Stem cell interactions with the injured brain

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Cover illustration

After engraftment neural stem cells (green) integrated into the organotypic culture and formed gap-junctional couplings (here visualized by stainings against connexin 43; white) to the injured and nestin (red) positive host cells. Cell nuclei are marked with DAPI (blue). As described in this thesis communication via gap junctions underlies early functional and beneficial interactions between grafted neural stem cells and the host.

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To Johan

ABSTRACT

Neurodegenerative diseases such as Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis and acute neurological disorders such as brain ischemia and traumatic injury yearly affect millions of people. Neural stem cell (NSC) grafting is an emerging strategy to treat and potentially cure these conditions for which today no effective remedies exist. The restoration of function might occur both by replacement of lost neural cell populations and by rescue of host cells at risk. In this thesis we have investigated the early interactions between grafted NSCs and the injured brain and characterized potential mechanisms that underlie the functional improvements seen after NSCs grafting. For these aims, we employed an organotypic culture (OC) system to model injured neural host tissue and grafted both murine and human NSCs to this model. First, we recognized that the OC was a suitable model system to study the early interactions between NSCs and the host. After NSC grafting we observed a reduced host cell damage using metrics like astrogliosis, apoptosis and necrosis. The grafted NSCs also integrated functionally and participated in host calcium signaling networks. Employing a combination of immunohistochemistry, RNA interference, pharmacological blockers, calcium imaging and dye coupling assays we identified gap-junctional graft-host couplings as the mechanism that conveyed both the beneficial impact on the host and the early functional interactions. We recognized that gap junction expression in the grafted NSCs and the injured host cells were highly dynamic processes. The investigations of the graft and host gap junction expression indicated a temporal window of opportunity for successful NSC engraftment. Finally, we noticed that graft-host gap-junctional couplings could be increased by treating the human embryonic stem cells with a Rho-associated kinase inhibitor. This was paralleled by an increased beneficial impact on the damaged host cells. The main conclusion in this thesis is that that gap-junctional coupling appears to be one of the first steps by which graft and host cells establish functional and beneficial interactions. This precedes the formation of more complex communication like chemical synapses. The direct cell-to-cell contact allows reciprocal exchange of a multitude of different health promoting substances and neutralization of pathological processes by diffusion of harmful substances. Increased knowledge of the exact molecular mechanisms involved in the interplay between the graft and host, and also how to direct them, can ultimately benefit the potential future use of NSCs grafts for the treatment of neurodegenerative disorders.

LIST OF PUBLICATIONS

This thesis is based upon the following papers, which will be referred to by their Roman numerals:

I. **Linda Maria Jäderstad**, Johan Jäderstad and Eric Herlenius. Graft and host interactions following transplantation of neural stem cells to organotypic striatal cultures. *Regenerative Medicine,* November 2010, Vol. 5, No. 6, Pages 901-917.

II. Johan Jäderstad*, **Linda Maria Jäderstad*,** Jianxue Li[†], Satyan Chintawar[†,] Carmen Salto, Massimo Pandolfo, Vaclav Ourednik, Yang D. Teng, Richard L. Sidman, Ernest Arenas, Evan Y. Snyder, Eric Herlenius. Communication via gap junctions underlies early functional & beneficial interactions between grafted neural stem cells & the host. *Proceedings of the National Academy of Sciences USA*, 2010, Vol. 107, No. 11, Pages 5184-5189.

III. Johan Jäderstad*, **Linda Maria Jäderstad*,** Eric Herlenius. Dynamic changes in connexin expression following engraftment of neural stem cells to striatal tissue. *Experimental Cell Research*, Jan 2011, Vol. 317, No. 1, Pages 70-81.

IV. **Linda Maria Jäderstad**, Yen-Fu Cheng, Rouknuddin Ali, Eric Herlenius, Lars Ährlund-Richter. Rho-associated kinase inhibition enhances gap-junctional communication between grafted human embryonic stem cells and the host. *Manuscript*.

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CONTENTS

LIST OF ABBREVIATIONS

18-α-GA	18-α-glycyrrhetinic acid
ALS	Amyotrophic lateral sclerosis
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BDNF	Brain-derived growth factor
β-gal	β-galactosidase
CBX	Carbenoxolone
CC-3	Cleaved caspase-3
CNS	Central nervous system
Cx	Connexin
Da	Dalton
DAPI	4',6-diamino-2-phenylindole
DiI	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GBSS	Gey's Balanced Salt Solution
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acicid protein
GFP	Green fluorescent protein
GJIC	Gap-junctional intercellular communication
hESC	Human embryonic stem cell
hNSC	Human neural stem cell
HD	Huntington's disease
Hsp	Heat chock protein
LY	Lucifer yellow
Map2ab	Microtubule associated protein 2ab
MMC	Mitomycin C
mNSC	Murine neural stem cell
NB/B27	Neurobasal^{TM} medium supplemented with B27^{TM}
NE	Neuroepithelial cells

NeuN	Neuronal nuclei
NSC	Neural stem cell
NGF	Nerve growth factor
nr mouse	*Nervous* mouse
NT-3	Neurotrophin-3
OC	Organotypic culture
P	Postnatal day
P2R	P2 purinergic receptors
PBS	Phosphate buffered saline
PD	Parkinson's disease
PL	Purkinje layer
PFA	Paraformaldehyde
PI	Propidium iodide
PN	Purkinje neurons
RD	Rhodamine Dextran
R_m	Membrane resistance
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROCK	Rho-associated kinase
ROCKi	Rho-associated kinase inhibitor
ROS	Reactive oxygen species
SCA-1	Spinocerebellar ataxia type 1
SGZ	Subgranular zone
SOD1	Superoxide dismutase 1
SVZ	Subventricular zone
tPA	Tissue plasminogen activator
TrkC	Tyrosine kinase receptor C
V_m	Resting membrane potential
VZ	Ventricular zone

1 INTRODUCTION

The initial part of the thesis will briefly summarize the current state of the field and some of the most important obstacles that remain before neural stem cell transplantations can be routinely used in the clinic. The emphasis will be on the topics specifically addressed in the included studies (*I-IV*).

