washed again in PBS, followed by an incubation with the primary antibody diluted in blocking solution at room temperature over night. Staining with the secondary antibody was performed as described.

3.9.2. Immunohistochemistry

Fixed and cryoprotected brain tissue of transplanted animals (see 3.8.2) was sectioned with a cryostat in 35 to 40 µm thick brain slices. Sections were permeabilized/blocked with 10% FCS for 1 hour. 0.1% Triton X-100 was added to the blocking solutions for stainings of intracellular markers. Incubation with primary antibody was for 16 hours followed by washing steps with PBS for at least 2 hours. Incubation with secondary antibodies was for 2 hours. All staining steps were performed at room temperature.
For immunohistochemical analysis of slice cultures, cultures were fixed in 4% paraformaldehyde for 4 hours and subsequently washed several times with PBS. Slices were permeabilized/blocked with 0.1% Triton X-100 + 10% FCS for 6 hours at 25°C. Incubation with primary antibody was for 16 hours at room temperature, followed by washing steps with PBS for 5 hours. Incubation with the secondary antibodies was for 2 hours at room temperature.
All antibodies, sources and dilutions are listed in the Material section (2.6.1 and 2.6.2).

3.10. RT-PCR

For reverse transcriptase polymerase chain reaction (RT-PCR) triplicate total messenger RNA (mRNA) samples were isolated using an mRNA extraction kit (see 2.7.2), following the supplier's instructions. 0.5 to 1 µg total mRNA were used for reverse transcription with the iScript cDNA synthesis kit (see 2.7.2) following the manufacturer's protocol. PCR reactions were run in at least triplicates using Taq Polymerase. In order to compare the expression levels of different genes, all data were normalized to GAPDH by performing 15, 20 and 25 cycles. PCR conditions and cycle numbers were then adjusted to each primer pair for specific DNA amplification on commercially available human fetal brain tissue (single donor,

female, 19 weeks of gestation). The selected number of cycles varied from 28 to 35 cycles depending on the particular cDNA abundance with denaturation at 94°C for 1 minute, annealing temperatures of 58°C to 63°C for 1 minute according to the primers, and elongation at 72°C for 2 minutes. Omission of transcriptase during RT or cDNA sample during PCR served as negative controls. All reactions were performed on a T3 Thermocycler. Primers used are listed in the Material section (2.7.1).

The components of the PCR reaction were as follows:

Master Mix (MM):

MM	Stock	Final Concentration	Volume in µl (total 10 ml)
PCR Buffer	10 x	2 x	2000
$MgCl_2$	50 mM	6 mM	600
dNTPs	100 mM	0.4 mM	40 (160)
dH_2O			7240

Primer Mix: Primer concentration 100 µM = 100 pmol/µl

Primer-Mix	Volume in µl
Primer forward	20 µl
Primer reverse	20 µl
dH_2O	360 µl

PCR-reaction:

12.5 µl Master Mix

8.3 µl dH_2O

3 µl Primer-Mix

1 µl cDNA (100 ng/µl)

0.2 µl Taq

25 µl

After the PCR-reaction was performed, 4 µl of the 6 x loading buffer was added to each tube. The samples were electrophoretically separated on an agarose gel (1.5% agarose in TAE-buffer + 1 µl ethidiumbromide/ 10 ml buffer) at 100 V for approximately 20 minutes. The agarose gel was exposed to UV-light in a gel documentation system to visualize DNA bands.

3.11. Electrophysiological recordings of purified neurons*

Cells grown on 13 mm diameter plastic coverslips (Nunc) or on hippocampal rat slice cultures were transferred to a chamber that was mounted to a x-y stage and continuously superfused with aCSF at 1–2 ml/min. This aCSF contained the following (in mM): 140 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 D-glucose, and 10 Hepes/NaOH (pH 7.35, 305-315 mosmol/kg). Recordings were performed at room temperature. Cells were visualized using an upright microscope equipped with near-infrared differential interference contrast (IR-DIC) and 40 x water immersion objective (Zeiss). In slice cultures transplanted cells were identified by their EGFP fluorescence (Opitz *et al.*, 2007). Whole cell current-clamp and voltage-clamp recording was carried out with an Axopatch-200B amplifier (Axon Instruments, Foster City, CA) that was interfaced by an A/D-converter (Digidata 1320, Axon) to a PC running PClamp software (vers. 9, Axon). For recordings of membrane potential or current, the patch pipette (tip resistance 3–5 MΩ) contained the following (in mM): 120 potassium gluconate (C6H11O7K), 20 KCl, 10 NaCl, 10 EGTA, 1 CaCl2, 4 Mg ATP, and 0.4 Na GTP, 10 HEPES/KOH (pH 7.2, 280-290 mosmol/kg). Command potential in voltage-clamp recordings was corrected for a 13 mV junction potential. For some recordings of postsynaptic currents, another pipette filling solution was used (in mM): 110 cesium methanesulfonate (CH3O3SCs), 10 CsCl, 10 TEA-Cl, 5 QX-314 Cl, 10 EGTA, 1 CaCl2, 4 Mg ATP, and 0.4 Na GTP (pH 7.2, 280-290 mosmol/kg). For the latter solution holding potential was corrected for a 9 mV junction potential. Signals were filtered at 2 kHz and recorded at a rate of 10 kHz.

* All functional studies were performed by T. Opitz

4. RESULTS

4.1. Generation and validation of a lineage selection protocol to derive pure cultures of immature neurons from hES cells

4.1.1. Expression profile of doublecortin at different stages of neural differentiation in lt-hESNSC

A key prerequisite for the application of hES cells in neural repair is the generation of defined neural cell types at high purity. Neuronal replacement might benefit from the availability of purified populations of immature neurons capable of integrating into host tissue. An attractive candidate marker for immature neurons is Doublecortin (DCX), a 40 kDa protein that associates with and stabilizes microtubules (Francis *et al.*, 1999; Gleeson *et al.*, 1999; Taylor *et al.*, 2000). *In vivo*, the expression of DCX is restricted to newborn and migrating neurons during CNS development and zones of adult neurogenesis (Chelly, 1998; Gleeson *et al.*, 1998; LoTurco, 2004; Rao & Shetty, 2004; Couillard-Despres *et al.*, 2005; Bernreuther *et al.*, 2006). The expression is initiated with neuronal determination and is described to be down-regulated during neuronal maturation (Kempermann *et al.*, 2004; Couillard-Despres *et al.*, 2005). To investigate whether *in vitro* generated neural precursors derived from hES cells reflect a DCX expression pattern comparable to the *in vivo* situation, a recently described population of long-term self-renewing hES cell-derived neural stem cells (lt-hESNSC; see 1.1.3) was investigated for the expression of DCX under proliferating and differentiating conditions by immunocytochemistry. This cell population was derived by mechanical isolation of neural rosettes from plated embryoid bodies and subsequent proliferation in the presence of FGF2 and EGF (Koch *et al.*, 2006). The method yields a homogeneous monolayer of neuroepithelial stem cells, expressing nestin but not DCX. Under proliferating conditions, expression of DCX was restricted to very occasional spontaneously differentiating neurons (< 1%, Figure 4.1 a). When differentiation was induced by growth factor withdrawal, DCX expression increased alongside the appearance of the neuronal marker beta III-tubulin (Figure 4.1 b). At this stage, DCX expression was restricted to cells with polar neuronal morphology. Upon further differentiation DCX expression could also be observed in more mature neurons expressing MAP2ab (Figure 4.1 c). DCX was barely detectable in neurons expressing the mature neuronal marker NeuN (Figure 4.1 d), suggesting that, in agreement with what is observed during normal development *in vivo*, DCX is down-regulated in terminally differentiated neurons. Thus, DCX is specifically expressed in young immature neurons during differentiation of lt-hESNSC. Expression of the astrocytic protein GFAP or the oligodendroglial antigen O4 could not be detected in DCX expressing cells. Ki67

immunostaining and BrdU labelling showed no evidence of cell proliferation within the DCX-positive population (Figure 4.1 f-g). Taken together, DCX-expression in this system delineates an early stage of postmitotic neuronal differentiation.

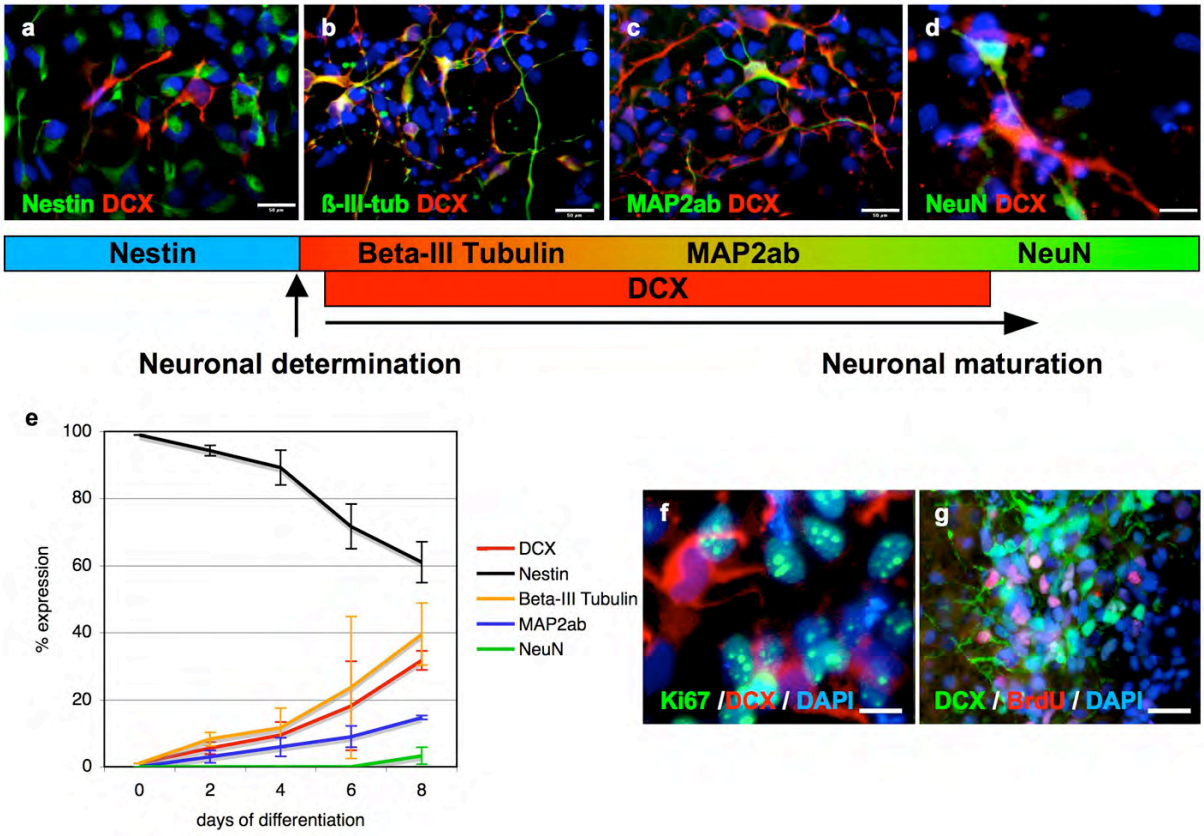

Figure 4.1: Expression profile of DCX in lt-hESNSC during *in vitro* differentiation. (a) Cultures of proliferating lt-hESNSC exhibited only very occasional DCX-positive neurons. The expression of DCX and nestin was mutually exclusive. **(b)** Two days after induction of differentiation, DCX was co-expressed in emerging beta-III tubulin-positive neurons. **(c)** At later stages of neuronal differentiation, DCX-positive neurons co-expressed MAP2ab (12 days of differentiation). **(d)** DCX was hardly detectable in NeuN-positive cells, suggesting down-regulation upon terminal differentiation. **(e)** Quantification of marker expression during the first 8 days of *in vitro* differentiation of lt-hESNSC. **(f)** Ki67 staining and **(g)** BrdU labelling revealed no evidence of cell proliferation in DCX-positive neurons. Scale bars: a-c,g 50 µm d,f 25 µm.

4.1.2. Lt-hESNSC stably expressing a doublecortin reporter/selection marker

Based on the highly selective expression of DCX in neurons, lt-hESNSC were transfected with a construct carrying the EGFP gene under the control of the human DCX promoter alongside with a constitutively expressed neomycin resistance gene. Transfected cells were replated and subsequently selected in the presence of 50 µg/ml G418 for 3 weeks. Clones were picked and further expanded. Faithful co-expression of the DCX protein and the EGFP reporter was investigated on separately plated cells of each clone following 8 days of differentiation. Four out of 27 clones analysed showed strong and exclusive expression of the EGFP transgene in DCX immunoreactive neurons. The remaining clones showed either background expression in non-neuronal cells (n=5) or an EGFP expression level insufficient

for cell sorting (n=18). A detailed *in vitro* differentiation study of the four clones without background expression revealed that the EGFP expression in the proliferating stage was restricted to sporadic spontaneously differentiating cells with immature neuronal morphology (Figure 4.2 a). When stained for DCX, differentiating cells showed co-expression of DCX and EGFP (Figure 4.2 b). Co-expression of the neural precursor marker nestin and of EGFP was largely exclusive (Figure 4.2 c). EGFP-labelled cells were consistently positive for beta-III tubulin (Figure 4.2 d). With further maturation, EGFP-positive cells also co-expressed MAP2ab (Figure 4.2 e). Co-expression of EGFP with DCX during the different stages of differentiation indicates that the expression of EGFP in the selected clones reflects the endogenous expression profile of the DCX protein in lt-hESNSC (see 4.1.1). Consolidated clones of DCX-EGFP-transduced cells could be further propagated without losing their neurogenic potential. For example, cells expanded in FGF2 and EGF for 4 and 11 passages and subsequently differentiated for 7 days yielded 26±2.2% and 25±2.5% EGFP-positive, DCX-immunolabeled neurons, respectively. Expanded clones could be frozen and stored in liquid nitrogen without loss of their proliferative and neurogenic potential.

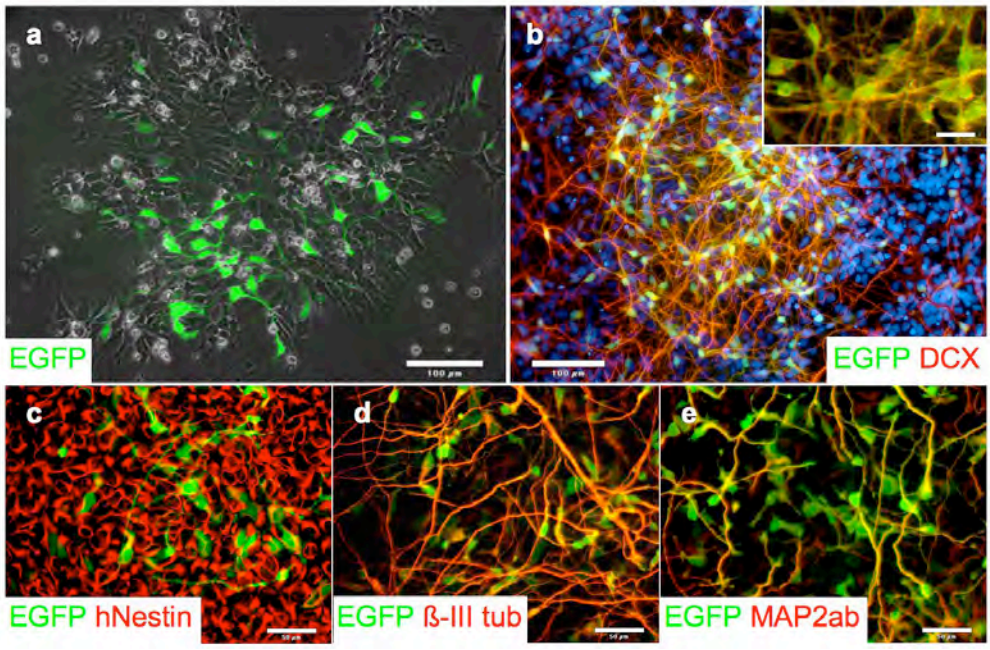

Figure 4.2: Expression of the DCX-EGFP transgene in differentiating lt-hESNSC. (a) EGFP-positive cells alongside still undifferentiated precursors. **(b)** Co-expression of EGFP and DCX protein confirmed faithful recapitulation of DCX expression by the reporter construct. **(c-e)** DCX-EGFP-positive cells were negative for nestin **(c)** but co-expressed the neuronal markers beta-III tubulin **(d)** and MAP2ab **(e)**. Cells were captured during **(a)** proliferation and at **(c)** 2, **(b,d)** 8 and **(e)** 12 days of *in vitro* differentiation. Scale bars: a-b 100 µm, c-e 50 µm.

4.1.3. Purification of DCX-EGFP-positive neurons by FACS

A key challenge in stem cell technology is the generation of purified sub-lineages. Because the *in vitro* data revealed a highly neuron-specific EGFP-fluorescence within the selected

clones, a question to address was whether these cells can be exploited for the generation of pure neuronal cultures by fluorescence activated cell sorting (FACS). A major issue in neuronal lineage selection is the identification of a time point where the desired neurons are present in sufficient numbers but still immature enough to tolerate sorting, replating or cryopreservation. Quantitative analysis of DCX-EGFP-positive cells showed a continuous increase in the numbers of EGFP-positive cells within the first 3 weeks of differentiation (from 28±2.8% by day 7 up to 55±6% by day 21). Within the first week of differentiation, the DCX-EGFP-positive cells still displayed an immature morphology with most of them exhibiting only a few unbranched processes. Less than 50% of the EGFP-positive cells expressed the more mature neuronal marker MAP2ab, indicating a still immature stage of differentiation. Based on these observations, day 8±1 was chosen as an ideal time window for sorting. Within this time period, an average of 31±2,8% of the cells expressed the DCX-EGFP transgene.