1.1 STEM CELLS

1.1.1 Definition

A stem cell is defined by its capacity for self-renewal and the ability to generate more differentiated cells (Potten et al. 1990). The regenerative capacity originates from the innate capability for symmetric cell division resulting in two identical copies of itself, i.e. two "clonogenic" stem cells. The stem cell can also undergo asymmetric cell division, which will result in one identical daughter cell and one with more restricted potential for differentiation.

1.1.2 Lineage

Embryonic stem cells (ESC) are derived from the inner cell mass of preimplantation embryos (Martin 1981). Similar to the inner cell mass, the *in vitro* cultured ESCs show a pluripotency to form cells of all three germ layers, i.e. endo- meso- and ectoderm. Tissue-specific ("adult") stem cells are found in tissues of the developed organism, like bone marrow (Till et al. 1963), intestine (Cheng et al. 1974), skin (Cotsarelis et al. 1990), lung (Kajstura et al. 2011) and brain (Messier et al. 1958; Altman et al. 1965). Such stem cells can differentiate into specialized cell types of the tissue from which they came. While the origin of ESCs is known, the origin of adult stem cells is less known. One hypothesis is that they escape linage restriction in the early embryo and later on colonize their special niches. An alternative and perhaps more widely accepted hypothesis is that adult stem cells are derived after the somatic linage specification by the dedifferentiation of fate determined cells (Kriegstein et al. 2009).

1.2 NEURAL STEM CELLS

1.2.1 Neural stem cells during development

The formation of the central nervous system (CNS) begins with the process of neurulation, where the neural plate folds to become the neural tube which fluid-filled center later will give rise to the ventricular system and spinal canal. The neural tube is

made up by primary progenitors called neuroepithelial (NE) cells. NE cells are radially organized and highly polarized cells that touch both the ventricular and basal side of the neural tube. During early embryonic development the NE cells undergo symmetric, proliferative divisions in order to increase the pool of progenitors (McConnell 1995; Rakic 1995). The symmetrical cell division occurs at the ventricular surface, which creates a proliferative ventricular zone (VZ). During interphase the NE cells pull their nuclei towards the pial surface by a process called interkinetic nuclear migration. In later developmental stages the NE cells switch to asymmetrical cell division that generates a daughter stem cell and a more fate restricted neural progenitor. At the onset of the neurogenesis the NE cells give rise to a distinct cell type, the radial glial cells (Kriegstein et al. 2009), that successively during development replace the NE cells. Like the NE cells, the radial glial cells remain in contact with the ventricular and the pial surface but in contrast they are more fate restricted and generate progenitors for either astrocytes, oligodendrocytes or neurons (Malatesta et al. 2000; Noctor et al. 2001; Noctor et al. 2004). The intermediate progenitor cells originating from the asymmetrical division of the radial glial cells typically undergo subsequent symmetric divisions to generate terminally differentiated cells. The newly born postmitotic cells in the ventricular zone migrate radially along the radial glial processes and consequently, most of the cells in the brain are derived from the radial glial cells. The accumulation of progenitor cell derivatives that migrates out from the VZ transforms the neuroepithelium into a tissue with numerous cell layers (Haubensak et al. 2004; Noctor et al. 2004; Merkle et al. 2006). In mammals, radial glial cells exist during the cortical development. At the end of this developmental period, the radial glial cells lose their ventricular attachment and migrate toward the cortical plate where they finally differentiate into astrocytes (Noctor et al. 2008).

1.2.2 Neural stem cells in the adult brain

There are two major sites for neurogenesis in the adult mammalian brain, the subventricular zone (SVZ) (Doetsch et al. 1999) and the dentate gyrus subgranular zone (SGZ) of the hippocampus (Gage 2000). The neurogenic cells which reside in these areas are considered to be remnants of the embryonic radial glial cells (Merkle et al. 2004).

The SVZ contains neural stem cells (NSCs) that line the lateral walls of the lateral ventricles. These SVZ cells generate neuroblasts which migrate long distances in chains to their final destination, the olfactory bulb, where they differentiate into interneurons

(Lois et al. 1994; Belluzzi et al. 2003; Curtis et al. 2007). The hypothesized functional role for the newborn neurons in the olfactory bulb is to contribute to odor memory and olfactory discrimination (Rochefort et al. 2002). The primary precursor of new hippocampal neurons is located in the subgranular zone that lies at the interface of the granule cell layer and the hilus. When born, the precursors migrate a relatively short distance into the granule cell layer where they differentiate into hippocampal granule cells. This adult neurogenesis in the hippocampus has been linked to learning, memory and mood regulation (Altman 1969; Gould et al. 1992; Winocur et al. 2006). Why neurogenesis in certain confined areas persists into adulthood is not fully understood. In the physiologically intact brain, one theory suggests that plasticity is needed in systems important for survival, like the ability to detect signs of danger such as smoke or contaminated food and to remember these inputs in order to avoid them in the future. In the diseased brain, adult neurogenesis is hypothesized to generate a constant source of new neurons that will help the brain to self-repair. Currently, evidence for adult neurogenesis in other brain regions, including the neocortex (Altman 1963), striatum (Dayer et al. 2005), amygdala (Bernier et al. 2002) and substantia nigra (Zhao et al. 2003) exist, but it is not conclusively demonstrated and awaits further investigation (Gould 2007).

1.3 BRAIN INJURY

1.3.1 Acute injury

Acute brain injury typically affects entire sections of the brain parenchyma including all the consisting neural cells, with a near complete loss of the supporting architecture and circuitry often as well as a deterioration of the local blood circulation. The acute insult is associated with excitotoxicity, a pathological process which results in the death of neurons by, most importantly, excessive glutamate release and sustained elevations of intracellular calcium levels (Olney 1969). Glutamate is the most common neurotransmitter in the brain but in situations of massive neuronal death, the concentration of glutamate reach pathological levels and it becomes toxic due to the excessive and prolonged activation of its receptor (Lucas et al. 1957). This leads to the influx of calcium to the intracellular compartment and subsequently the activation of calcium-dependent catabolic enzymes which will damage different cell structures such as components of the cytoskeleton, the membrane and the DNA (Manev et al. 1989).

1.3.1.1 Ischemic stroke

Prolonged interruption of the blood supply leads to deprivation of oxygen and nutrients to energy demanding neurons. The affected neurons become dysfunctional within a few seconds and show signs of structural damage after just two minutes (Murphy et al. 2008) due to inability to maintain a normal ion gradient which results in an imbalance in the water and ion homeostasis. This ultimately leads to cell damage or death through multiple processes like excitotoxicity, acidotoxicity, oxidative stress and inflammation. The pathological processes affect not only neurons, but also other non-neuronal cells which causes a focal degeneration of the brain parenchyma. The processes have their own distinct time frames, some occurring within minutes, others within hours and days which ultimately leads to the impairment of sensation, movement or cognitive functions executed by the affected area (Doyle et al. 2008).

1.3.2 Chronic injury

Neurodegenerative disorders are characterized by the slow progressive death of neurons, which ultimately leads to a decline of functions performed by the diseased region. The degeneration can be focal, with the selective death of a specific neuronal population such as in Parkinson's disease (PD) and Huntington's disease (HD), or multifocal with a widespread loss of many different neurons as in Alzheimer's disease (AD). The pathophysiology is often viewed as cellular dysfunction due to pathological protein assembly or aggregate formation (Bruijn et al. 1998; Orr et al. 2000; Walker et al. 2000; Sherman et al. 2001; Ross et al. 2004), oxidative stress (Simonian et al. 1996; Wiedau-Pazos et al. 1996; Jenner 2003), mitochondrial dysfunction (Boillee et al. 2006; Lin et al. 2006; Turner et al. 2007), ion dyshomeostasis (Roy et al. 1998) or excitotoxicity (Doble 1999; Hardingham et al. 2010). The initial insult will successively lead to the activation of the immune system that will detect and destroy diseased neurons (Block et al. 2007; Tansey et al. 2007). The inflammatory response contributes to the clinical manifestations, and participates in the development and progression of the disease (Wyss-Coray et al. 2002; Block et al. 2005; Mrak et al. 2005; Lindvall et al. 2010).

1.3.2.1 Parkinson's disease

Parkinson's disease is an untreatable and progressive disease that yearly affects over 4 million of people worldwide (Dorsey et al. 2007). It is the second most common cause of neurodegeneration after Alzheimer's disease (Hirtz et al. 2007) and the clinical

features involves resting tremor, muscle rigidity, bradykinesia and postural instability (Jankovic 2008). The characteristic motor symptoms observed in patients with PD are due to the selective death of dopaminergic neuron in the substantia nigra pars compacta and the decreased dopaminergic innervation of the striatum which results in deficits in dopaminergic neurotransmission. The PD affected dopaminergic neurons exhibit cytoplasmic inclusions called Lewy bodies that are rich in α-synuclein (Chu et al. 2007; Uversky 2007), a protein needed for normal synaptic function but in affected neurons are misfolded and hypothesized to cause neuronal stress and death. The most important risk factor is age, but there exists a genetic predisposition for acquiring the disease. The exact etiology is however unknown. There is currently no cure for PD. The primary goal of existing treatment is threefold; delay the progression of the disease, achieve symptomatic relief and to cause as few adverse effects as possible (Barone 2010).

1.3.2.2 Huntington's disease

Huntington's disease is an autosomal dominant inherited disease, which originally was called Huntington's chorea (the Greek word for dance) due to its display of motor dysfunction like involuntary movements, bradykinesia, dystonia and rigidity. In addition to motor disturbances, HD also causes cognitive impairment, psychiatric disorders and slowing of intellectual processes (Gil et al. 2008). The disease is caused by a mutation in the HD gene which encodes for huntingtin, a protein which cellular functions is not fully understood but essential for normal brain development (Young 2003; Walker 2007).

The mutant protein exhibit a neurotoxic effect through toxic gain of function, i.e. protein misfolding and aggregation resulting in disturbed normal cell homeostasis and a pathogenic cascade that subsequently leads to cell destruction (Tobin et al. 2000). The pathological process shows a striatal selectively and in the advanced stages of HD, a nearly complete loss of striatal medium spiny neurons is observed (Halliday et al. 1998). The treatment for HD is symptomatic, aiming to reduce and postpone motor dysfunction, cognitive decline and psychiatric disturbances.

1.3.2.3 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is characterized by the selective death of upper and lower motor neurons (Cleveland et al. 2001; Rothstein 2009). The most prominent clinical ALS characteristics are muscle weakness and wasting leading to death, typically due to respiratory failure within three years after diagnosis (Eisen 2009). The

etiology is unknown, but likely there are a multitude of different genetic and environmental factors that in combination cause the disease. To date, few genetic or environmental risks have been discovered (Gordon 2011). A minority of all ALS cases are due to mutations in the superoxide dismutase 1 (SOD1) enzyme which normally catalyses the conversion of reactive oxygen species (ROS) to less harmful substances. The mutant SOD1 protein has a susceptibility to form aggregates (Shaw et al. 2007) due to protein misfolding (Nordlund et al. 2008) which leads to increased cellular vulnerability and subsequently death. Treatment is aiming at postpone ventilator-dependence, symptomatic relief and palliative care.

1.4 BRAIN REPAIR

1.4.1 Recruitment of endogenous neural stem cells

It is estimated that between 0.03% and 0.3% of the neuronal populations in rodent hippocampus and SVZ function as stem cells (Peterson 2002). Considering the extraordinary potential for a single stem cell, this low percentage might still provide a considerable regenerative capacity. Although the presence of active germinal zones in the adult brain is well established, it is generally considered that the regeneration provided by these areas is ineffective and insufficient during brain injury. The limited ability for self-repair is hypothesized to depend on the restricted locations of NSCs and limitations imposed by the surrounding microenvironment (Martino et al. 2006). NSCs are however found *in situ* in response to various insults such as ischemia (Jin et al. 2001; Arvidsson et al. 2002; Tonchev et al. 2003), trauma (Gould et al. 1997), epilepsy (Zhao et al. 2008) and neurodegeneration (Fallon et al. 2000). A number of studies indicated that neurogenesis is part of a self-repair process in several disease states like HD (Curtis et al. 2003), AD (Jin et al. 2004) and in a rodent model of ALS (Chi et al. 2006). Interestingly, neurogenesis in PD is in contrast decreased (Hoglinger et al. 2004).