Preparative FACS was performed with EGFP fluorescence intensity as the only sorting criterion. The population of EGFP-positive neurons was readily distinguishable from other cell types (undifferentiated or glial cells) by virtue of their EGFP-fluorescence (Figure 4.3 a). Re-analysis of the sorted population showed efficient enrichment of the EGFP-positive cells (Figure 4.3 b). Following replating, most of the cells survived, exhibited strong EGFP fluorescence (Figure 4.3 c), and were negative for nestin (Figure 4.3 d). Twenty-four hours after plating up to 95% (92.3±2.5%) of the cells were found to co-express beta-III tubulin and EGFP (Figure 4.3 e). The replated cells initiated neurite outgrowth within the first 24 hours after attachment (Figures 4.3 c-f). Four days after FACSorting the cells exhibited distinct neuronal phenotypes with extension of long neurites forming a network-like architecture (Figure 4.3 g).

Plated neurons survived up to 3 weeks in standard media (containing B27 supplement, but no neurotrophic factors). Supplementation of the media with BDNF and GDNF promoted the development of complex neuronal networks, and enhanced the survival of neurons (Figure 4.3 h). Neurotrophic factors are also produced from astrocytes. Therefore, another method to supplement standard media (containing B27 supplement, but no extra added neurotrophic factors) is to use astrocytic conditioned media or to directly co-culture the neurons on astrocytes (see 3.5). Quantification performed 3 weeks after plating and propagation in astrocyte-conditioned medium revealed that 40±4% of the neurons had survived. These neurons could be further maintained for a total period of more than 2 months without overt cell loss.

After further differentiation the majority of the neurons acquired a GABAergic phenotype. Immunohistochemical analysis performed 3 weeks after FACSorting and replating in astrocyte-conditioned medium revealed that 61±5.4% of the purified neurons expressed

GABA. These cells displayed a bipolar to multipolar morphology and were partially positive for GAD67 (Figure 4.3 i-j). Other neuronal subtypes, such as tyrosine hydroxylase (TH) positive neurons were detected only very occasionally (< 0.1%, Figure 4.3 k). Differentiation towards a GABAergic neuronal phenotype was independent of the passage number of the DCX-EGFP lt-hESNSC reporter cell line.

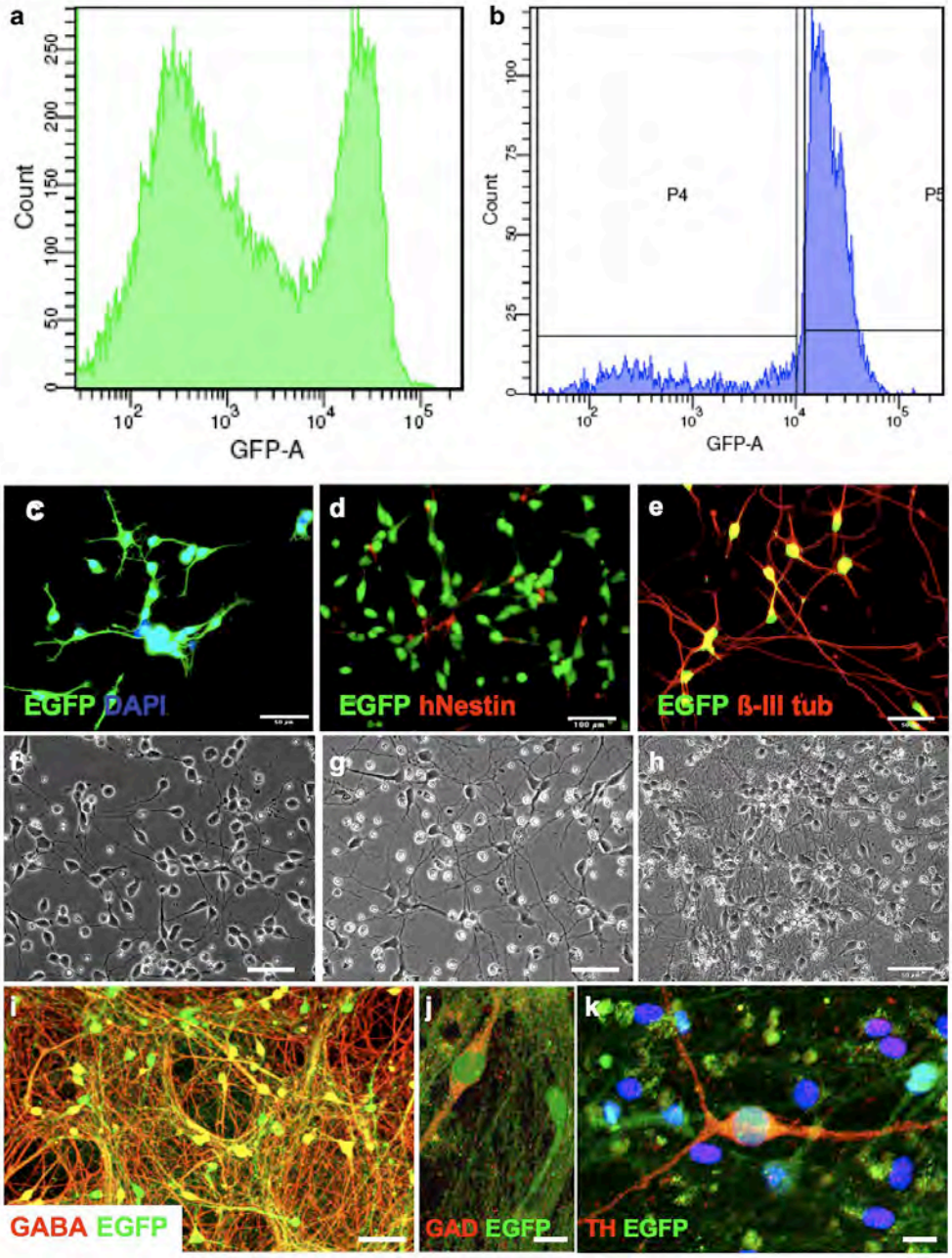

Figure 4.3: FACS-based lineage selection of DCX-EGFP-positive human neurons. (a) After 7-9 days of *in vitro* differentiation, a dominant fraction of fluorescent cells was detected in the DCX-EGFP cultures by FACS. **(b)** Re-analysis revealed enrichment of EGFP-positive cells up to 95%. **(c-d)** Twenty-four hours after FACS and replating, sorted cells **(c)** maintained strong EGFP fluorescence, **(d)** were largely negative for nestin and **(e)** positive for beta-III tubulin. **(f-h)** Neurite outgrowth 24 hours **(f)**, 4 days **(g)** and 1 month **(h)** after replating. **(h)** Cells were propagated as shared media cultures with primary mouse astrocytes to promote long-term survival. **(i-j)** Expression of GABA **(i)** and GAD67 **(j)** three weeks after FACSorting and differentiation in astrocyte-conditioned medium. **(k)** Occasional neurons were found to express TH. Note: down-regulation of EGFP in this neuron might be due to advanced differentiation. Scale bars: c,e-h 50 µm, d and i 100 µm, j-k 10 µm.

The expression profile of DCX in lt-hESNSC revealed that DCX is down-regulated in more differentiated neurons. To enable cell detection by fluorescent label beyond down-regulation of the DCX-EGFP transgene a lt-hESNSC clone labelled with EGFP under a ubiquitous promoter (Figure 4.4 a; provided by Philipp Koch) was transfected with a construct carrying a DsRED2 gene as fluorescence detection marker under the control of the human DCX promoter. Comparable to the DCX-EGFP transfected and selected lt-hESNSC the transfected cells were selected in the presence of 50 µg/ml G418 for 3 weeks. Clones were picked and further expanded as described for the DCX-EGFP transfected cells. *In vitro* differentiation of the selected clones showed comparable results to the ones obtained for the DCX-EGFP construct with DsRED2 expression only detectable in cells with neuronal morphology (Figure 4.4 b). The population of DsRED2- and EGFP-positive cells was distinguishable by FACS resulting in two fluorescent peaks, from the cells that were expressing only EGFP (Figure 4.4 c). DCX-DsRED2 sorted cells could be replated and further cultured (Figure 4.4 d-e). This labelling system should allow the identification of DCX-DsRED2:PGK-EGFP purified cells by virtue of their EGFP-fluorescence in more differentiated stages where DCX is down-regulated.

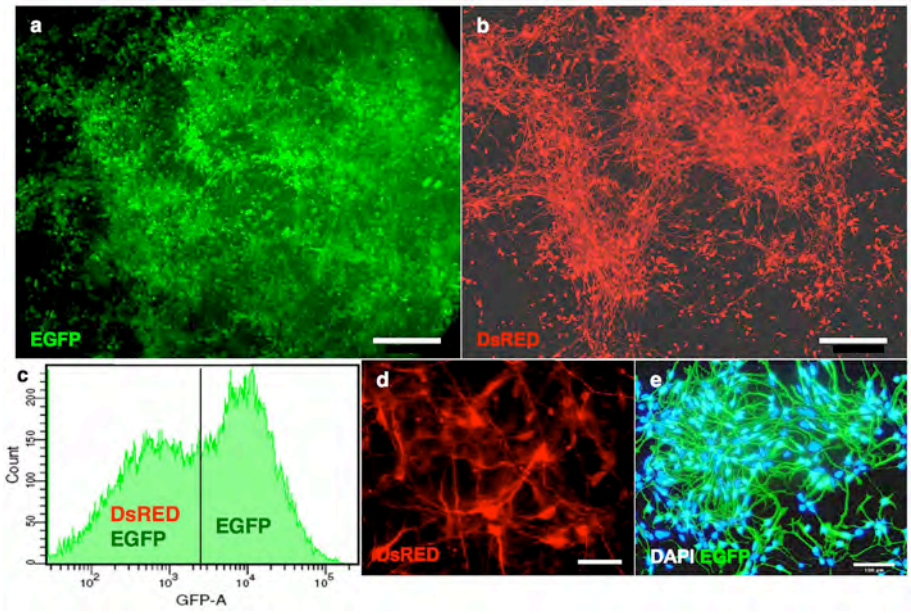

Figure 4.4: FACS-based lineage selection of DCX-DsRED2:PGK-EGFP-positive human neurons. After 7-9 days of *in vitro* differentiation, lt-hESNSC **(a)** ubiquitously expressing EGFP **(b)** co-expressed DsRED2 in cells showing neuronal morphology. **(c)** The DsRED2-positive cells could be detected by FACS. Twenty-four hours after FACS and replating, sorted cells maintained strong **(d)** DsRED2 and **(e)** EGFP fluorescence. Scale bars: a-b 200 µm, d 50µm and e 100 µm.

4.1.4. Functional maturation of purified hES cell-derived neurons

To assay functionality of the selected cells, whole-cell patch-clamp recordings were performed on DCX-sorted neurons*. To facilitate long-term survival and functional maturation, the DCX-EGFP sorted neurons were co-cultured for 4 weeks with primary mouse astrocytes. Neurons gained fast transient inward currents sensitive to the selective Na+ channel blocker tetrodotoxin (TTx, 300 nM) and complex outward currents. The latter could be shown to consist of at least two components: a fast activating and inactivating one, sensitive to 3 mM 4-aminopyridine (4-AP) and resembling characteristics of A-type K+ current; and a slowly activating sustained component reminiscent of delayed rectifier type (n=3, Figure 4.5 a-d). All neurons tested (n=13) fired repetitive action potentials upon sustained depolarization (Figure 4.5 e). Furthermore, following application (500 ms) of either the AMPA/KA receptor agonist kainic acid or GABA receptor agonist muscimol, the neurons displayed surface expression of AMPA/kainate and $GABA_A$ receptors (n=3, Figure 4.5 f) as a prerequisite for the formation of glutamatergic and GABAergic synapses.

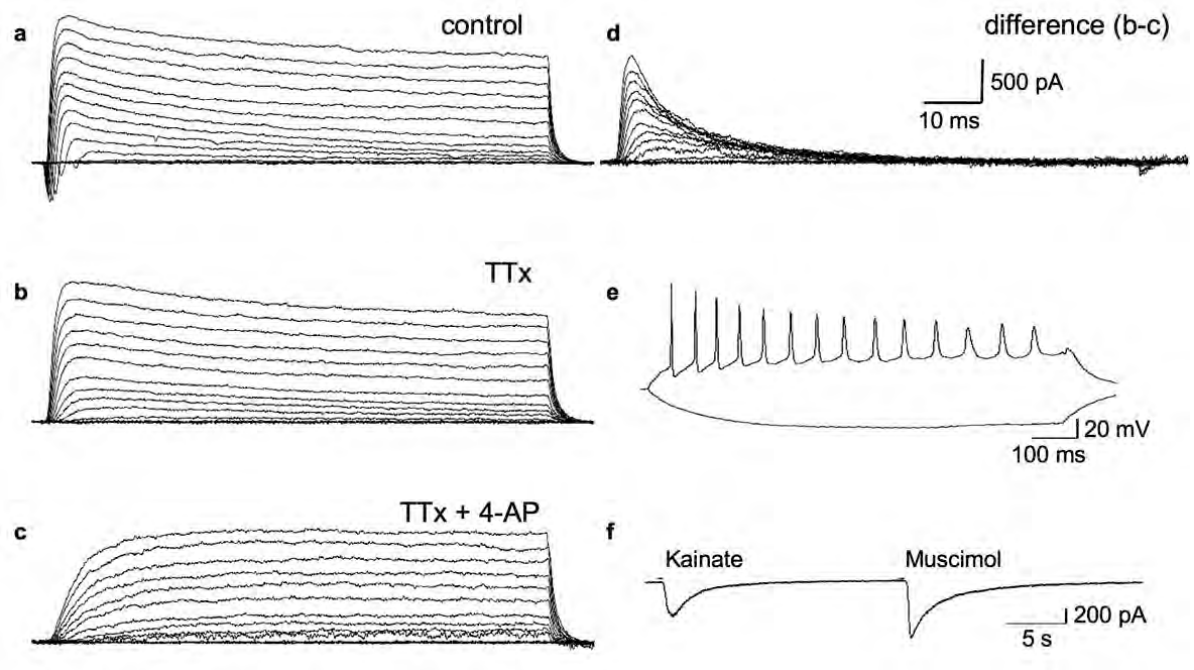

Figure 4.5: Electrophysiological properties of DCX-EGFP purified neurons differentiated 4 weeks *in vitro*. Voltage-dependent whole cell current **(a)** consisted of a fast transient inward component, which could be blocked by 300 nM TTx and **(b)** a complex outward current. **(c)** The latter consisted of a 4-AP insensitive, slow activating, and during the test pulse (90 ms) non-inactivating component. **(d)** Digital subtraction of the traces shown in b and c revealed a 4-AP-sensitive fast activating and inactivating current. **(e)** Neurons displaying the current pattern shown in a, were able to fire multiple action potentials upon long-lasting (1 s) depolarizing current injection. **(f)** Brief (500 ms) application of either the AMPA/KA receptor agonist kainic acid or $GABA_A$ receptor agonist muscimol elicited a clear current response.

* All functional studies were performed by T. Opitz

To further explore the ability of the selected neurons to engage in synaptic circuits, purified neurons were transplanted onto hippocampal slice cultures prepared from P10 rats, a paradigm known to promote long-term survival and functional maturation of ES cell-derived neurons (Benninger *et al.*, 2003). As EGFP down-regulation indicates a more mature neuronal stage, functional integration was evaluated by patch-clamp recordings 4 weeks after transplantation, selectively on those neurons with lower EGFP expression (Figure 4.6 a-c). The cells exhibited both transient and persistent outward, and large amplitude transient inward currents. All of the neurons recorded (n=9) were able to fire repetitive action potentials upon long-lasting depolarization. In addition, spontaneous postsynaptic currents (PSCs) could be recorded in 8 out of 9 neurons. In some recordings (n=3) a Cs^+-based pipette solution for a closer characterization of those PSCs was used. Recordings at different holding potentials revealed a reversal potential of postsynaptic currents between -20 and -60 mV (Figure 4.6 a). Large PSCs in cells located in the CA1 region could be evoked by electrical stimulation with a stimtrode placed in the stratum radiatum (Figure 4.6 b). Those evoked PSCs reversed polarity at -48 mV (Figure 4.6 c), close to the calculated ECl of -43 mV, suggesting synaptic input via $GABA_A$ receptors. Taken together, the data indicate that the population of human cells selected with the DCX-EGFP transgene may acquire functional properties of mature neurons upon *in vitro* differentiation.