1.4.2 Neural stem cell engraftment

Neural stem cell grafting holds great promise as a potential cure for several diseases that today are incurable. As discussed by (Lindvall et al. 2010) several major obstacles remains before stem cell grafting can be routinely used. The different conditions stem cells have the potential to treat are heterogeneous in their nature and exhibit great variability in severity. Patients suffering from PD have normal life expectancy and there exist drugs which are safe and effective. In contrast, ALS is a rapidly progressing

disease that always leads to death within a few years after symptomatic onset. While the severity of a disease like ALS might justify the potential risks of NSC transplantation, like tumor formation (Brustle et al. 1997; Deacon et al. 1998; Wernig et al. 2004; Gruen et al. 2006), dyskinesia (Freed et al. 2001; Politis et al. 2010), allodynia (Hofstetter et al. 2005) and seizures, these side effects are unacceptable for a PD patient.

Clinical stem cell transplantation in neurodegenerative diseases states is still experimental, but preliminary trials have showed some functional benefit. The first applications of stem cell grafting to patients were attempted in PD, where two patients received embryonic substantia nigra tissue grafted to the striatum. However, the procedure provided temporary and modest functional recovery (Lindvall et al. 1989). This led to changes in used protocol were the age of donors was reduced, more fetal tissue was grafted and measurements were undertaken to reduce brain trauma in connection with the transplantation procedure. These measurements resulted in a significant functional recovery in the third and fourth patient to be grafted (Lindvall et al. 1990). Today, graft-induced dyskinesias have suspended the clinical trials.

The second neurodegenerative disease to be addressed was HD. Limited data suggest symptomatic amelioration and some retrieval of motor and cognitive functions which are stabilized at least for six years after transplantation with embryonic striatal tissue (Bachoud-Levi et al. 2000a; Bachoud-Levi et al. 2000b). The first clinical trial with transplantation of NSCs to patients with stroke has recently been initiated (Lindvall et al. 2011). Previous transplantation of pre-differentiated NSCs derivates to patients with stroke have resulted in an improved cognitive and motor function (Kondziolka et al. 2000; Stilley et al. 2004). Application of NSC transplantations to ALS patients also is in its infancy. A phase I trial is currently ongoing to test whether the injection to the spinal cord is safe (Aboody et al. 2011).

The mechanisms underlying the beneficial graft and host interplay and how this reciprocal interaction can lead to restored function needs to be better understood before safe transition to the clinic. This is one of the most important goals of stem cell research today.

1.4.3 Repair mechanisms

1.4.3.1 Replacement of lost cells

The functional improvements following NSC engraftment have traditionally been attributed to replacement of missing neurons. The success of NSC engraftment is likely

depending on the complexity of the affected circuitry. When specific neuronal populations is affected like in PD and ALS, successful engraftment would require both selective replacement of lost neurons and the reestablishment of the original connections with proximal and distant host afferent and efferent fibers. It is known that when NSCs are grafted to a brain region were a certain neuronal subgroup is degenerated they respond by shifting their pattern of differentiation towards the missing cell type (Rosario et al. 1997; Snyder et al. 1997; Flax et al. 1998) but a complete reconstitution of neuronal connectivity is difficult to achieve. More extensive requirements have to be met when the cell replacement is aimed to treat focal lesions that cause loss of several different cell types, such as in ischemic stroke. In this case, transplanted cells should be able to populate a post-infarct cavity and to regenerate all the lost neural cell types in appropriate relative numbers, and to reestablish long distance connections with the host circuitry and to restore a functional vascular compartment.

1.4.3.2 Rescue of damaged host cells

There is a growing body of evidence indicating that NSCs can provide direct support to host cells at risk (Ourednik et al. 2002; Li et al. 2006a; Chintawar et al. 2009). These processes may even be of equal importance as host cell replacement. The host cell rescue and reactivation of function after NSC grafting may in part be attributed to the NSCs capacity to secrete diffusible factors needed for neuronal health and in part due to their ability to form direct cell-to-cell contacts to dysfunctional host cells and through the intercellular contacts mediate exchange of molecules that promote survival and neutralize pathological processes. Some of the more extensively described diffusible factors delivered by NSCs to damaged neighboring host cells are growth factors like brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) (Lu et al. 2003; Pluchino et al. 2003; Lee et al. 2007; Madhavan et al. 2008) which through receptor mediated actions contribute to cell homeostasis and survival.

Formation of direct cell-to-cell gap-junctional channels between graft and host cells allows direct exchange of several different molecules like adenosine, adenosine triphosphate (ATP), ions, amino acids, glutathione, genetic material and polypeptides which together with a diffusion and buffering of harmful substances which both enable grafted NSCs to restore and retain normal host cell homeostasis (Li et al. 2006a; Rouach et al. 2008; Chintawar et al. 2009; Goodenough et al. 2009).

2 AIMS

The main objective of this thesis was to investigate early mechanisms underlying cell level interactions between graft and host cells following NSC engraftment to CNS tissue.

The more specific objectives of the thesis were

- To establish and optimize an *ex vivo* organotypic slice culture model suitable to study cell level graft and host interactions.
- To characterize the morphological integration of implanted NSCs.
- To estimate host cell effects induced by the grafted NSCs.
- To evaluate the influence of growth factors on host cell survival and differentiation of grafted stem cells.
- To investigate if communication via gap junctions underlies the early functional and beneficial interactions between grafted NSCs and the host.
- To study the dynamic changes in gap junction expression following engraftment of NSCs and based on these findings evaluate potentially important factors for the optimal timing of the NSC engraftment.
- To test if pre-treatment with a Rho-associated kinase inhibitor can enhance gap-junctional communication between grafted stem cells and the host and if this pharmacologically enhanced communication is associated with increased beneficial effects on the host.

3   METHODS

This chapter contains a brief summary of the most important methods used. A complete listing and descriptions that are more detailed are to be found in the articles included in this thesis.