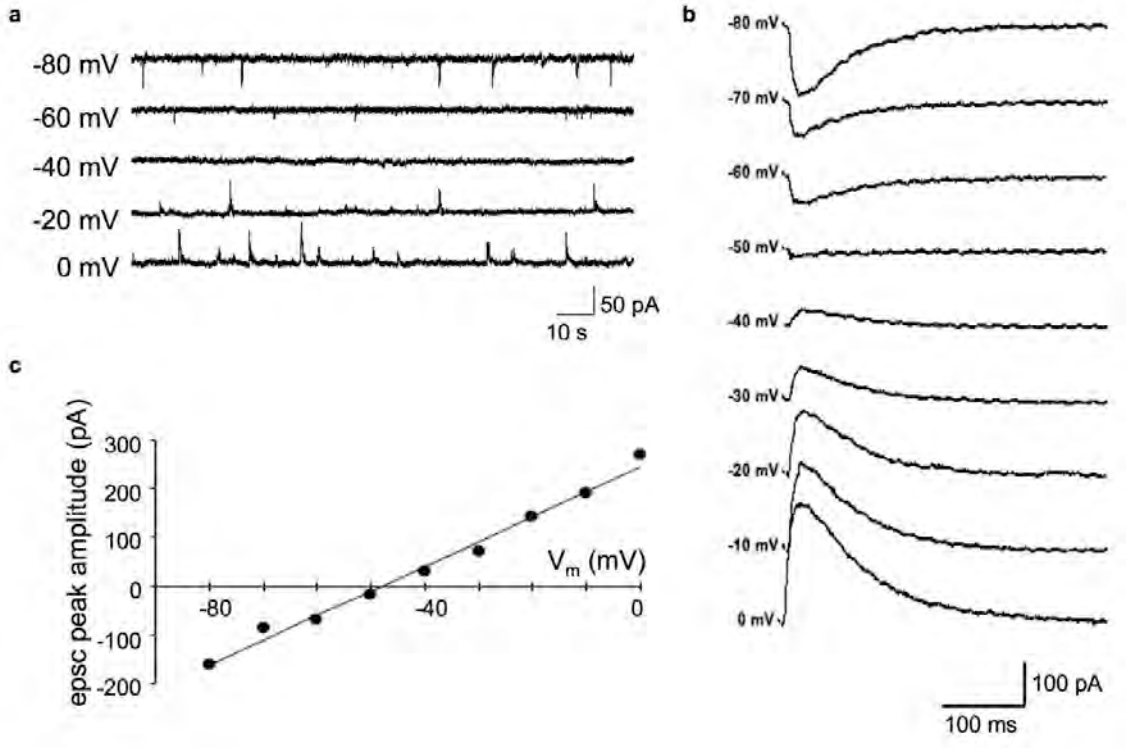

Figure 4.6: Postsynaptic currents in DCX-EGFP-selected neurons propagated on hippocampal rat slice cultures. (a) Four weeks after deposition, PSCs could be observed which reversed between -60 and -20 mV. **(b)** Electrical stimulation of presynaptic fibers evoked monosynaptic PSCs. **(c)** Plotting PSC amplitude over holding potential revealed a reversal potential of -48 mV.

4.2. Generation of an efficient cryopreservation protocol for human neurons

4.2.1. Cryopreservation of purified hES cell-derived neurons

An essential prerequisite for the effective use of hES cell-derived neurons for further biological and pharmacological applications is the development of efficient cryopreservation protocols. When applying conventional slow-cooling and thawing protocols (e.g. a -1°C/min cooling rate and 37°C thawing temperature) involving commercial serum-free freezing formulations or standard protocols composed of 90% FCS and 10% dimethyl sulfoxide (DMSO), sorted DCX-EGFP-positive cells exhibited relatively low survival rates between 30% and 35% directly after thawing analysed by trypan blue exclusion assays. Therefore different freezing solutions were formulated including one containing DMSO, the cryoprotectant myo-inositol, polyvinyl-alcohol as ice-controlling agent, and serum replacement (KO-SR) with an osmolarity of 300-400 mOsm/kg (see Materials & Methods). Using the latter, the post-thawing survival could be enhanced up to 47% (40±5%) (Figure 4.7 d).

To further increase post-thaw survival, cells were pre-treated with the general caspase inhibitor z-VAD-fmk 30 minutes before freezing. Apoptosis mediated by a caspase-dependent mechanism was shown to significantly contribute to cryopreservation-associated cell loss. Furthermore, caspase inhibition has been successfully employed to improve cell survival upon freezing of undifferentiated hES cells and other cell types (Stroh *et al.*, 2002; Heng *et al.*, 2006). Pre-incubation of DCX-EGFP-sorted neurons with 500 nM z-VAD-fmk for 30 minutes significantly reduced the number of cells undergoing cell death, resulting in an increased survival rate up to 83% (70.1%±13.1%), as detected by trypan blue exclusion assays directly after thawing (Figure 4.7 d). Furthermore, it was tested whether z-VAD-fmk treatment prior to cryopreservation also protects the neurons within the first 20 hours after thawing and replating. To quantify caspase-mediated apoptosis 20 hours after thawing and replating, a luminescent caspase-assay was performed which measures the caspase-3 and -7 activities. Both caspase-3 and caspase-7 play key roles in mediating apoptosis of mammalian cells (Thornberry *et al.*, 1997; Le *et al.*, 2002). A reduction by 3.5 fold in caspase-3 and -7 activities could be measured in z-VAD-fmk-treated neurons compared to untreated control cells (Figure 4.7 e).

To show that the effect of the general caspase inhibitor was only temporary, treated and untreated thawed neurons were further cultured for four days, and subsequently incubated with 250 nM staurosporine for 3 hours, followed by a luminescent caspase assay. At this time point no significant difference in the induction of caspase-3 and -7 activity was detected between z-VAD-fmk treated and non-treated cells.

Two days after thawing and plating, z-VAD-fmk-treated cells still showed strong expression of EGFP (Figure 4.7 a), a neuronal morphology with prominent neurites, and immunoreactivity to DCX and beta-III tubulin (Figure 4.7 b-c). Maintenance of functionality was further confirmed by patch-clamp analysis. This analysis showed that thawed neurons co-cultured for four weeks with mouse primary astrocytes were able to generate functional ion channels, to fire repetitive action potentials, and to display functional AMPA and GABA receptors. Taken together, these data indicate that cryopreservation does not impair the physiological properties of the cells (Figure 4.7 f-h).

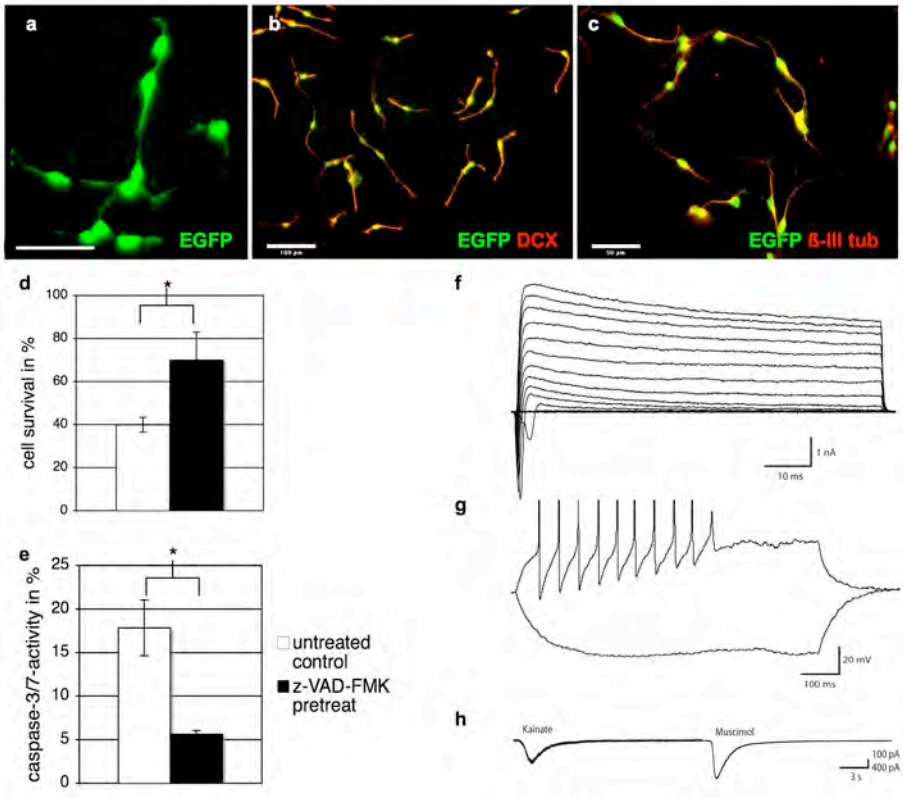

Figure 4.7: Cryopreservation of purified hES cell-derived neurons. (a-c) Two days after thawing, sorted and z-VAD-fmk treated cells maintained **(a)** strong expression of the DCX-EGFP transgene, **(b)** the DCX protein and **(c)** the neuronal marker beta-III tubulin. **(d)** Quantification of the post-thaw cell survival of cryopreserved DCX-EGFP-sorted cells with and without pre-treatment with z-VAD-fmk (* $P < 0,05$). **(e)** Quantification of caspase-3 and -7 activity of cryopreserved DCX-EGFP sorted cells with and without z-VAD-fmk pre-treatment as detected 20 hours after cryopreservation and replating (* $P < 0.01$). **(d-e)** Bars depict the mean fraction of cells in the different cell populations. **(f)** Thawed neurons displayed voltage-dependent whole cell current and **(g)** were able to fire multiple action potentials upon long-lasting (1 s) depolarizing current injection. **(h)** Application (500 ms) of either the AMPA/KA receptor agonist kainic acid or GABA receptor agonist muscimol, elicited a clear current response. Scale bars: c and e 50 µm, d 100 µm.

4.2.2. Transplantation of purified and cryopreserved neurons into the neonatal rodent brain

To analyze whether cryopreservation impairs *in vivo* survival and integration capacity of hES cell-derived neurons, 100 000 freshly thawed neurons were injected unilaterally into the ventricles of the developing brain of P2 rats. Two weeks after transplantation, animals were sacrificed and their brains explanted for immunohistochemical analysis. DCX-EGFP-expressing cells showed distribution in a variety of host brain regions, including the rostral migratory stream, the olfactory bulb and the hippocampus (n=7) (Figure 4.8 a-h). EGFP-positive cells displayed a clear neuronal morphology with delineation of neurites (Figure 4.8 b,g). All engrafted EGFP-positive donor cells co-expressed a human specific nuclear antigen (Figure 4.8 b,d,f-h). Transplanted neurons found in the rostral migratory stream displayed a bipolar morphology with an elongated leading process, oriented towards the olfactory bulb, which is suggestive of a migratory phenotype (Figure 4.8 b). In contrast, cells that reached the olfactory bulb displayed a multipolar morphology with extension of branched neurites (Figure 4.8 c-d). Some EGFP-positive neurons were located in the dentate gyrus (Figure 4.8 f,h), as well as between the CA1 region and the dentate gyrus (Figure 4.8 g) of the hippocampus. These data indicate that FACS and cryopreservation do not affect survival and integration of hES cell-derived neurons *in vivo*.

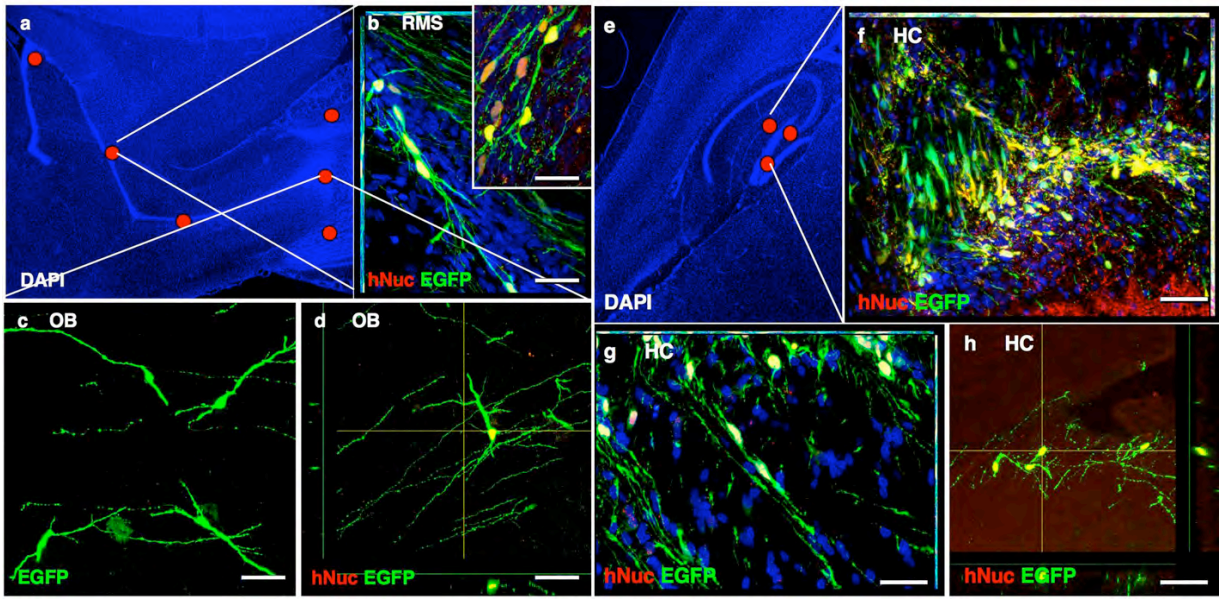

Figure 4.8: Purified and cryopreserved hES cell-derived neurons following *in vivo* engraftment into the P2 rat brain. (a) Schematic representation (red) of the distribution of DCX-EGFP-positive cells along the SVZ, the rostral migratory stream (RMS), the olfactory bulb (OB) and **(e)** in the hippocampus (HC). Co-expression of **(b)** EGFP and the human specific nuclei antigen (hNuc) in transplanted cells migrating along the rostral migratory stream, **(c)** within the olfactory bulb and **(d-h)** within the hippocampus. Scale bars: b-c,g 50 µm, d,f,h 100 µm.

4.3. Enhanced migration of purified human neurons

The expression of DCX in the developing and adult brain is associated with a strong migratory potential (Bai *et al.*, 2003; Tanaka *et al.*, 2004). During development, newborn DCX-positive neurons leave the VZ and migrate towards the apical surface of the developing cortex. In the adult brain DCX-positive neurons, born in the SVZ or the dentate gyrus, migrate towards the olfactory bulb and the outer layer of the dentate gyrus, respectively. To study whether the DCX-EGFP purified human neurons share an enhanced migration potential with their *in vivo* counterparts, different *in vitro* and *in vivo* migration assays were performed. To that end, DCX-EGFP purified human neurons (also termed hES-N), derived from 8±1 days pre-differentiated lt-hESNSC expressing the EGFP reporter gene under the control of the DCX promoter, were investigated. Migration of the purified neurons was compared with that of the 8±1 days pre-differentiated lt-hESNSC expressing the EGFP reporter gene under the control of the DCX promoter which were not purified, i.e. termed to as hES-NSC+N. This population consisted of 31±2.8% immature DCX-EGFP-positive neurons and 69±2.8% other cell types, most of which expressed nestin (Figure 4.1 e) and showed a precursor morphology.

4.3.1. *In vitro* migration of purified human neurons

In a first set of experiments, 100 000 hES-N as well as 100 000 hES-NSC+N were plated on a matrigel scaffold (see 3.7.2). This scaffold mimics the *in vivo* extracellular matrix and has been shown to promote migration *in vitro* (Durbec *et al.*, 2008). Two days after plating the hES-N showed a distribution across a large area with strong migration of individual neurons from the plating site (Figure 4.9 c-d). In contrast, the hES-NSC+N control population formed spherical clusters and hardly any cell migrated away from these clusters. Instead, only limited axonal outgrowth could be observed (Figure 4.9 a-b). Interestingly, the large number of DCX-EGFP-positive neurons within the hES-NSC+N control cells, which have the potential to migrate as a pure population, remained confined to the spherical clusters and did not migrate out on the matrigel scaffold.

A similar result was observed when a Boyden chamber migration assay was performed using medium not containing additional chemoattractants (see 3.7.1). A significant higher number of EGFP-positive neurons from the hES-N were found to reach the lower side of the chamber compared to the EGFP-positive neurons within the hES-NSC+N control-population 20 hours after plating (Figure 4.9 e). Again, only limited migration of DCX-EGFP-positive neurons in the control population was observed. Thus, the purified DCX-EGFP-positive immature neurons show a strong migratory phenotype.