3.1   STEM CELLS

3.1.1   Murine neural stem cells

Two clones from the C17.2 cell line were employed to model the behavior of NSCs (Snyder et al. 1992). The, by now, well characterized C17.2 cell line was originally derived from postnatal day (P) 4 mouse cerebellum and retrovirally transfected with the myc gene to preserve multipotency, self renewal and an undifferentiated state *in vivo* (Parker et al. 2005). It is further engineered to express the β-galactosidase encoding *lacZ* gene (Snyder et al. 1992) and green fluorescent protein (GFP) for recognition of grafted cells (Ourednik et al. 2002; Lu et al. 2003). To study the effects of grafting NSCs in various stages of differentiation, a C17.2 subclone that routinely yields a high proportion of young neurons was utilized. This subclone has been genetically modified to secrete large amounts of neurotrophin (NT)-3 which operate upon both NSCs and host cells in an autocrine/ paracrine fashion (Lu et al. 2003). Throughout the thesis the NSC clone overexpressing NT-3 will be referred to as 'NSC-NT-3' and the clone expressing only GFP as 'NSC-GFP'.

In addition to the C17.2 cell line, another NSC preparation was used in the spinocerebellar ataxia type 1 (SCA1) mouse model employed in *Study II* (described in section 3.8.2). These NSCs were derived from the SVZ of four to eight week old FVB/N mice and the cells were engineered to express GFP to enable recognition (Gritti et al. 1999). The mouse neural stem cells (mNSCs) were cultured as previously described (Gritti et al. 1999; Lu et al. 2003) and detailed in *Study II*.

3.1.2   Human neural stem cells

Three different preparations of human neural stem cells (hNSCs) were used, none of which were genetically modified or carried any transgenes. In *Study I* and *II* HFB2050 (Flax et al. 1998), an hNSC line isolated from the telencephalic ventricular germinal zone of a 13-week-old fetal cadaver, was used. In *Study II*, HFB11ws6 and HCX11ws6 (Carpenter 1999; Castelo-Branco et al. 2003; Piao et al. 2006; Akesson et al. 2007) both derived from cerebral cortex and subcortical regions of an 11-week-old fetal

cadaver were used for characterization of their calcium homeostasis, dye coupling and connexin expression. The different preparations of hNSCs were maintained as previously described (Brewer et al. 1993; Flax et al. 1998) and detailed in *Study I* and *II*.

3.1.3 Human embryonic stem cells

In *Study IV* the H181 cell line (Hovatta et al. 2003), derived from discarded blastocysts after *in vitro* fertilizations, were used as graft material to study if less mature embryonic stem cells were associated with the same beneficial impact on the host as more differentiated neural stem cells. The human embryonic stem cells (hESCs) were not genetically manipulated and had a normal karyotype. The hESCs were maintained and cultured as previously described (Imreh et al. 2004) and detailed in *Study IV*.

3.1.3.1 *Pre-treating hESC with Rho-associated kinase inhibitor*

In *Study IV*, the Rho-associated kinase inhibitor (ROCKi) Y-27632 was used in order to enhance the connexin expression in hESCs. This was done by exposing the hESCs to 10 μM Y-27632 in cell culture medium 8 hours prior to engraftment to the organotypic culture (Anderson et al. 2002; Watanabe et al. 2007).

3.2 ORGANOTYPIC CULTURES

To study graft and host interactions, roller-drum organotypic cultures (OCs) were employed (Gahwiler 1981). The roller-drum technique for culturing nervous tissue was introduced 65 years ago (Hogue 1947), and has since then mostly been used for anatomical (Gahwiler et al. 1997), pathological (Kaal et al. 2000; Testa et al. 2005), developmental (Becq et al. 2005; Liu et al. 2005) and pharmacological studies (Corse et al. 1999; Ray et al. 2000; Sundstrom et al. 2005). Lately, the membrane interface method (Stoppini et al. 1991) where the brain slices rest on a porous membrane at the interface between medium and a humidified atmosphere, has grown more popular. The membrane interface method preserves the three-dimensional structure and the tissue architecture in a way that the roller-drum setup is unable to achieve. Instead, the roller-drum technique facilitates flattening of the brain slice due to the constant rotation. During time in culture the tissue transforms from a thick opaque slice to a highly accessible thin layer of cells. Although the original structure is changed, the neuronal connectivity, cell type composition and cell-to-cell interactions are preserved (Gahwiler et al. 1997). Due to the thinning of the slice to a near monolayer of cells, the roller-

drum setup provides pristine optical conditions and ideal circumstances to study cell level graft and host interactions, which is unachievable using any other model. The major drawbacks of the OC system are the absence of the immunological response and a functional vascular system (Lossi et al. 2009).

Organotypic striatal cultures were prepared as previously described (Gahwiler 1981). Briefly, newborn Sprague-Dawley rats were killed by decapitation at P 2 to 6 and the striatum was dissected out and stored in Gey's Balanced Salt Solution (GBSS). The striatal tissue was cut in 250 μm thick coronal slices using a McIlwain tissue chopper and transferred to fresh GBSS to allow tissue debris and potentially toxic substances, such as excitatory amino acids, to diffuse away. The tissue slices were carefully separated and subsequently moved to tissue water. Tissue slices were then placed on 12 x 24 mm glass cover slips and embedded in reconstituted chicken plasma coagulated by bovine thrombin. Cover slips containing the organotypic culture were finally placed in Falcon tubes with 1.1 ml medium (for medium composition, see section 3.2.1). The cultures were grown at 37°C in 5% CO_2 in a roller-drum device exposing the cells to gaseous and liquid phase every 30 seconds. The OCs were evaluated after 7, 14, 21 and 28 days *in vitro* (DIV). In *Study IV,* all cultures were evaluated at 21 DIV.