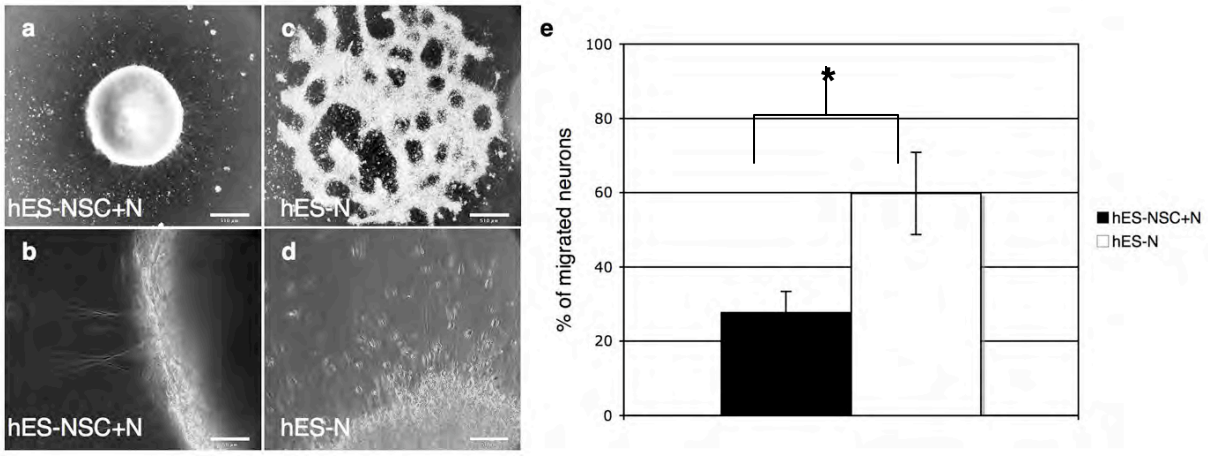

Figure 4.9: ***In vitro*** **migration-assays of purified human neurons. (a-d)** Matrigel migration assay. **(a)** Sphere-like structures of plated hES-NSC+N showing **(b)** axonal outgrowth but no single cell migration and **(c)** centrifugal distribution of plated hES-N **(d)** reflecting strong single cell migration. **(e)** Mini-chamber migration monitored after 20 hours in culture towards medium. Bars represent percentage of neurons counted on the lower side of the migration chamber (see 3.7.1) either from plated hES-NSC+N (black), or from hES-N (white). Cell numbers were normalised to the number of DCX-EGFP-positive plated cells (* $P < 0.05$). Scale bar: b-e 510 µm.

4.3.2. Migration of purified human neurons on hippocampal rat slice cultures

The above made observations raised an interesting question: Why do the purified human neurons alone have enhanced migration capacity whereas the same neurons within the hES-NSC+N control cells do not show this property?

To investigate whether the enhanced migration capacity of purified neurons is conserved in an environment closer to an *in vivo* system consisting of different cell types and migration cues, DCX-EGFP-positive neurons purified by FACS (hES-N) and control cells, which were not purified but contained DCX-EGFP-positive cells as well as less differentiated cells (hES-NSC+N), were deposited on hippocampal slice cultures generated from day 9 to 10 postnatal rats (Benninger *et al.*, 2003; Opitz *et al.*, 2007). Migration capacity was assessed 7 (n=40) and 14 (n=14) days after spotting. In this experiment the enthorinal cortex was chosen as transplantation site, because from here projections of the perforant path approach the dentate gyrus serving as potential guiding structures for migrating cells (Benninger *et al.*, 2003). Within 7 days hES-N were observed all over the hippocampal slice culture including the perforant path. Among other properties transplanted hES-N showed adaptation to the morphological structures of the slice with orientation orthogonal to the cortical structure: the CA1 and CA3 region of the pyramidal cell layer, and the granule cell layer of the dentate gyrus (Figure 4.10 a). By 14 days post transplantation many neurons showed distribution along the neuronal layers, the CA1 and CA3 region of the slice, displaying extensive intercellular axonal connections (Figure 4.10 b). Because of the extensive migration of the hES-N the deposition site of the cells on the slice could hardly be detected 7 days post

transplantation. In contrast, hES-NSC+N formed dense cores at the transplantation site (Figure 4.10 d). 14 days post transplantation the neurons within the hES-NSC+N transplanted cells generated numerous axonal projections into the hippocampus over long distances, but hardly migrated out of the transplantation core (Figure 4.10 e). The axonal projections could, however, be frequently traced within several 100 µm away from the transplantation core (Figure 4.10 e). The significant difference in migration capacity observed in the horizontal xy-axis was also observed in the vertical z-axis. Seven days after transplantation the number of EGFP-positive neurons that reached a depth of 175 µm and 350 µm in the vertical z-axis were quantified in z-stacks (Figure 4.10 c,f,i). Whereas an average of 1.25±0.9 of the neurons within hES-NSC+N and 13.44±7.7 of the hES-N reached a depth of 175 µm per mm^2, none of the neurons within the hES-NSC+N group but 6.5±4.39 of the hES-N migrated to a depth of 350 µm per mm^2. Migrating cells expressed the neuronal marker DCX, as well as human specific nuclear antigen (Figure 4.10 g-h).

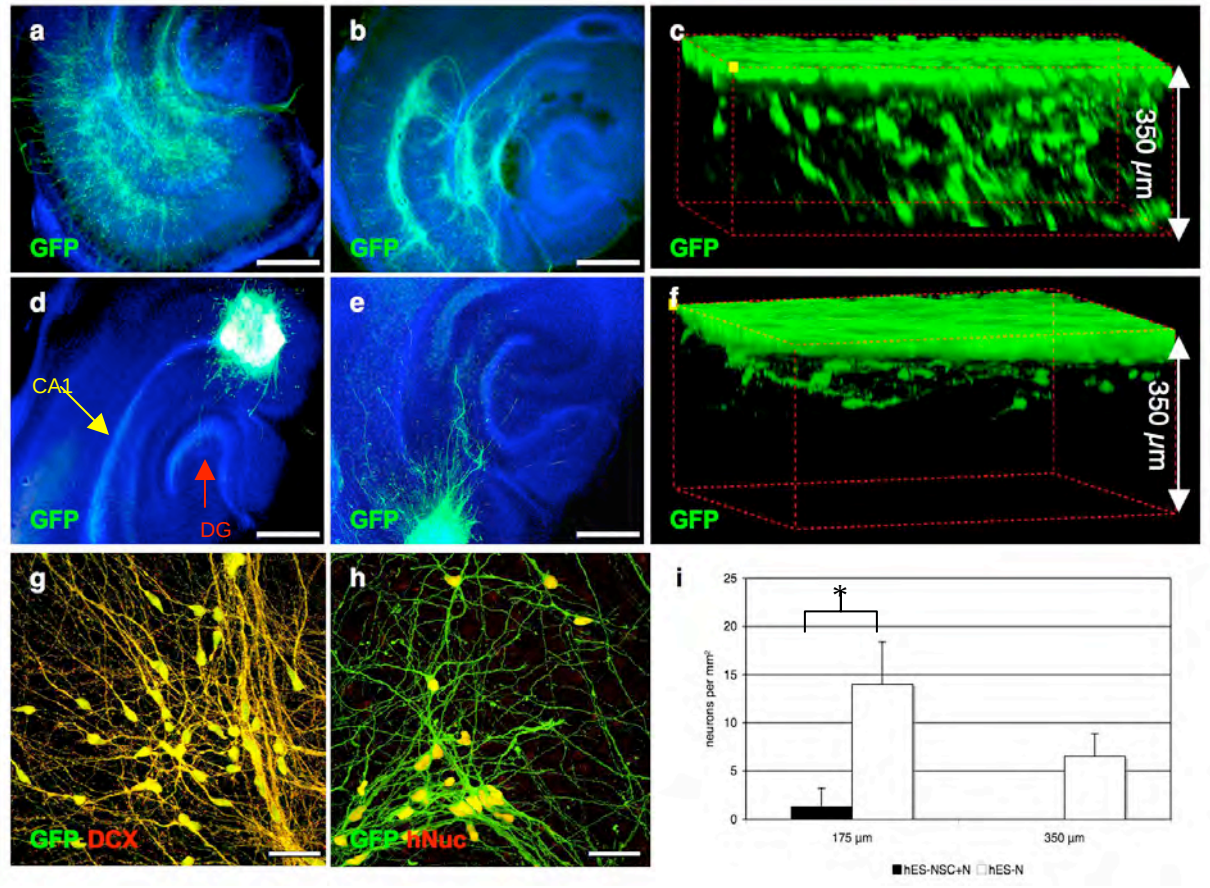

Figure 4.10: Migration of hES-N versus hES-NSC+N on hippocampal slice cultures. Distribution of transplanted neurons within **(a,b)** hES-N and **(d,e)** hES-NSC+N on hippocampal slices cultured **(a,d)** 7 and **(b,e)** 14 days after transplantation. **(d)** The red arrow marks the dentate gyrus (DG) and the yellow arrow the CA1 region of the hippocampal slice. 3D reconstruction of migrating neurons 7 days post transplantation **(c)** of hES-N and **(f)** of hES-NSC+N. **(g)** EGFP/DCX and **(h)** EGFP/hNuc co-staining 7 days post transplantation on hippocampal slices. **(i)** Quantification of the total numbers of cells reaching migration depths of 175- and 350 µm in the z-axis per mm^2. Data based on n=4 per condition. Bars depict the mean fraction of cells in the different cell populations (* $P < 0.005$). Scale bars: a-b and d-e 850 µm, g-h 50 µm.

Thus, DCX-EGFP sorted neurons showed enhanced migratory ability also in hippocampal slice cultures. Furthermore, the distribution of the transplanted hES-N over the hippocampal slice cultures indicates that transplanted neurons might use hippocampal structures as guiding cues as they distributed along the neuronal layers including the CA1 and CA3 region of the hippocampal slice, whereas neurons within transplanted hES-NSC+N did not show these characteristics.

4.3.3. *In vivo* migration of purified human neurons in the CNS of adult rats

On the basis of the above-presented data of the strongly enhanced migration of hES-N *in vitro* and on hippocampal slice cultures, a central question was whether this migratory ability is maintained in an *in vivo* environment. To address this question, 100 000 FACS-purified DCX-EGFP-positive neurons (hES-N) or control cells which were not purified but contained DCX-EGFP-positive cells as well as less differentiated cells (hES-NSC+N) were transplanted into the striatum (n=16) or the SVZ close to the rostral migratory stream (n=15) of adult rats. Eight days post transplantation the animals were sacrificed and the brains were analyzed (Figure 4.11). In the animals that received hES-NSC+N either transplantaed into the striatum or the SVZ, the grafts formed densely packed clusters with very few cells migrating out of the transplantation site (Figure 4.11 b). In the animals that received the hES-N, however, the grafts consisted of only small transplantation cores and large numbers of migrating cells in the surrounding brain parenchyma (Figure 4.11 c-d,f). The hES-N were primarily oriented towards and along preexisting fiber tracks such as the corpus callosum (Figure 4.11 a,c-d). The hES-N transplanted in the SVZ also appeared to follow pre-existing migration tracts of the rostral migratory stream (Figure 4.11 f). Migrating cells displayed bipolar morphology with a leading process towards their direction of movement and relatively small somata, indicating active migration (Figure 4.11 d,f).

Quantification of the total number of EGFP positive cells in the corpus callosum (see 3.8.2) amounted to an average of 534±108.70 cells per animal which received hES-N whereas no EGFP positive cells were found in the corpus callosum in the animals which received the hES-NSC+N. These data demonstrate that the strong migration capacity of the hES-N is maintained *in vivo*. The distribution of the cells further suggested that hES-N might be sensitive to pre-existing migration cues. As already observed in other paradigms, the hES-NSC+N showed a strong tendency to form clusters with the DCX-EGFP-positive cells remaining insight these clusters. One possibility to explain these phenomenon is that the DCX-EGFP-positive neurons are attracted by factors secreted from the surrounding undifferentiated progenitor population which is present in the hES-NSC+N cell population and

that this “self attraction” of the transplantation core might be the reason for the only limited migration of DCX-EGFP-positive neurons away from the core in the control cell population.

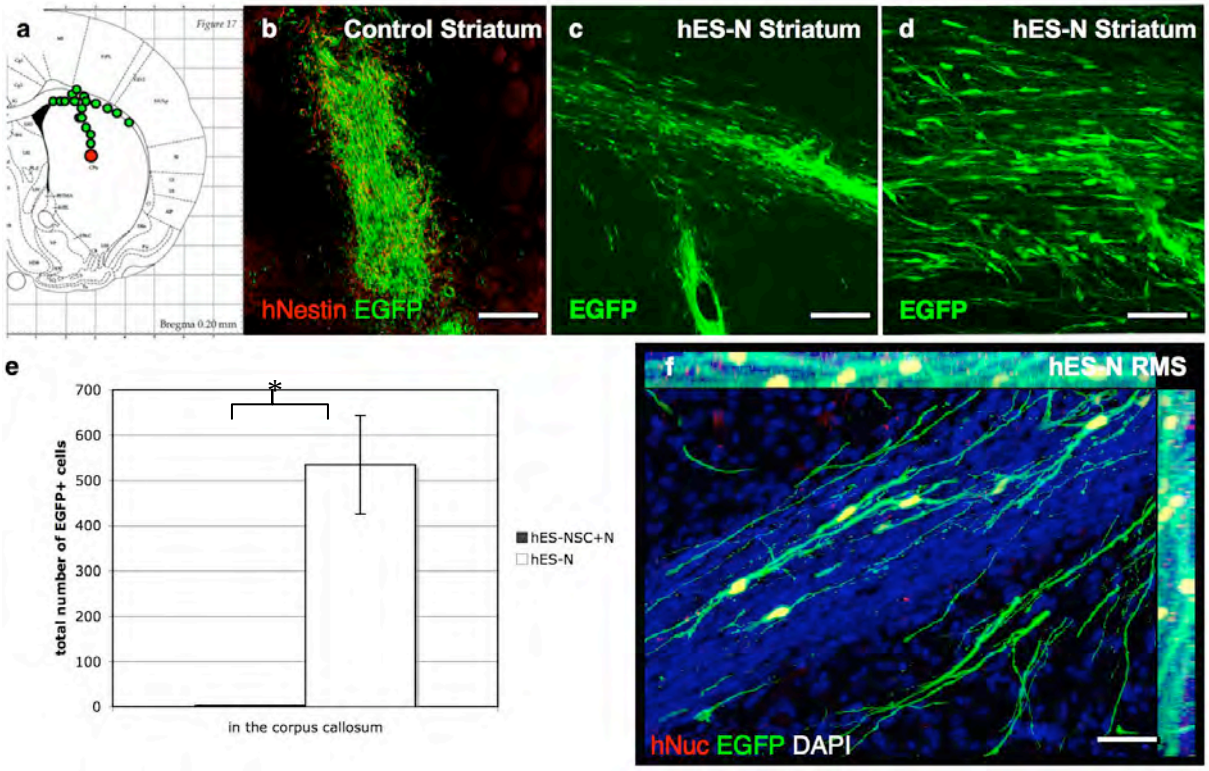

Figure 4.11: Migration of DCX-EGFP-positive cells *in vivo*. (a) Schematic representation of a coronal section through the brain of a transplanted animal illustrating the transplantation site (red) and the distribution of hES-N (green) 8 days post grafting. **(b)** Core formation of transplanted hES-NSC+N. Note that these grafts consist of EGFP-positive neuronal cells and nestin positive precursors. **(c)** HES-N showed a strong tendency to migrate away from the transplantation site towards the corpus callosum and **(d)** appeared to follow preexisting fiber tracks of the corpus callosum. **(e)** Quantification of EGFP-positive neurons found in the corpus callosum within transplants of hES-NSC+N (black) and hES-N (white). Bars depict the mean fraction of cells in the different cell populations (* $P < 0.007$). **(f)** EGFP-positive neurons transplanted at the SVZ migrate along the rostral migratory stream co-expressing the human Nuclei antigen (red). Scale bars: b-c 200 µm, d 100 µm, f 50 µm.

4.4. Interaction between neural stem/progenitor cells and immature neurons

A central issue in neural replacement therapy is the question of how grafted cells can be directed to particular targets within the CNS. Currently this question is far from being solved. Knowledge of signals controlling grafted neuronal progenitor migration might be of major importance for future transplantation approaches. Assessing neuronal migration within a pure neuronal culture, as well as within a cell mixture of neurons and less differentiated neural stem/progenitor cells, may help to understand some of the graft-intrinsic mechanisms (i.e., cell-cell interactions, attraction of neurons by the graft itself) that underlie NSC graft formation and restricted neuronal migration following NSC transplantation.

4.4.1. Chemoattraction between neural stem/progenitor cells and immature neurons

The previous *in vitro* and *in vivo* migration studies showed that DCX-positive neurons derived from human ES cells have an enhanced migration potential as a “purified population” (hES-N), whereas only restricted migration occurs in a “non-purified” cell population (hES-NSC+N) consisting of neurons and less differentiated neural stem/progenitor cells. This might be due to an interaction existing between neurons and the yet undifferentiated neural stem/progenitor cells. In principle this interaction could be based on a direct cell-cell contact or mediated by soluble factors. To test whether soluble factors secreted from the neural stem/progenitor cell population may attract immature neurons, a mini-chamber migration assay was performed as described in 4.3.1. In this specific case though, proliferating NSCs were plated in the bottom well of the migration chamber, and either DCX-EGFP-positive neurons purified by FACS (hES-N) or control cells which were not purified, but contained DCX-EGFP-positive cells as well as less differentiated neural cells (hES-NSC+N) were plated in the upper well. In this setting 70% of the DCX-EGFP-positive neurons within the hES-NSC+N population, and 92% of the DCX-EGFP-positive hES-N migrated towards the stem cell population within 20 hours (Figure 4.12). Compared to the previously performed mini-chamber migration assay (see 4.3.1), in which 60% of the hES-N and 28% of the hES-NSC+N migrated towards the medium in the lower well, the number of migrating neurons increased by 32% and 42%, respectively. Thus, proliferating NSCs appear to express at least one soluble factor, which has a strong chemoattractive effect on immature neurons.

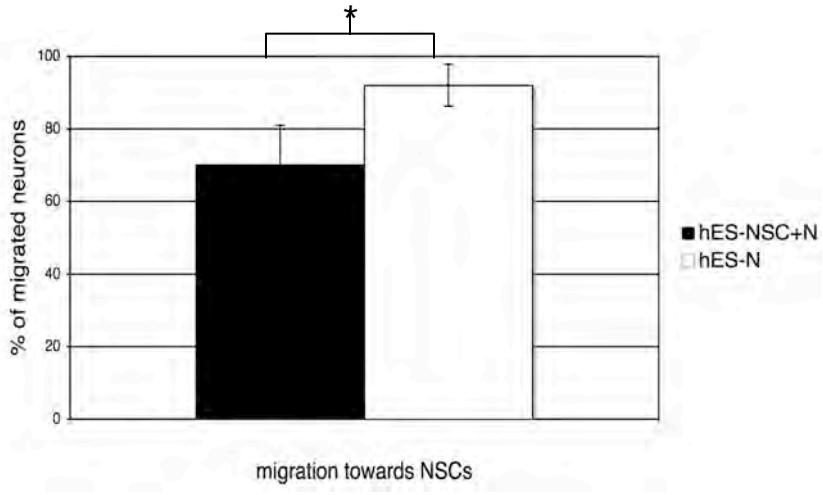

Figure 4.12: Migration of neurons towards NSCs monitored after 20 hours in culture. Bars represent percentage of migrated neurons either from plated hES-N (white) or hES-NSC+N (black). Cell numbers were normalised to the number of DCX-EGFP-positive plated cells (* $P < 0.05$).

4.4.2. Migration of immature neurons in a cell mixture with neural stem/progenitor cells on hippocampal rat slice cultures and in the CNS of adult rats

Taking in account that DCX-EGFP-positive neurons are highly migratory once purified from the surrounding undifferentiated- or less differentiated neural stem/progenitor cells (see 4.3), and that factors secreted by the neural stem/progenitor cells may be responsible for an inhibitory effect on the migration of these neurons, we assessed whether increasing or reducing the ratio of neural stem/progenitor cells within a neural/neuronal population correlates with the restriction or enhancement in migration of the DCX-EGFP expressing neurons, respectively. To address this issue the migration of the purified neurons (hES-N) and the non-purified cells (hES-NSC+N) (see 4.3.2), which consist of about 30% immature DCX-EGFP-positive neurons and about 70% neural stem/progenitor cells (see 4.3), termed hES-NSC70+N30 should be compared to a third population consisting of 30% neural stem/progenitor cells and 70% immature DCX-EGFP-positive neurons. To generate such a population 70% of the hES-N was artificially mixed together with 30% of proliferating nestin positive NSCs, further named: hES-NSC30+N70. HES-NSC30+N70 were deposited on the enthorinal cortex of hippocampal slices, as performed in 4.3.2 (n=4). After 7 days in culture, the hES-NSC30+N70 showed migration of the EGFP-positive neurons towards various regions of the slice including the pyramidal cell layer (Figure 4.13 b,e). The migration of hES-NSC30+N70 on the hippocampla slice was compared to the migration of hES-N and hES-NSC70+N30. This comparison revealed that hES-N showed the strongest single cell migration of EGFP-positive neurons away from the transplantation site (Figure 4.13 e-f). Whereas some migration was observed in the case of hES-NSC30+N70 (Figure 4.13 c-d),

hardly any single cell migration could be detected in the group of the hES-NSC70+N30 (Figure 4.13 a-b).

To quantify the difference in the migration rate for the three populations, EGFP-positive neurons were counted that had migrated within the hippocampal slice reaching a depth of 175 µm and 350 µm in the z-axis per mm^2. Whereas an average of 1.25±0.9, 4.75±2.87 and 13.44±7.7 of hES-NSC70+N30, hES-NSC30+N70 and hES-N reached a depth of 175 µm, respectively (Figure 4.13 g), none of the neurons within the hES-NSC70+N30 group but 1.25±1.2 of the hES- NSC30+N70 and 6.5 ± 4.39 of the hES-N migrated to a depth of 350 µm per mm^2.

To complete this slice experiment a final mixing ratio of 50% hES-N together with 50% proliferating nestin positive NSC was transplanted. Following 7 days in culture, migration of EGFP-positive neurons out of this mixed cell population was indistinguishable from the migration of hES-NSC70+N30 transplanted cells. These data indicate that an increase in neural stem/progenitor cell content within the neuronal/neural cell mixtures corresponds to a decrease in neuronal migration out of the transplantation site. As few as 30% of neural stem/progenitors cells appear sufficient to significantly reduce neuronal migration from the transplantation core.

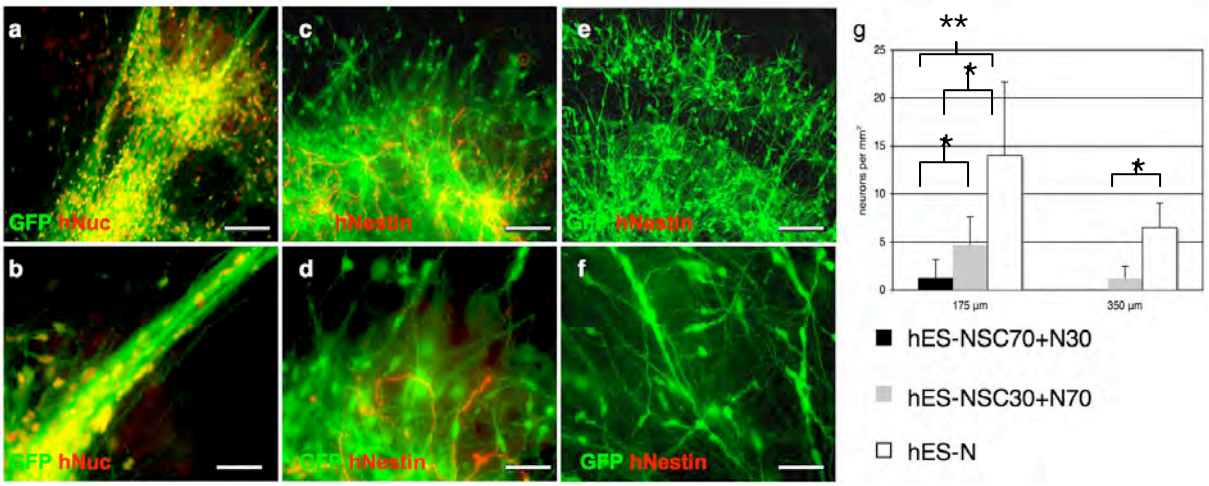

Figure 4.13: Migration of neurons out of "mixed populations". Migration 7 days after transplantation on hippocampal rat slice culture of **(a-b)** hES-NSC70+N30, **(c-d)** hES-NSC30+N70 and **(e-f)** hES-N. **(g)** Quantification of the total number of cells reaching migration depths of 175- and 350 µm in the z-axis per mm^2 within hES-NSC70+N30 (black), hES-NSC30+N70 (grey) and hES-N (white). Bars represent the mean fraction of DCX-EGFP-positive migrated neurons in the different cell populations (* $P < 0.05$; ** $P < 0.01$). Scale bars: a,c,e 100 µm, b,d,f 50 µm.

A similar result was observed following transplantation of the hES-NSC30+N70 (consisting of 70% purified neurons and 30% proliferating, nestin positive NSC) into the striatum of adult rats, as performed in 4.3.3 (n=4). The total number of EGFP-positive neurons in the corpus callosum was quantified eight days post transplantation (see 3.8.2) and compared to the

number of EGFP positive neurons in the corpus callosum from the transplanted hES-N, as well as from the transplanted hES-NSC70+N30. None of the neurons of the hES-NSC70+N30 versus 31.3±7.64 neurons of the hES-NSC30+N70 versus 534±108.70 neurons of the hES-N were found in the corpus callosum (Figure 4.14). Notably, the decrease in cell numbers of EGFP positive cells in the corpus callosum of hES-N, correlates with the increase in the fraction of neural stem/progenitor cells within the transplanted cells.

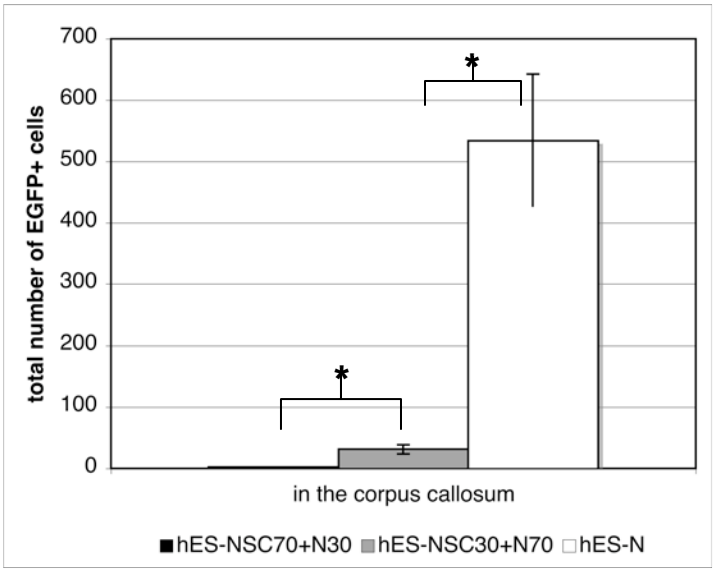

Figure 4.14: *In vivo* migration of neurons out of "mixed populations". Quantification of transplanted hES-NSC70+N30 (black), hES-NSC30+N70 (grey) and hES-N (white) found in the corpus callosum 8 days post transplantation. Bars represent the mean fraction of migrated cells in the different cell populations (* $P < 0.01$).

4.4.3. Soluble factors with chemoattractive effect on immature neurons

The previous results suggest that neural stem/progenitor cells attract immature neurons while neurons do not seem to show attraction to each other. The results from the Boyden chamber migration assay (see 4.4.1) further indicate that the attraction is due to at least one soluble factor. Therefore, the response of neurons to several soluble factors known to be present in the NSC niche, or described to be neuronal chemoattractants were tested. More specifically, DCX-EGFP-positive neurons purified by FACS (hES-N) were plated in the upper well of a Boyden chamber and either medium (as negative control) or plated lt-hESNSC (as positive control) or agarose beads pre-soaked with BDNF, EGF, SDF1, SCF, PDGF, FGF2 or VEGF were placed in the bottom well (see 3.7.1). In this experiment hES-N showed pronounced migration towards beads releasing PDGF, FGF2 and VEGF and towards plated lt-hESNSC (Figure 4.15). Migration of neurons was less pronounced in the presence of other factors or medium control.

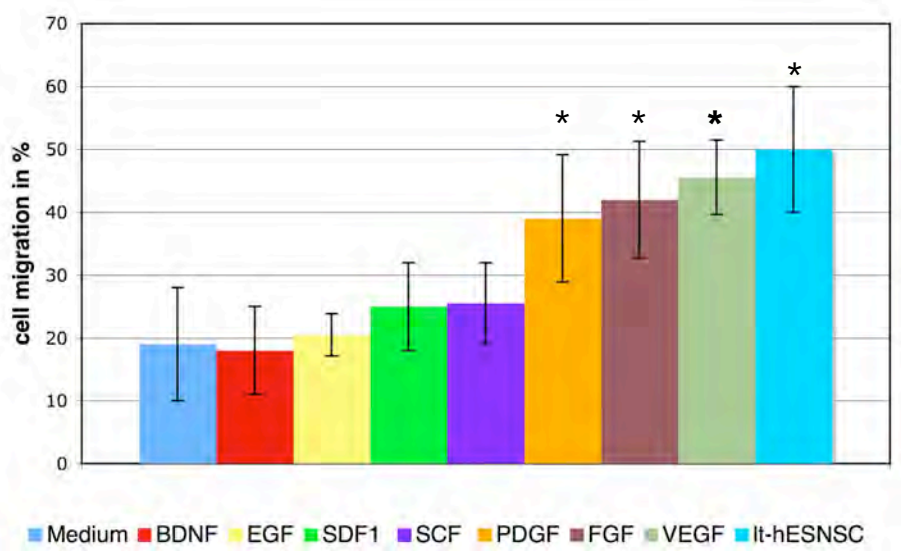

Figure 4.15: Mini-chamber migration assay. Migration of hES-N monitored after 20 hours in culture towards either medium or plated lt-hESNSC or agarose beads pre-soaked with BDNF, EGF, SDF1, SCF, PDGF, FGF2 or VEGF. Bars represent percentage of migrated neurons (* $P < 0.03$; Statistical significance was determined in relation to medium control).

4.4.4. Expression profile of chemoattractants and their receptors in neural stem/progenitor cells and immature neurons

To determine whether the previously tested chemoattractants and their respective receptors are expressed by neural stem/progenitor cells and/or immature neurons, RT-PCR using RNA isolated from neural stem/progenitor cells and hES-N, respectively were performed. The expression profile revealed that neural stem/progenitor cells strongly express transcripts for BDNF, EGF, SCF, PDGF (isoforms B, C and D), FGF2 and VEGF (Figure 4.16 a). In contrast hES-N showed weak expression of the transcripts for EGF, SCF, PDGF (isoforms A, B, C and D), FGF2 and VEGF and a strong expression for BDNF. Furthermore, hES-N strongly express transcripts for the BDNF receptor trkB, the SCF receptor c-kit, the PDGF receptors α and β, the FGF receptors 1 to 4 and the VEGF receptors Flt1, Flk/KDR and Flt4, as well as the co-receptor NRP1 (Figure 4.16 b). With exception of PDGF receptor α and β, FGF receptor 4 and the VEGF receptor Flk/KDR, transcripts for the above mentioned receptors were also expressed by the neural stem/progenitor cells (Figure 4.16 b). These data show that several soluble chemoattractants are expressed in neural stem/progenitor cells, whereas neurons express the receptors associated to these factors. Considering the significant chemoattractive effect of VEGF, FGF2 and PDGF observed in the chamber migration assay (Figure 4.15), these factors represent interesting candidates to test for a role in core formation of progenitor-containing neural grafts.

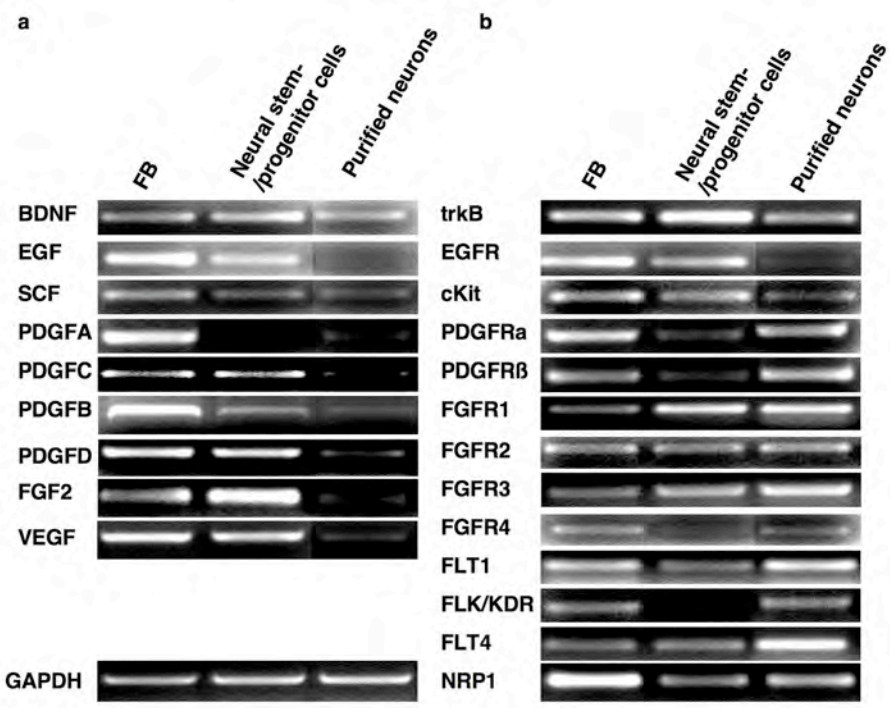

Figure 4.16: Expression of chemoattractants and their respective receptor sets in neural stem/progenitor cells and immature neurons. RT-PCR analysis of the expression of the RNA transcript for **(a)** chemoattractants and **(b)** the respective receptor sets in neural stem/progenitor cells and "purified neurons" (hES-N). Human fetal brain tissue (19 weeks of gestation) (FB) served as control.

4.4.5. Interaction with chemoattractants expressed by neural stem/progenitor cells *in vitro*

To assess whether FGF2 and VEGF play a role in preventing migration of immature neurons out of a cell mixture with neural stem/progenitor cells, different agents known to interfere with these signaling pathways were tested. SU5402 is a known pharmacological inhibitor of the tyrosine kinase activity of FGF receptor 1, as well as FGF-induced phosphorylation of the downstream kinases ERK1 and ERK2 (Mohammadi *et al.*, 1997). To interfere specifically with the VEGF pathway an anti-human VEGF receptor 2 antibody (VEGF R2-AB), known to neutralize the bioactivity of VEGF receptor Flk/KDR (Ferrara & Davis-Smyth, 1997) was chosen. Furthermore the recombinant human protein endostatin, a cleavage product of collagen XVIII (Marneros & Olsen, 2005), which has been reported to have anti-angiogenesis effects (O'Reilly *et al.*, 1997; Marneros & Olsen, 2001) and which inhibits endothelial cell migration in response to FGF2 and VEGF (Eriksson *et al.*, 2003) was tested, although, no effect on neural cells was so far reported.