3.2.1 Maintenance of organotypic cultures

Grafted and non-grafted cultures were maintained in organotypic culture medium consisting of 55% Dulbecco's Modified Eagle Medium with 25mM HEPES, Sodium Pyruvate and 1000 mg/L glucose, 32.5% Hanks Balanced Salt, 10% Fetal Bovine Serum, 1.5% D-(+)Glucose 20% solution, 1% HEPES and 1% Penicillin/ Streptomycin/ Fucidin. Additionally, to test whether external cues could direct differentiation and promote a neuronal fate in grafted mNSCs, a subset of grafted cultures were grown in Neurobasal culture medium supplemented with B27 (Brewer et al. 1993). This serum-free medium was originally developed to enhance survival of hippocampal neurons but has gained wide recognition for its differentiation-inductive properties in a number of CNS tissues such as the striatum, cerebellum and substantia nigra (Brewer 1995). Neurobasal/B27 medium was prepared by mixing Neurobasal™ with 2% B27 supplement, 1% L-glutamine (200 mM) and 1% Penicillin/ Streptomycin/ Fucidin. Throughout the thesis, this culture medium is abbreviated 'NB/B27 medium' and the organotypic culture medium is abbreviated 'OC medium'. The culture medium was changed every third day.

3.3 ENGRAFTMENT OF STEM CELLS

In *Study I-III*, NSCs were grafted to the OCs after 48-hour exposure to 1 µM of the alkylating agent Mitomycin C (MMC). The mNSCs were grafted as a cell suspension with a concentration of 300-500 cells/µL. A total of 10 µL cell suspension was dispersed within 1 mm from the striatal tissue, resulting in $3-5 \times 10^3$ NSCs per OC and a 1:40-50 NSC:OC cell ratio.

In *Study IV*, colonies of hESCs where carefully removed from the underlying layer of feeder cells with a sterile needle after treatment with Dispase (10 mg/ml). Four to five colonies, approximately 2×10^4 cells, per OC were added in close vicinity to the striatal tissue that resulted in a 1:200 hESCs:OC cell ratio.

To allow adherence of the grafted cells, cover slips were left in a horizontal position at 37°C in 5% CO_2 for 30-45 minutes before returning them to the Falcon tubes. For descriptions of the grafting procedures used for the *in vivo* models, see *Study II*.

3.4 RECOGNITION OF GRAFTED STEM CELLS

Grafted mNSCs were identified by their GFP expression, whereas an antibody against Heat chock protein (Hsp) 27 was used for detection of human NSCs and ESCs. The Hsp27 staining was visualized using fluorescent secondary antibodies labeled with Alexa 488 or 546. Immunohistochemical protocols are described below. In *Study II*, a subset of HFB2050 cells were pre-labeled with the membrane bound fluorescent dye 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) (Honig et al. 1986).

3.5 IMMUNOHISTOCHEMISTRY

Standard immunohistochemical protocols were applied. In summary, grafted and non-grafted cultures were fixed with 4% paraformaldehyde (PFA) for 5-10 minutes and then rinsed twice with phosphate buffered saline (PBS). The cultures were incubated with the primary antibodies in a humidity chamber over night at 4°C. After rinsing the primary labeled OCs three times with PBS, they were incubated with fluorescent-tagged secondary antibodies in the same humidity chamber at room temperature for one hour. After rinsing three times with PBS the labeled OCs were finally counterstained with DAPI nuclear stain.

3.5.1 Proliferation, gliosis and apoptosis

To assess the effects of stem cell grafting on host cell viability three different antibodies were used, glial fibrillary acidic protein (GFAP), cleaved caspase-3 (CC-3) and Ki67. The preparation of OCs in itself includes a traumatic perturbation involving axotomy and excitotoxicity with subsequent reactive astrogliosis. The astrocytic marker GFAP was used to estimate the progression of the reactive astrogliosis and the evolution of the injury afflicted to the OC during establishment. CC-3 immunostaining was performed as a supplement to GFAP labeling as a metric for cell viability. Caspase-3 is a zymogen that is activated upon cleavage, both by the extrinsic and intrinsic apoptotic pathway. The activation of caspase-3 ultimately leads to apoptosis. In *Study IV* the proliferation marker Ki67, which labels cells during late G1, S, G2 and M phases of the cell cycle, was used to assess host and graft cell well-being.

3.5.1.1 Other techniques for evaluation of host cell damage

In order to assess cell viability on single cell level, propidium iodine (PI) staining was preformed. For PI staining, unfixed cultures were quickly rinsed in PBS and incubated in 20 μg/ mL PI for 10 minutes at room temperature, followed by fixation with 4% PFA in PBS for 5 minutes. PI binds to DNA of necrotic or late apoptotic cells and when it is bound to nucleic acid, its red fluorescence is enhanced 20-30 fold.

Evaluation of the overall OC viability was performed by judgment of transparency, color and boarder morphology. Cultures which after 7 DIV were pale, had an opaque appearance and distinct borders were considered to be non-viable and were discarded. To validate the accuracy of this method, a double-blind trial with trypan blue was preformed and accuracy was found to be 100%.

3.5.2 Neural markers

To identify neurons three different antibodies were used; Tuj1, Map2ab and synaptotagmin. Tuj1 labels class III β-tubulin, which is present in immature neurons. Map2ab labels slightly more mature neurons, which expression peaks at the time for axonal growth, but before synaptic activity. Synaptotagmin labeling is indicative of mature neurons capable of chemical synaptic transmission. For information about the neuronal markers used in the *in vivo* models, see *Study II*.

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3.5.3 Connexin markers

To evaluate the presence of connexins (Cx), the substrate for gap junction formation, we used two different antibodies directed towards Cx26 and Cx43. Gap junctions are formed by the consolidation of two connexons, each consisting of six connexin subunits, on adjacent cells. Gap junctions containing Cx26 are present in neural precursor cells (Leung et al. 2002; Montoro et al. 2004) while gap junctions containing Cx43 are apparent in ESCs (Worsdorfer et al. 2008), neuronal progenitor cells (Rouach et al. 2002; Khan et al. 2007), radial glial cells (Dermietzel et al. 1989) and mature astrocytes (Rozental et al. 2000).

3.6 FLOW CYTOMETRY

Flow cytometry was used in *Study I* to determine the GFP expression in mNSCs and in *Study III* to evaluate the temporal changes in connexin expression in graft and host cells. Flow cytometry allows rapid quantification of large number of cells. For preparation of single cells, expanded mNSCs were trypsinized, fixed with 4% PFA, centrifuged and resuspended in PBS. Grafted OCs were trypsinized for 10 minutes and then gently removed from the cover slip with a cell scraper. The tissue was then carefully triturated with a pipette and 10% fetal calf serum was added to inactivate the trypsin. For fixation, 4% PFA was added to give a final concentration of 2% for 15 minutes. The suspension was centrifuged and resuspended in PBS. For immunocytochemical labeling, primary antibodies were added for one hour and thereafter secondary antibodies for 30 minutes. After each step, the suspension was centrifuged and resuspended in PBS.

The cell fluorescence signals were determined using a FACSort flow cytometer (Becton Dickinson, San Jose, CA). A primary gate based on physical parameters (forward and side light scatter) was set to separate cells from debris. Negative and positive controls were used to set the detection range. In order to decide GFP expression in mNSC a native C17.2 clone with no GFP expression was used as a negative control. As a positive control, mNSCs with a well known and by microscope verified strong GFP expression in all cells (the NSC-NT-3 clone) was used. For the red spectrum, the background levels in both NSCs and OC cells were estimated by treating the cells with the secondary antibody and by omitting the primary antibody. The upper detection range was adjusted using fluorescent beads with colors exactly corresponding to the used secondary labels. Using this approach, cells within the first decade in the

fluorescence plots were considered as negative and cells within the second to third decades were considered as positive for the investigated parameters.

3.7 FUNCTIONAL CHARACTERIZATION

3.7.1 Calcium imaging

Calcium imaging was performed in *Study II* to investigate how grafted NSCs participate in and affect host calcium based network activity. To estimate intracellular Ca^{2+} levels fluorescent Ca^{2+} indicators were used. These indicators change their light absorption and/or emission characteristics upon Ca^{2+} binding. We used the calcium indicator Fluo-4 to evaluate overall activity and host cell-to-cell communication in non-grafted cultures and the indicator Fura-2 for ratiometric imaging. For a detailed description of the methodology, see *Study II*.

3.7.2 Dye transfer

3.7.2.1 Scrape loading

In addition to calcium imaging, scrape loading experiments were performed to evaluate the functionality of gap-junctional couplings between the NSCs, hESC and between grafted NSC derivates and host cells. Expanded hESCs in *Study IV*, or grafted OCs in *Study II* and *III* were immersed in a dye solution containing 0.5 mg/ml Lucifer Yellow (LY) and 0.5 mg/ml Rhodamin-Dextran (RD) diluted in PBS for one minute. A tear was made through the hESC colony/grafted OC using a surgical blade. The preparation was then incubated for 30 to 60 minutes to allow dye spread. Due to its high molecular weight (10.000 Da) RD is too large to be transferred between neighboring cells via gap junctions and thus serves as a control dye whereas the low molecular weight dye LY (457.2 Da) easily passes through gap-junctional couplings (Stewart 1981) (see also Fig. 2a, *Study III*). The amount of dye spreading was expressed as the ratio between LY and RD filled cells. In *Study IV* the amount of dye spreading was estimated based on the dye spread area instead of the number of cells.

3.7.2.2 Dye transfer from pre-loaded neural stem cells

To study gap-junctional coupling between grafted NSC progeny and host cells in a non-invasive fashion, dye transfer from DiI labeled and calcein pre-loaded NSCs was investigated in *Study II* using a previously described protocol (Goldberg et al. 1995). Similarly to LY, calcein is able to pass through gap junctions due to its small size (995 Da), while the dye DiI is bound to the membrane of the grafted cell. Hence, if

functional gap junctions are established, calcein is transferred from the DiI positive grafted NSCs to neighboring DiI negative OC cells. The amount of dye spreading was expressed as the ratio of calcein and DiI positive cells. For further details, see *Study II*.

3.7.2.3 Dye transfer following microinjection

To further test the presence and functionality of gap-junctional couplings between graft and host cells, dye transfer after micropipette dye injection directly into the grafted NSCs was studied in subset of grafted cultures in *Study II*. Grafted NSCs were injected with 0.1% LY and RD using glass micropipettes. After patching the grafted cells, dye-spread was photographed repeatedly over a 30 minute time period. Dye-spread was thereafter also evaluated in fixed slices using a confocal laser scanning microscope and digital three-dimensional reconstruction.

3.7.3 Whole cell patch clamp electrophysiology

To determine neuronal membrane properties in *Study II*, basic whole-cell patch-clamp recordings were performed in expanded NSCs, NSC progeny and OC cells. The resting membrane potential (Vm), membrane resistance (Rm) and ability to generate action potentials were determined in examined cells. The protocols are detailed in *Study II*.

3.7.4 Gap junction inhibition

To examine whether the gap-junctional couplings could be blocked and to estimate the associated effects on the graft-host interplay we utilized both pharmacological blockers and RNA interference (RNAi). The pharmacological agents 18-α-glycyrrheetinic acid (18-α-GA) and carbenoxolone (CBX) was used to block gap junction function in *Study II* and *Study II, III* and *IV*, respectively. However, these substances are non specific and to avoid off target effects the more specific RNAi method targeting Cx 26 and Cx43 was used (Spray et al. 2006). For a detailed description, see *Study II*.

3.7.5 Western blot

To detect presence of Cx26 and Cx43 in mNSCs and in hNSCs, routine immunoblotting was performed in *Study II* as previously described (Salomonsson et al. 2002). Immunoblotting was also made in purpose to estimate the level of Cx43 protein suppression in RNAi treated cells. For details, see *Study II*.

3.8 *IN VIVO* MODELS

In order to investigate if the beneficial effects seen after mNSC engraftment to OCs were also present *in vivo*, three different *in vivo* models were investigated in *Study II*. This was done in cooperation with Jianxue Li and Richard L. Sidman at Harvard Medical School (the nervous mouse model), Satyan Chintawar and Massimo Pandolfo at Université Libre de Bruxelles (the spinocerebellar ataxia type 1 mouse model) and Yang D. Teng at Harvard Medical School (the contused cervical spinal cord rat model).