To study whether any of these three molecules (SU5402, VEGF R2-AB or endostatin) had an effect on the neuronal migration of immature neurons in the presence of neural stem/ progenitor cells, Boyden chamber assays were performed. HES-NSC70+N30 which pre-

incubated for 30 minutes in 30 µl Cytocon™Buffer II with either 20 µM SU5402, 10 µl VEGF R2-AB stock solution (see 2.5.1), 2 µl endostatin stock solution (see 2.5.1) or with the equivalent solvents, were placed in the upper well. The lower wells contained medium and either plain agarose beads or agarose beads soaked with PDGF as attractant. A significantly enhanced migration of immature neurons towards media was observed when pre-incubating the cell mixture with the VEGF R2-AB or endostatin. The chemoattractive gradient, which was caused by the agarose beads releasing PDGF, additionally enhanced the effect of the VEGF-R2-AB and endostatin pre-incubation (Figure 4.17). In contrast the SU5402 did not show a significant effect on the migration of immature neurons compared to the untreated control cell population.

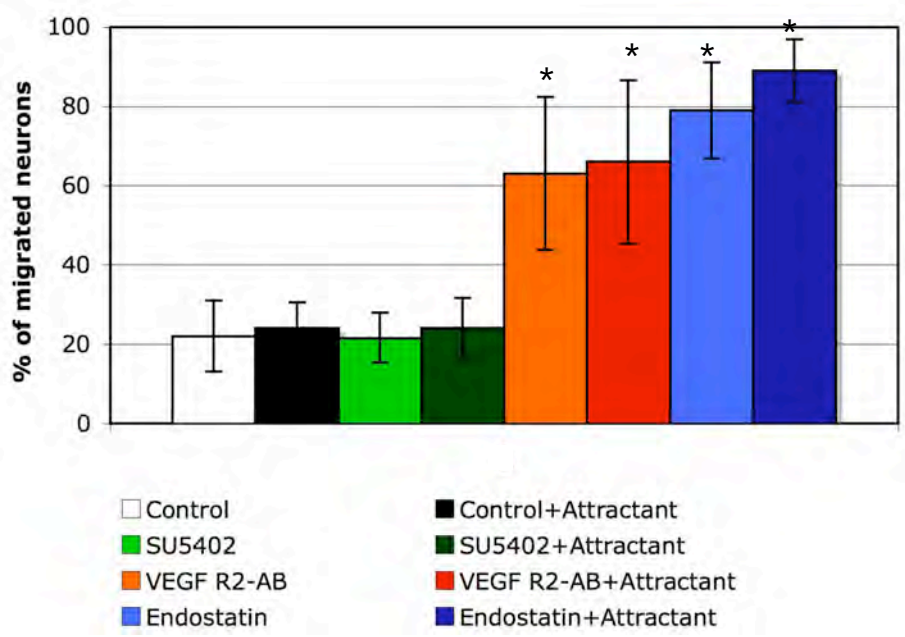

Figure 4.17: Mini-chamber migration of hES-NSC70+N30 in the presence of SU5402, VEGF R2-AB or endostatin. Bars represent the percentage of migrated neurons after 20 hours in culture towards medium or a chemoattractant either with or without SU5402, VEGF R2-AB or endostatin treatment. Cell numbers were normalised to the number of EGFP positive plated cells (* $P \leq 0.02$; statistical significance was determined in relation to the control).

The data of this Boyden chamber assay suggest that interfering with chemoattractive factors expressed by neural stem/progenitor cells can partially influence the auto-attractive effect of a mixed neural/neuronal population.

4.4.6. Interaction with chemoattractants expressed by neural stem/progenitor cells on hippocampal rat slice cultures

The previous results showed that pre-incubation of hES-NSC70+N30 with endostatin resulted in the most prominent enhancement of migration in a Boyden chamber assay (see 4.4.4). To further investigate whether endostatin can also interfere with the chemoattractive effect of neural stem/progenitor cells on immature neurons in a context closer to an *in vivo* situation, hippocampal rat slice culture experiments were performed. HES-NSC70+N30 were pre-incubated for 30 minutes in 30 µl Cytocon^{TM}-Buffer II with either 2 µl stock solution endostatin (n=9) or with 2 µl of the solvent of endostatin (citric-phosphate buffer) as control (n=9), and then deposited on the entorhinal cortex of hippocampal slice cultures. HES-N were transplanted as positive control (n=9). In this experiment, slices were further cultured for 18 days after deposition of the cells as a clearer significant difference of migration was expected with extended co-culture duration. 1 µl/ml endostatin stock solution was continuously applied every day to the slice media. When analyzing the cultures at day 18, endostatin treated cells showed an enhanced migration horizontally over the slice, compared to the control population. Yet this migration appeared more limited than the migration of purified neurons (Figure 4.18 a,c,e). Migration of single EGFP-positive neurons up to 800 µm away from the transplantation core was frequently observed in the endostatin treated transplants, whereas the cells from the untreated control transplants never migrated further than 500 µm from the transplantation core. This enhancement of migration was also present along the z-axis, as the endostatin treated cells frequently reached depths of 350 µm (Figure 4.18 d) whereas the non-treated cells never exceed 200 µm (Figure 4.18 b). These data, although preliminary, suggest that interference with the chemoattraction between neural stem/progenitor cells and immature neurons via applying endostatin might indeed enhance the migration and integration capacity of mixed neural grafts. However, when compared to hES-N only, the migration and integration capacity of endostatin treated cells appeared to be only mildly enhanced, indicating that by solely applying endostatin, a comparable enhancement of migration as seen in the hES-N might not be achievable (Figure 4.18 e-f).

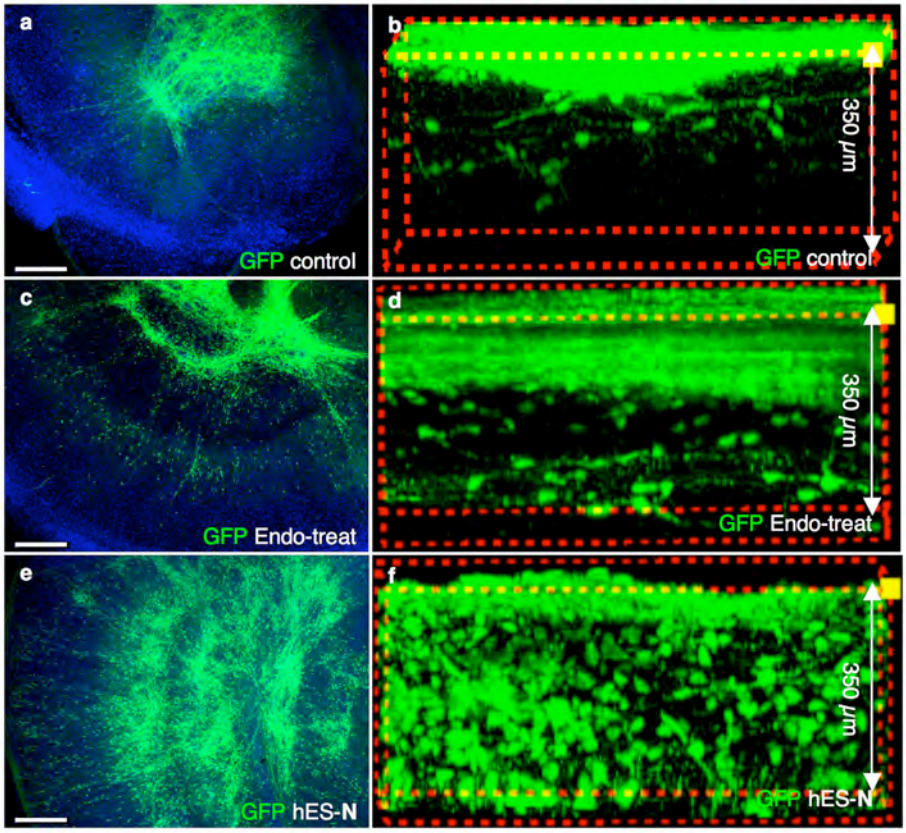

Figure 4.18: Neuronal migration of a hES-NSC70+N30 control population, a hES-NSC70+N30 endostatin treated population and hES-N on hippocampal rat slice cultures. 18 days after transplantation **(c,d)** endostatin treated cells showed an enhanced migration and integration capacity as compared to **(a,b)** control cells both in the xy- as well as the z-axis. However, migration as observed in **(e,f)** hES-N could not be achieved. Slices (a,c,e) were DAPI and EGFP stained post fixation. Scale bars: a,c,e 500 µm.

5. DISCUSSION

5.1. Genetic lineage selection of hES cell derived neurons

A fundamental prerequisite for CNS cell replacement strategies is the generation of donor cells in high purity to avoid the contamination risk with insufficiently differentiated cells leading to tumour formation. In order to achieve this, tools for the generation of pure lineage specific cell types, or of specific postmitotic sub-types need to be developed. In the present study a doublecortin-based lineage selection approach has been established for the enrichment of human ES cell derived immature neurons.

5.1.1. Surface bound versus genetic lineage selection

Several markers have been so far explored as potential lineage selection candidates for the isolation of specific cell types. Surface bound epitopes are widely used for the selection of a cellular subtype, especially in the field of the hematopoietic research (Herzenberg *et al.*, 2002; Perez & Nolan, 2006). Immuno-isolation of neural lineages has successfully been applied to neurons (Jungling *et al.*, 2003; Schmandt *et al.*, 2005) and glia (Malatesta *et al.*, 2000) in the mouse system. Recently, Pruszak and colleagues published a comprehensive analysis on the applicability of surface marker-based selection of human embryonic stem cell-derived neural lineages (Pruszak *et al.*, 2007). Application of surface markers for lineage selection has several advantages, for instance the isolation of cell types without genetic manipulation, and the easy application to any cellular source. However, most of the known surface markers lack specificity. For example N-CAM is expressed in neural progenitors and neurons but also in other cell types such as hematopoietic natural killer (NK)-cells or neuroendocrine cells (Seldeslagh & Lauweryns, 1997; Koch *et al.*, 2006; Pruszak *et al.*, 2007). NG2 is expressed in oligodendrocyte progenitors (Polito & Reynolds, 2005) but also in melanocytes (Lim *et al.*, 2007). CD133 is expressed in NSCs (Uchida *et al.*, 2000) but also in several stem cells of different origin (Jaatinen *et al.*, 2006). Moreover, the surface marker-based selection strongly depends on the specificity of the individual antibodies and is associated with high costs when facing large-scale applications.

Genetic lineage selection either by a fluorescent protein or by an antibiotic resistance gene is another widely used method. Transgenic murine ES cells and mouse strains harbouring a marker gene have been successfully employed to monitor and isolate multiple cell types including neuroepithelial progenitors (Aubert *et al.*, 2003), neurons (Wernig *et al.*, 2002) or defined neuronal subtypes (Wichterle *et al.*, 2002; Thompson *et al.*, 2006). Random insertion of specific promoter-EGFP constructs proved to be a reliable tool for lineage selection in a

cell culture based system (Roy *et al.*, 2000a; Wang *et al.*, 2000; Glaser *et al.*, 2005; Schmandt *et al.*, 2005). On the one hand, the transgene-based lineage selection has the disadvantage that cells need to be genetically manipulated and thereby carry artificial genome alterations. On the other hand, the beauty of this system lies in its reliability and simplicity once established. Furthermore, genetic lineage selection has proven to be highly specific and led to isolation of lineages, cell-types and even defined cellular sub-types (Herzenberg *et al.*, 2002; Goldman, 2005).

5.1.2. Doublecortin as candidate marker for the selection of immature neurons

The present study investigated and further characterized a candidate marker for a genetic lineage selection approach to specifically isolate immature neurons derived from human ES cells. Doublecortin (DCX), a 40 kDa microtubule-associated protein (Francis *et al.*, 1999; Gleeson *et al.*, 1999; Taylor *et al.*, 2000) appeared to be an interesting potential candidate marker, as it is known to be exclusively expressed in newborn and immature neurons in the developing and adult brain *in vivo* (see 1.2.3) (Chelly, 1998; Gleeson *et al.*, 1998; LoTurco, 2004; Rao & Shetty, 2004; Couillard-Despres *et al.*, 2005; Bernreuther *et al.*, 2006). In addition, DCX is one of the earliest markers for neuronal differentiation (Magavi & Macklis, 2008), and thus offers the possibility to identify and isolate neurons in a very early developmental state. The immature state of neurons is particularly important for a lineage selection approach because of several reasons. First of all, recent studies showed that only immature neurons survive the mechanical manipulation, which is fundamental for FACS-based isolation, i.e. enzymatic digestion to single cell suspensions, and pressure during the sorting procedure (Pruszak *et al.*, 2007). Secondly, this differentiation state is important in the context of reconstructive approaches, as only immature neurons possess sufficient plasticity to survive and integrate after transplantation (Bjorklund & Lindvall, 2000; Brederlau *et al.*, 2006).

In this study, the exclusive expression pattern of DCX in immature neurons could be confirmed in the human ES cell-based cell culture paradigm of lt-hESNSC (see 1.1.3). This was shown by the co-expression of DCX with markers expressed in immature neurons such as beta-III tubulin, the lacking co-expression with the neural progenitor marker nestin and the barely detectable co-expression with the more mature neuronal marker NeuN (see Figure 4.1). Additionally, GFAP-positive astrocytes or O4-positive oligodendrocytes did not express DCX. These data revealed that the observed dynamics of DCX expression in an *in vitro* cell culture system largely resemble the highly specific expression pattern of DCX in immature neurons *in vivo*.

5.1.3. Establishment of a DCX-EGFP lineage selection system

Based on the specific expression of DCX in lt-hESNSC derived immature neurons, a lineage selection system using DCX has been established. As DCX is an intracellular marker, surface based immuno-isolation could not be applied. Instead, a construct carrying an EGFP under the control of a 3.5 kb human DCX promoter sequence (kindly provided by Sebastien Couillard-Després and Ludwig Aigner, see 2.4) was used to generate a DCX-EGFP lt-hESNSC reporter cell line. One limitation of stable transfection of cell lines with constructs carrying a minimum consensus promoter sequence is the frequently observed background expression of the reporter gene due to random insertion into the genome (Glaser *et al.*, 2005; Schmandt *et al.*, 2005). In contrast to e.g. large constructs carrying up to 250 kb of the DNA sequence around the promoter region such as bacterial artificial chromosomes (BACs), the expression specificity of these small reporter constructs is strongly influenced by the locus they integrate in. In line with this, unspecific EGFP expression was observed in most of the clones generated in this study. Careful characterization of the clones to show the faithful co-expression of the DCX protein and the EGFP transgene was therefore an essential prerequisite for the further applicability of the established cell lines. The comparative analysis of the expression of endogenous DCX and EGFP identified four clones which faithfully reflect an immature neuronal phenotype by the EGFP transgene expression (see Figure 4.2). The established clones showed to have a constant proliferation and differentiation potential over multiple passages and could be frozen in liquid nitrogen without loss of their specific EGFP transgene expression.

Although FACS for neural cells has been used in studies of animal models and even for hES cell derived neurons (Pruszak *et al.*, 2007) it has not been standardized for neuronal cell types most likely due to the problems in using such fragile cells. An optimal model should guarantee high efficiency, high purity as well as maximum post FACS survival. As discussed before the broad expression of DCX in an immature neuronal state was a major prerequisite. Limited efficiencies and restricted survival observed in other studies were often due to the fact, that the chosen markers stained later developmental states or just subsets of neurons (Dittgen *et al.*, 2004; Hedlund *et al.*, 2007). However, also within the DCX-EGFP system efficiencies had to be traded against the survival. Longer differentiation times of the DCX-EGFP lt-hESNSC reporter cell line resulted in an increase in DCX-EGFP-positive cells but also increased the maturation stage of the DCX-EGFP-positive neurons. Thus the appropriate time point of differentiation had to be defined at which DCX-EGFP-positive cells were present in sufficient numbers, yet still immature enough to survive the mechanical manipulation associated with the sorting procedure. Differentiation studies suggested day

8±1 as an optimal time window as at this time point in fact, on average 31±2.8% of the cells expressed the DCX-EGFP transgene, while less than 50% of them co-expressed the more mature neuronal marker MAP2ab (see 4.1.3). At this time point the sorting procedure was highly efficient. Starting by day 8±1 of differentiation from three to four 10 cm cell culture plastic dishes with a total of 8 x 10^7 cells, a typical FACS session of 4 to 5 hours yielded more than 2 x 10^7 purified neurons. Sorted cells were amenable to replating; thus they survived the sorting procedure. Characterisation of replated neuronal cells showed that over 95% of them co-expressed beta-III tubulin and EGFP, indicating that also high purity could be achieved by the DCX-EGFP based lineage selection.

Thinking about further applications of a DCX-EGFP lineage selection such as transplantation studies with follow up times of several months, one limitation of a DCX-EGFP reporter line is that the EGFP transgene might be down-regulated during neuronal maturation *in vivo*, making it more difficult to identify transplanted cells thereafter. To overcome this problem a double reporter line was established expressing EGFP under control of a ubiquitous promoter and DsRED2 under control of the DCX promoter (DCX-dsRED2:PGK-EGFP). The DCX-dsRED2:PGK-EGFP reporter line also showed faithful overlap of the dsRED2 reporter and the endogenous DCX protein. Most importantly DCX-DsRED2 positive cells could be distinguished by FACS from only EGFP-positive cells and DCX-dsRED2 positive neurons could be isolated in comparable purity to DCX-EGFP-positive neurons (Figure 4.4).

5.1.4. Characterization of the DCX-EGFP purified neurons

For further use of the DCX-EGFP purified neurons in various biological and pharmacological applications it was of major importance to control that neither the genetic manipulation, nor the FACSorting had an influence on the characteristics and the functional properties of the purified cells. To that end immunocytochemistry-based phenotype analysis and functional characterisation of DCX-EGFP purified neurons were required.

Immunocytochemistry-based phenotype analysis of the purified neurons showed that most neurons acquired a GABAergic phenotype. The huge proportion of GABAergic differentiation is in agreement with the notion that growth factor-expanded neural precursors tend to acquire a mainly GABAergic phenotype (Jain *et al.*, 2003; Conti *et al.*, 2005; Koch *et al.*, 2006; Zhang, 2006) and with the data of Koch and co-workers (Koch *et al.*, in revision) who in detail investigated in the differentiation pattern of lt-hESNSC. Thus neither the genetic manipulation of the lt-hESNSC with the DCX-EGFP construct nor the FACSorting seemed to significantly influence the neuronal phenotype of the cells. However, it would be of major interest whether other neuronal phenotypes such as glutamatergic neurons, serotoninergic neurons,

dopaminergic neurons or motoneurons could be isolated using the same parameters established for the mainly GABAergic cells. Future experiments using extrinsic morphogens or intrinsic key transcription factors to guide lt-hESNSC towards these specific neuronal subtypes in combination with the DCX-EGFP lineage selection approach might then also facilitate the isolation of these important disease-related cell types.

In addition to the immunocytochemical analysis, functional characteristics of the selected cells were assayed using whole-cell patch-clamp recordings. The analysis revealed that the purified neurons were able to develop into mature and functional neurons. They expressed sodium and different kinds of potassium channels, were able to generate repetitive action potentials upon long-lasting depolarisation and displayed surface expression of AMPA/kainate and $GABA_A$ receptors as a prerequisite for the formation of glutamatergic and GABAergic synapses and evoked PSCs. After placing the sorted neurons onto hippocampal rat slices, a paradigm, which promotes long-term survival and functional maturation of ES cell-derived neurons (Benninger *et al.*, 2003) postsynaptic currents could be identified indicating functional connections with the slice. These studies revealed that genetic manipulation and FACSorting do not impair the functional properties of the cells. Interestingly, mature ion channel and repetitive action potential generation could be demonstrated as early as four weeks post sorting. Long maturation times of human ES cell derived neurons are a major problem for studying neuronal networks *in vitro*. Recent publications show that network generation is depending on mature astrocytes and typically requires eight weeks after the initiation of differentiation by growth factor withdrawal (Johnson *et al.*, 2007; Wu *et al.*, 2007). To facilitate long-term survival, DCX-EGFP selected neurons were also placed on astrocytic layers or hippocampal slice cultures. However, network integration and postsynaptic currents were detectable as early as four weeks after sorting (i.e. five weeks after growth factor withdrawal) in 8 out of 9 cells. Further experiments are needed to consolidate this observation. One might speculate that the very early appearance of mature functional properties is due to the fact that DCX-EGFP selected cells were placed on the slices as pure neuronal population, which might accelerate maturation.

In conclusion, a DCX-EGFP based lineage selection system is a robust method to isolate immature neurons out of differentiating lt-hESNSC cultures and the purification of DCX-EGFP-positive neurons does not impair the functional properties of the cells.

5.2. Efficient cryopreservation of purified human neurons

For subsequent applications of hES cell-derived neurons in neural restoration or pharmacological screening, it would be desirable to have purified young neurons stored and available on demand. Unfortunately, mature neurons are profoundly sensitive to cryopreservation. The immature state of the selected DCX-EGFP-positive neurons was an important prerequisite for their amenability to cryopreservation.

Commercially available freezing media only led to moderate post freezing survival of the cells. To optimize cryopreservation of DCX-positive neurons a formulation was composed which was supplemented with myo-inositol and polyvinyl alcohol. Myo-inositol is found in cyanobacteria, algae, fungi and plants, where it functions as an osmolyte to tolerate or avoid freezing (Kostal & Savory, 1996; Yancey, 2005). Polyvinyl alcohol is well known to prevent or delay ice nucleation (Asada *et al.*, 2002). However, even with the optimized freezing medium post-freeze survival remained < 48%, and many cells did not survive the first 24 hours after thawing. Thus, pre-incubation of the cells with the general caspase inhibitor z-VAD-fmk was included, which resulted in a further increase in post-thaw cell survival rate. Apoptosis mediated through a caspase-dependent mechanism has been shown to be responsible for a significant cell loss following cryopreservation of hES-cells (Heng *et al.*, 2006; Heng *et al.*, 2007). In addition, NSCs undergo apoptosis via the mitochondrial pathway with caspase-3 serving as the executioner caspase in the apoptotic machinery (Sleeper *et al.*, 2002). Although the general caspase inhibitor z-VAD-fmk used in this study has a half-life of less than 24 hours in cell culture media (Alessandri *et al.*, 2006), this was sufficient to decrease caspase-3 and -7 activity in the z-VAD-fmk pre-treated cells as measured in the caspase-3/7 assay performed 20 hours post thawing. Thus the general caspase inhibitor z-VAD-fmk might protect the cells from undergoing apoptosis during the cryopreservation process and within the first hours after thawing and plating. However, pre-incubation with a pharmacological caspase inhibitor might also impair possible read-outs in pharmacological studies. To analyze whether the caspase inhibition is temporary, staurosporine treatment of the cells was applied four days after thawing, showing no difference in induced apoptosis between z-VAD-fmk-treated and non-treated cells. Therefore, the z-VAD-fmk treatment should not impair pharmacological toxicity studies in cryopreserved hES cell-derived neurons. Furthermore, cryopreserved, thawed and replated neurons co-expressed beta-III tubulin and EGFP and, most importantly, maintained functionality after thawing and further culturing, comparable to their non-cryopreserved counterparts. Another important aspect for further application is that cryopreserved and thawed neurons survive transplantation into the neonatal rat brain. Transplanted DCX-EGFP expressing cells showed distribution in a variety of host brain

regions where they appeared to morphologically integrate. Furthermore, they displayed a neuronal phenotype with delineation of neurites (see 4.2.2). Thus, cryopreservation of DCX-EGFP-purified postmitotic immature neurons appears to be highly efficient and does not impair the functional properties of the cells. Further electrophysiological studies are necessary to really prove that the cryopreserved neurons can develop functional properties *in vivo* and integrate into the existing circuitries.

5.3. Enhanced migration of human neurons as pure population

In the developing as well as in the adult brain DCX expression is closely associated with an immature migratory neuronal phenotype (Chelly, 1998; des Portes *et al.*, 1998; Gleeson *et al.*, 1998; Francis *et al.*, 1999; LoTurco, 2004; Rao & Shetty, 2004; Couillard-Despres *et al.*, 2005; Bernreuther *et al.*, 2006). Analysis of genetically induced malformations in the mammalian brain showed that DCX plays an essential role in the regulation of neuronal migration, as mutations of this gene cause disorganization of the layers in the cerebral cortex (see 1.2.3) (Feng & Walsh, 2001). Thus, an interesting question was whether the DCX-EGFP purified human neurons have a strong migration potential. Indeed, when studying the migration capacity of DCX-EGFP-positive neurons in a variety of *in vitro* and *in vivo* migration assays, DCX-positive neurons appeared to be highly migratory.

5.3.1. Migration of DCX-EGFP positive neurons as pure population and within a neural stem/progenitor cell containing population

In a matrigel and transfilter-based migration assay DCX-EGFP purified neurons (hES-N) showed strong migration towards the bottom well of the transwell filter and distributed across a large area on the gel matrix. In contrast, the non-purified control population (hES-NSC+N) - consisting of about 30% DCX-EGFP-positive immature neurons and 70% other cell types, most of which expressing nestin - displayed only limited migration in the transwell filter assay and formed sphere-like cell clusters on the gel matrix (see Figure 4.9). This observation was interesting, as it indicates that DCX-EGFP-positive neurons have an enhanced migratory potential as a purified population only. In contrast, the corresponding DCX-EGFP-positive neurons within the non-purified control population (hES-NSC+N) did not show such a strong migratory behaviour although they should in principle have the same migration potential as the purified neurons.

Similar results were received when transplanting hES-N or hES-NSC+N on hippocampal rat slice cultures. The hES-N showed strong migration and integration potential whereas the

hES-NSC+N only displayed limited migration and formed the already in the matrigel assay described sphere-like clusters with axonal outgrowth only (see Figure 4.10).

To ensure that the observations from the *in vitro* migration assays were not only *in vitro* cell culture artifacts, *in vivo* migration studies following transplantation of hES-N and hES-NSC+N into the brain of adult rats were performed. Several previous studies analysed the migration and integration potential of hES cell-derived neurons *in vivo*. Former studies, in which hES cell-derived neural cells were transplanted into rat brains, described clusters of donor cells at the site of engraftment one week after transplantation, the so called transplantation cores (Reubinoff *et al.*, 2001). Tabar (Tabar *et al.*, 2005) and co-workers investigated *in vivo* migration of hES cell-derived neural precursors transplanted into the rostral migratory stream of adult rats and found that about one fourth of the transplanted cells migrated out of the transplantation core within 11 weeks. In comparison, when transplanting DCX-EGFP purified cells into the striatum of adult rats, a large amount of the cells migrated out of the transplantation core within 8 days. Similar results have been achieved following transplantation into the rostral migratory stream, where transplanted neurons morphologically orientated to and migrated towards the olfactory bulb within 8 days (see Figure 4.11). However, when a mixed population (hES-NSC+N) was transplanted it was observed that the cells formed densely packed clusters at the transplantation site with only restricted migration of DCX-EGFP-positive neurons out of the transplantation core.

Cluster formation and limited migration and integration have been topics in neurobiological research for many years. A major challenge in therapeutic transplantation of donor cells for neural damage repair is to achieve functional integration of the donor cells into the host tissue. Limited integration due to restricted emigration of the transplanted cells, which mainly remain located at the grafted site (Guzman *et al.*, 2008) is a widely discussed issue (see 1.1.3) and described in many different studies using primary cells or ES cell derived neural progeny (Fricker *et al.*, 1999; Tabar *et al.*, 2005; Roy *et al.*, 2006). It is argued that this core formation of neural transplants is due to physical or molecular barriers caused by glial scarring at the lesion site following transplantation (Reier *et al.*, 1983; Rudge & Silver, 1990). Successful axonal outgrowth is known to be associated with minimal up-regulation of proteoglycans within the extracellular matrix of reactive glial cells at the transplantation site (Davies *et al.*, 1997). This might also restrict migration of transplanted neuronal progenitors. It was suggested that the up-regulation of proteoglycans might be avoided by using micro-transplants that minimize scarring by injecting minimal volumes of dissociated cells (Nikkhah *et al.*, 1995; Davies *et al.*, 1997). Glial scarring at the transplantation site is, however, not the only possible explanation for the formation of transplantation cores. As in the transplantation paradigm used in the present study, the total number of transplanted hES-N or hES-NSC+N

cells was equal, as was the transplantation methodology. The intriguing question arising at this point was why immature neurons only migrate as purified populations, whereas they display only very restricted migration when mixed with other cell types, most of which expressing nestin. As there must be factors involved in causing core formation other than transplantation size or glial scar formation, one potential factor might be direct or indirect cell-cell-interactions between the different transplanted cell types.

5.4. Mechanisms causing core formation of neural stem/progenitor cell containing transplants

Experiments performed in the context of this study provided first evidence that one possible putative mechanism underlying cluster formation and restricted emigration of donor neurons from neural stem/progenitor-containing grafts might be chemoattractive interactions between the transplanted neural stem/progenitor cells and immature neurons. *In vitro*, human neurons showed a pronounced migration towards undifferentiated lt-hESNSC in a transfilter migration assay (see Figure 4.12) indicating that lt-hESNSC express at least one soluble factor having a chemoattractive effect on neurons. In addition, migration assays of cell mixtures composed of neural/neuronal cells in different ratios in rodent CNS tissue revealed that the extent of neuronal migration away from the core appears to be highly dependent and inversely proportional to the number of neural stem/progenitor cells present in the transplanted population, with a proportion of about 30% neural stem/progenitors being sufficient to almost completely inhibit migration away from the transplantation site. According to these data it appears that interaction between neural stem/progenitors and neurons might indeed be one reason for the observed core formation in neural transplants. One possibility to avoid core formation would thus be to transplant purified immature neurons (hES-N). However, considering that most neural transplants from either ES cells or from fetal tissue contain a considerable amount of neural stem/progenitor cells, and taking into account that establishing a lineage selection system to purify immature neurons might not be feasible for primary cultures, it would be of great value to gain more insight into the mechanisms responsible for this suggested “auto-attraction” phenomenon. Once uncovered, interference with such auto-attractive mechanisms may be effectively used to enhance neuronal migration and integration of transplanted neural populations without the need of lineage selection.

5.4.1. Analysis of chemoattractive factors and associated receptors in neural stem/progenitor cells and immature neurons

Considering a possible interaction between *in vitro* generated neural stem/progenitor cells and immature neurons based on indirect cell-cell signalling, one might speculate that this signalling shares similarities to those signals present in the NSC niche. *In vivo* migration of immature progenitors out of the NSC niche is regulated by a complex interaction of intrinsic and extrinsic factors including repulsive and attractive cues (Fuchs *et al.*, 2004). The interplay of the extrinsic and intrinsic cues modulates the NSC niche, allowing NSCs to persist under physiological normal conditions and recruiting immature progenitors to a specific site under challenging physiological conditions (Scadden, 2006). Intrinsic factors and attractants, which function in the NSC niche *in vivo,* might also be involved in building up a "niche-like structure" within the grafts of *in vitro* generated NSCs. This might result in an chemo-attraction of the neurons by the neural stem/progenitor cells causing the observed core formation phenomenon. In the literature, many factors have been described to have a chemoattractive effect on neurons including VEGF (Zhang *et al.*, 2003), GDNF (Paratcha *et al.*, 2006) and FGF (Shirasaki *et al.*, 2006). The microenvironmental niche signals comprise a number of those factors including VEGF and FGF2 (Doetsch, 2003). VEGF was originally identified as a major mediator of angiogenesis (Carmeliet *et al.*, 1996). Recently VEGF was also shown to stimulate neurogenesis (Jin *et al.*, 2002; Fabel *et al.*, 2003; Cao *et al.*, 2004; Schanzer *et al.*, 2004) and to act as a chemoattractant for FGF2 stimulated neural progenitors (Zhang *et al.*, 2003). In addition, previous studies suggest that both vascular cells and glial cells which both express VEGF and FGF2 (Heine *et al.*, 2005) may serve as a niche for NSCs (Song *et al.*, 2002; Shen *et al.*, 2004).

In the context of this study different potential chemoattractants for immature neurons were tested in a Boyden chamber assay (see Figure 4.15). In addition, mRNA expression levels of the tested factors and the corresponding receptors were analyzed in neural stem/progenitor cells and immature neurons by RT-PCR (see Figure 4.16). At least two potential candidate factors of which one or both might be involved in the auto-attraction phenomenon observed between neural stem/progenitor cells and derived neurons were identified, namely FGF2 and VEGF. Those two showed high expression in the neural stem/progenitor population, high expression of the corresponding receptors in the DCX-EGFP purified neurons and had a significant chemoattractive effect on DCX-EGFP-positive neurons in the Boyden chamber migration assay.

5.4.2. Interference with the chemoattractive mechanisms between neural stem/progenitor cells and immature neurons *in vitro*

In order to enhance neuronal migration out of mixed neural/neuronal populations one might have to interfere with the suggested auto-attraction between neural stem/progenitor cells and immature neurons without completely inhibiting neuronal migration. This approach should ideally, on the one hand reduce the auto-attractive mechanisms that keep the neurons in the transplantation cores and inhibit their migration from the grafts and, on the other hand achieve that the neurons become more susceptible to other environmental attractants. Three different potentially interesting candidate molecules were tested. One was SU5402, a known inhibitor of the tyrosine kinase activity of FGF receptor 1 as well as FGF-induced phosphorylation of the downstream kinases ERK1 and ERK2 (Mohammadi *et al.*, 1997). The second candidate molecule was the anti-human VEGF receptor 2 antibody (VEGF R2-AB), which neutralizes the bioactivity of VEGF receptor Flk/KDR (Ferrara & Davis-Smyth, 1997) which could be detected in immature neurons by RT-PCR (see Figure 4.16). Finally, endostatin, a 20 kDa C-terminal proteolytic fragment of collagen XVIII (Marneros & Olsen, 2005) was tested. This compound has been found to be a potent inhibitor of angiogenesis. *In vitro*, endostatin specifically inhibits endothelial cell proliferation (O'Reilly *et al.*, 1997). *In vivo*, the systemic administration of recombinant endostatin resulted in growth inhibition or regression in a number of different tumour models (O'Reilly *et al.*, 1997; Bergers *et al.*, 1999; Dhanabal *et al.*, 1999; Shi *et al.*, 2002). Functionally, endostatin was also suggested to be an inhibitor of the FGF2 and VEGF chemoattractant-mediated cell migration of endothelial cells (Yamaguchi *et al.*, 1999; Eriksson *et al.*, 2003). However the exact mechanisms mediating these effects are barely understood. It was not yet clear whether endostatin is inhibiting the migration in response to FGF2 and VEGF only or chemoattractant-mediated migration in general. In addition, no data exists on whether endostatin has similar effects on neural cells. Thus, endostatin appeared to be an interesting candidate to be tested in the present work with regard to a possible inhibitory function in chemoattractant-mediated auto-attraction.

To analyse the role of the different compounds *in vitro*, the substances were applied in a transfilter migration assay in which neuronal migration out of a neural/neuronal cell mixture was measured (see Figure 4.17). This study revealed that neural/neuronal cell mixtures treated with SU5402 did not show enhanced neuronal migration. This can have different reasons. First of all, it might be that the attraction of immature neurons towards FGF2 is not mediated via the FGF receptor1. Secondly, SU5402 might also inhibit other pathways essential for migration. Finally, it could also be that FGF2 is only playing a minor role in the suggested auto-attraction phenomenon. Treating the cell-mixture with an antibody binding to

the VEGF receptor Flk/KDR significantly increased migration in a transfilter assay. This finding would support the hypothesis that VEGF is involved in the auto-attraction phenomenon and that the chemoattractive effect of VEGF is at least in part mediated via the VEGF receptor Flk/KDR. The strongest enhancement in migration could be monitored when applying endostatin (see Figure 4.17). The effect could be augmented by adding an attractant other than FGF2 or VEGF such as PDGF to the lower well. This suggests that endostatin does not interfere with chemoattractant-mediated migration of neural cells in general. However, the exact role of the protein in cell migration remains unclear. Hence, by interacting with the chemoattraction between neural cell populations and their neuronal progeny it might be possible to enhance neuronal migration and integration.

However, preliminary data on the effect of endostatin on transplanted neural/neuronal populations on hippocampal rat slice cultures showed an only mild enhancement in neuronal migration out of the transplantation core (see Figure 4.18). This might be explained by a balance between intrinsic cell-cell interaction restricting neuronal migration out of neural/neuronal mixed cell transplants and extrinsic factors such as attractive cues recruiting transplanted immature neurons to specific sites. Interacting with some of the intrinsic mechanisms only by e.g. endostatin treatment might not be sufficient to enhance neuronal migration out of a mixed neural/neuronal population in a tissue context. An additional attractor might be necessary to mediate migration away from the core. Previous studies already showed that implanted neural progenitors migrate towards a stroke region (Imitola, *et al.,* 2004) and that this migration was directed due to signals provoked by the stroke (Guzmann, *et al.,* 2007). One attractive experiment would therefore be the transplantation of a cell mixture of neural stem/progenitor cells and immature neurons in the vicinity of a lesion or stroke model *in vivo*. In such an experiment, the transplanted animals might be also treated systemically with endostatin. The lesion or stroke might provide sufficient attractants for the transplanted and endostatin treated cells to overcome the remaining auto-attractive effect, thereby, enabling enhanced targeted migration.

5.5. Perspective

The here developed protocol for the DCX-EGFP based lineage selection represents a useful tool for the production of infinite numbers of pure immature human neuronal cultures. These cells are promising candidates for future transplantation strategies, basic research, and model systems for screening approaches. The cell-based reconstruction within the CNS depends on neuronal precursors that have the potential to migrate and integrate into the existing neuronal circuitries. Their post-mitotic stage of differentiation minimizes the risk of

tumour formation, an issue that always has to be considered talking about ES cells as donor populations. The purified cultures could also be used to study gene expression patterns or biochemical pathways specifical for human neurons. In principle, the DCX-EGFP based neuronal lineage selection approach should also be applicable to other human ES cell-like cells. In this context, recently described alternative sources of pluripotent cells from adult germline (Conrad *et al.*, 2008) or of reprogrammed somatic cells (Takahashi & Yamanaka, 2006; Okita *et al.*, 2007; Wernig *et al.*, 2007) which have an attractive potential for regenerative medicine and pharmaceutical studies might further extend potential applications of this technology. As an example, applying the DCX-EGFP based lineage selection on a patient specific iPS cell line, which carries a gene alteration for a neurodegenerative disease might enable to study the disease pathogenesis specifically in altered human neurons.

Moreover, the DCX-EGFP lineage selection technique coupled with the newly established efficient cryopreservation protocol provides interesting perspectives for the use of DCX-EGFP-selected cells in screening functional effects of pharmacological compounds, as it opens the possibility for having functional human neurons on demand.

The availability of a pure neuronal population enables to investigate neuronal migration in a highly controlled manner. The findings that neural stem/progenitor cells inhibit neuronal migration and the identification of potential candidates involved in the underlying mechanism, will be of great value for the field of neuronal transplantation, possibly leading to protocols facilitating enhanced integration of cells in the host tissue. Endostatin, an antiangiogenetic drug, was suggested to enhance the migration potential of neuronal cells in a transfilter assay. As many statins are available, this group of drugs applied to the patient might also enhance the migration and integration of neural transplants *in vivo*. Further studies will be required to determine whether and to what extent neural stem cell cores can be avoided by interacting with the discovered cell-interaction, as well as to identify to what extent the migrating cells become functionally active, and acquire region-specific properties. As the discovered cell-interaction do not seem to be the only attractants functioning in core formation, additional pathways and/or receptors have to be investigated. While it is clear that application to different disease models is required before considering potential clinical applications, these data suggest that interacting with the auto-attraction in neural grafts might lead to methods enabling enhanced integration into the host tissue and therefore represents a promising approach for neural repair.

6. ABSTRACT

In this study, a human Doublecortin (DCX) promoter-based lineage selection strategy is presented for the generation of purified human embryonic stem (hES) cell-derived immature neurons. Stable transfection of long-term self-renewing hES cell derived neural stem cells (lt-hESNSC) with a neuronal specific DCX-EGFP construct and subsequent selection allowed the generation of clonal hES cell-derived long-term self-renewing neural stem cell lines, which show specific and abundant expression of EGFP exclusively in immature neurons. Fluorescence activated cell sorting (FACS) enabled the enrichment of DCX-EGFP-positive immature human neurons at purities of up to over 95%. Selected neurons were amenable to replating and functionally mature *in vitro*.

Considering that the applicability of purified hES cell-derived neurons would largely benefit from an efficient cryopreservation technique, defined freezing conditions were devised involving caspase inhibition, which enabled the storage of hES cell-derived neurons, and yielded post thawing survival rates up to 83%. The cryopreserved neurons were amenable to replating, and developed physiological properties comparable to their non-cryopreserved counterparts. Combined with the established lineage selection protocol, this cryopreservation technique enabled the generation of human neurons in a ready-to-use format for a large variety of biomedical applications.

Migration studies in transwell chambers and transplantation into rodent CNS tissue revealed an enhanced migratory and integration potential of the DCX-EGFP purified immature neurons. In contrast, transplants comprising neural stem cells (NSCs) or a mixture of neurons and neural stem/progenitor cells showed restricted migration into the host tissue, accompanied by core formation. Many components are involved in the cellular and molecular mechanisms of neuronal migration, such as the extracellular matrix, integrins, cell adhesion molecules, cell junctions as well as soluble factors and their receptors. Experiments performed in the context of this study provided first evidence that one possible underlying mechanism for the effect of cluster formation and restricted emigration of donor neurons from neural stem/progenitor-containing grafts might be chemoattractive interactions between the transplanted neural stem/progenitor cells and the immature neurons. In detail, human neurons showed a pronounced chemoattractive migration towards undifferentiated NSCs *in vitro*. In addition, the two populations expressed complementary sets of chemoattractants and their respective receptors. Moreover, first results indicated that interfering with the suggested chemoattractive mechanisms might reduce this proposed auto-attraction between neural stem/progenitor cells and immature neurons. These preliminary data open the possibility to pharmacologically interfere with this auto-attraction mechanism. In particular,

the antiangiogenic drug endostatin, which interferes with the chemoattractant-mediated migration of endothelial cells, appears to be sufficient to induce enhanced migration out of mixed neural cell populations *in vitro*. These data should offer a starting point for the development of pharmacological strategies to enhance tissue integration in neural transplantation.

7. ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wurde eine Doublecortin (DCX)-Promoter basierte Linienselektions-Strategie für die Generierung aufgereinigter Neurone aus humanen embryonaler Stammzellen (hES) entwickelt. Mittels stabiler Transfektion hES Zell-abgeleiteter neuraler Stammzellen mit einem Konstrukt, welches das grün fluoreszierende Protein (EGFP) unter der Kontrolle des neuronalspezifischen DCX-Promoters exprimiert, wurden klonale hES Zell-abgeleitet neurale Stammzelllinien generiert, welche eine Zelltyp-spezifische Expression von EGFP in frühen, unreifen humanen Neuronen zeigten. Fluoreszenz-basierte durchflusszytometrische Zellsortierung (FACS) ermöglichte die Aufreinigung DCX-EGFP-positiver Neurone mit Reinheitsgraden von über 95%. Die selektionierten Neurone konnten replatiert werden und reiften *in vitro* funktionell aus.

Da die Anwendbarkeit aufgereinigter hES Zell-abgeleiteter Neurone von einer effizienten Kryokonservierungs-Technik profitieren würde, wurden definierte Einfrierbedingungen unter zu Hilfenahme von Caspaseinhibition entwickelt, welche in Überlebensraten von bis zu 83% resultierten. Die kryokonservierten Neurone konnten replatiert werden und entwickelten physiologische Eigenschaften, die mit denen nicht-kryokonservierten Kontrollzellen vergleichbar waren. In Kombination mit der etablierten Linienselektions-Methode ermöglichte diese Kryokonservierungs-Technik die Generierung von humanen Neuronen in einem “ready-to-use” Format, welches in einer Vielzahl von biomedizinischen Anwendungen genutzt werden kann.

Transwellkammern-basierte Migrationsstudien sowie Transplantationsexperimente in das zentrale Nervensystem von Nagern zeigten ein gesteigertes Migration- und Integrationspotential der selektionierten Zellen. Im Gegensatz hierzu zeigten Reinkulturen neuraler Stammzellen oder Mischkulturen bestehend aus Stammzellen und Neuronen ein eingeschränktes Migrationtsverhalten *in vitro* und im Wirtsgewebe. Hier fanden sich die Zellen ausschließlich im Bereich des Transplantationsortes in sogenannten Zellclustern. Die Migration von Neuronen *in vivo* wird durch multiple Faktoren beeinflusset, wobei extrazelluläre Matrix, Integrine, Zell-Adhäsionsmoleküle, Zell-Zell-Kontakte sowie lösliche Faktoren und deren Rezeptoren eine Rolle spielen.

Experimente im Rahmen dieser Studie ergaben Hinweise, dass die Clusterbildung und das eingeschränkte Einwandern von Spender-Neuronen in das Wirtsgehirn aus neuralen Stammzelltransplantaten auf eine chemoattraktive Interaktion zwischen den neuralen Stammzellen und den differenzierten Neuronen im Transplantat beruhen könnten. *In vitro* fand sich eine verstärkte chemoattraktive Migration von humanen Neuronen in Richtung neuraler Stammzellen. Zusätzlich konnte gezeigt werden, dass beide Populationen eine

komplementäre Expression von chemoattraktiven Faktoren und deren Rezeptoren aufwiesen.

Die Interaktion mit den angenommenen chemoattraktiven Mechanismen ermöglichte eine Reduktion der Autoattaktion zwischen neuralen Stammzellen und unreifen Neuronen. Hier weisen erste Daten auf eine Mögliche pharmakologische Interaktion durch das antiangiogenetisches Medikament Endostatin hin, welches in die chemokinetisch-vermittelte Migration endothelialer Zellen eingreift. Mit dieser Substanz behandelte Zellen wiesen ein verstärktes Migrationsverhalten neuronaler Zellen aus neuralen Mischpopulationen *in vitro* auf. Diese Daten stellen eine Mögliche pharmakologische Strategien zur Optimierung von Migration und Integration neuraler Transplantate dar.

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10. ERKLÄRUNG

Hiermit versichere ich, dass diese Dissertation von mir persönlich, selbständig und ohne jede unerlaubte Hilfe angefertigt wurde. Die Daten, die im Rahmen einer Kooperation gewonnen wurden, sind ausnahmslos gekennzeichnet. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind ebenfalls unter Angabe der Quelle gekennzeichnet.
Die vorliegende Arbeit wurde an keiner anderen Hochschule als Dissertation eingereicht. Ich habe früher noch keinen Promotionsversuch unternommen. Diese Dissertation wurde an der nachstehend aufgeführten Stelle auszugsweise veröffentlicht.

Ladewig J, Koch P, Endl E, Meiners B, Opitz T, Couillard-Despres S, Aigner L & Brustle O. (2008). Lineage selection of functional and cryopreservable human embryonic stem cell-derived neurons. *Stem Cells* **26**, 1705-1712.

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Publications

Ladewig J, Koch P, Brüstle O, (2008). Enhanced Tissue Integration of Purified Human Embryonic Stem Cell-Derived Neurons: Overcoming Inhibitory Auto-Attraction of Stem Cell-Containing Transplants. Manuscript in preparation.

Koch P, Opitz T, Steinbeck J, Ladewig J, Brüstle O. (2008). A Rosette-Type, Self-Renewing human ES Cell-Derived Neural Stem Cell with Potential for in vitro Instruction and Synaptic Integration. Manuscript in revision.

04/2008 Ladewig J, Koch P, Endl E, Meiners B, Opitz T, Couillard-Despres S, Aigner L & Brustle O. (2008). Lineage selection of functional and cryopreservable human embryonic stem cell-derived neurons. *Stem Cells* **26**, 1705-1712.

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Congress Contributions (Talk)

02/2008 Enhanced tissue integration of purified human ES cell-derived neurons: Overcoming inhibitory auto-attraction of stem cell-containing transplants (Jahrestreffen Bonner Forum Biomedizin 2008, BFB, Bad Breisig)

06/2007 DCX based lineage selection of human neuroblasts (ESTOOLS-First Annual Consortium Meeting, Brno, Czech Republic)

First Author Congress Contributions (Poster)

07/2008 Enhanced tissue integration of purified human ES cell-derived neuronal progenitors (2nd International Congress on Stem Cells and Tissue Formation, Dresden, Germany, Abstract-Viewer p. 210, 130)

06/2008	Promotion of neuronal integration in human ES cell-derived neural stem cell grafts (6th ISSCR Annual Meeting, Philadelphia, USA)
05/2008	Lineage Selection of Functional and Cryopreservable Human Embryonic Stem Cell-Derived Neurons, (ESTOOLS-First Annual Consortium Meeting, Budapest, Hungary)
02/2008	Enhanced tissue integration of purified human ES cell-derived neurons: Overcoming inhibitory auto-attraction of stem cell-containing transplants (First International Symposium on Human Embryonic Stem Cell research, FISH-ESC, Evry-Paris)
11/2007	Enhanced tissue integration of purified human ES cell-derived neuroblasts: Overcoming inhibitory auto-attraction of stem cell-containing transplants (37th Annual Meeting of the Society of Neuroscience, San Diego, USA, Abstract-Viewer 32.19/C25
10/2007	Enhanced tissue integration of purified human ES cell-derived neuroblasts: Overcoming inhibitory auto-attraction of stem cell-containing transplants (4th International Meeting of the Stem Cell Network North Rhine Westphalia, Düsseldorf, Abstract: Regenerative Medicine 2(6, Suppl.), S23, 2007)
06/2007	Enhanced tissue integration of purified human ES cell-derived neuroblasts: Overcoming inhibitory auto-attraction of stem cell-containing transplants (5th ISSCR Annual Meeting, Cairns, Queensland, Australia, ISSCR, 136: Mo272)
04/2007	Lineage selection of doublecortin-positive migratory human ES cell-derived neuroblasts (7th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany)
02/2007	Doublecortin-based lineage selection of migratory human ES cell-derived neuroblasts (11. Semester-Meeting des Bonner Forum Biomedizin, Bad Breisig)
11/2006	Lineage selection of doublecortin-positive migratory human ES cell-derived neuroblasts, (Neuro2006 Neuro-Vision, Düsseldorf, November 28, 2006)
10/2006	Doublecortin-based lineage selection of migratory human ES cell-derived (Neuroblasts. 36th Annual Meeting of the Society of Neuroscience, Atlanta, Georgia, Abstract-Viewer 787.17/B36)
10/2006	Lineage selection of doublecortin-positive migratory human ES cell-derived neuroblasts, (Erstes gemeinsames wissenschaftliches Symposium der medizinischen Fakultäten der Universitäten Köln und Bonn, Bonn)
09/2006	Lineage selection of doublecortin-positive migratory human ES cell-derived neuroblasts (EuroStemCell Summer School: Stem Cells & Regenerative Medicine, Hydra, Greece, Abstract: Vol. 1, page 26, Number 31)
05/2006	Lineage selection of doublecortin-positive human ES cell-derived neurons (3rd International Meeting of the Stem Cell Network North Rhine Westphalia, Münster, Program, Abstracts and Company Profiles 2006, page 89)
05/2006	Lineage selection of doublecortin-positive human ES cell-derived neurons (4th International Symposium on Neuroprotection and Neurorepair: Cerebral Ischemia and Stroke, Magdeburg, Abstracts 2006, page 24)