3.8.1 *Nervous* (*nr*) mouse model

The *nervous* (*nr*) mutation originally occurred in the BALB/cGr mouse strain (Li et al. 2006b). In this mutant mouse model, a majority of the Purkinje neurons (PNs) die by the forth to fifth postnatal weeks due to excessive tissue plasminogen activator activity (tPA). By P 21 all affected animals will exhibit characteristic like hyperactivity and ataxia. For details see (Li et al. 2006b).

3.8.2 Spinocerebellar ataxia type 1 mouse model

The spinocerebellar ataxia type 1 (SCA1) mouse model (Burright et al. 1995) recapitulate many pathological and behavioral characteristics of the human disease spinocerebellar ataxia type 1. SCA1 is a hereditary neurodegenerative disorders caused by the expansion of a CAG trinucleotide repeats which is translated into a polyglutamine tract in the ataxin-1 protein (Orr et al. 2007). The ataxin-1 is localized in cerebellar PNs and through a toxic gain of function the faulty protein destroys the affected cells (Bowman et al. 2007). The SCA1 mouse model develops a progressive motor disorder starting at about five weeks of age. For details see (Chintawar et al. 2009).

3.8.3 Contused cervical spinal cord rat model

Spinal cord injury was induced in Sprague-Dawley rats by creating a four millimeter long longitudinal cut along the midline of the spinal cord at cervical level 4-5. For details see (Teng et al. 2002).

3.9 ETHICAL PERMISSIONS

All animal experiments were performed in accordance with European Community guidelines and approved by the regional animal research ethics committees at

Karolinska Institutet, Harvard Medical School and Université Libre de Bruxelles respectively.

3.10 STATISTICAL METHODS

Statistical differences between groups with equal variance were determined by ANOVA and post hoc Bonferroni tests. Variance was determined by the Brown-Forsythe test. Categorical responses were compared with Fisher's Exact Test. Differences were considered to be statistical significant at $P<0.05^*$, $P<0.01^{**}$ and $P<0.001^{***}$. Data are presented as means ± SEM.

4 RESULTS AND DISCUSSON

This chapter summarizes the most important findings in the thesis with brief reflections and a final general discussion.

4.1 ORGANOTYPIC CULTURES AS A MODELL SYSTEM

In *Study I* we characterized the organotypic culture and evaluated if it was a suitable model system to study graft and host interactions. The culture system was analyzed weekly during 28 DIV. During this time period it gradually thinned from initially 250 µm to a thin layer of cells. The thinning of the OCs was mainly due to cell migration and cell death which was facilitated by the gravitational forces generated by the constant rotation in the roller drum device **[Fig. 1a, b]**.

The original tissue architecture was altered during the time in culture but most of the neuronal connectivity was retained which was demonstrated by preserved synaptotagmin stainings. The plasma and thrombin clot that initially covered the OCs dissolved during the first days *in vitro* but still caused some background fluorescence when the cultures were immunostained at 7 DIV.

At the time of OC establishment (0 DIV) nestin expression in cryosectioned striatal tissue was estimated to 10 ± 1% and GFAP expression to 4 ± 1%. This expression increased to 56 ± 4% respectively 63 ± 3% after 7 DIV, but returned to lover levels after 28 DIV, 26 ± 2% and 24 ± 3% respectively. The dynamics in nestin and GFAP expression can be viewed as metrics of cellular stress and correlates to the degree of injury inflicted upon the OCs during establishment (Frisen et al. 1995; Pekny et al. 2005). The feature of nestin and GFAP manifestations as response to injury, the reproducibility of the OC system, the unmatched optical conditions combined with the tissue thinning during time *in vitro,* provided an optimal platform to study cell level graft and host interactions.

4.2 STEM CELL ENGRAFTMENT

4.2.1 Neural stem cells before engraftment

In *Study I-III* we investigated the survival, proliferation and differentiation of the C17.2 clone. Thawing and expanding stem cells generated large quantities of undifferentiated mNSCs. The undifferentiated state of the mNSCs was reflected by their nestin expression. The majority of both the NSC-GFP and NSC-NT-3 cells expressed the stem and progenitor cell marker nestin. The NSC-NT-3 clone was already at the time

Tissue flattening following OC establishment

a 1 DIV

b 28 DIV
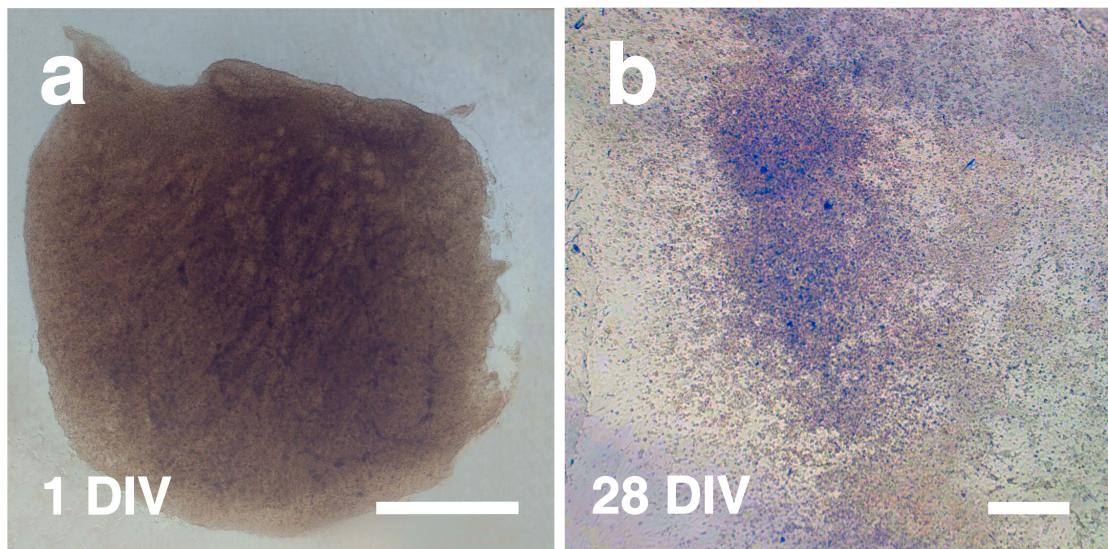
Integration of grafted neural stem cells
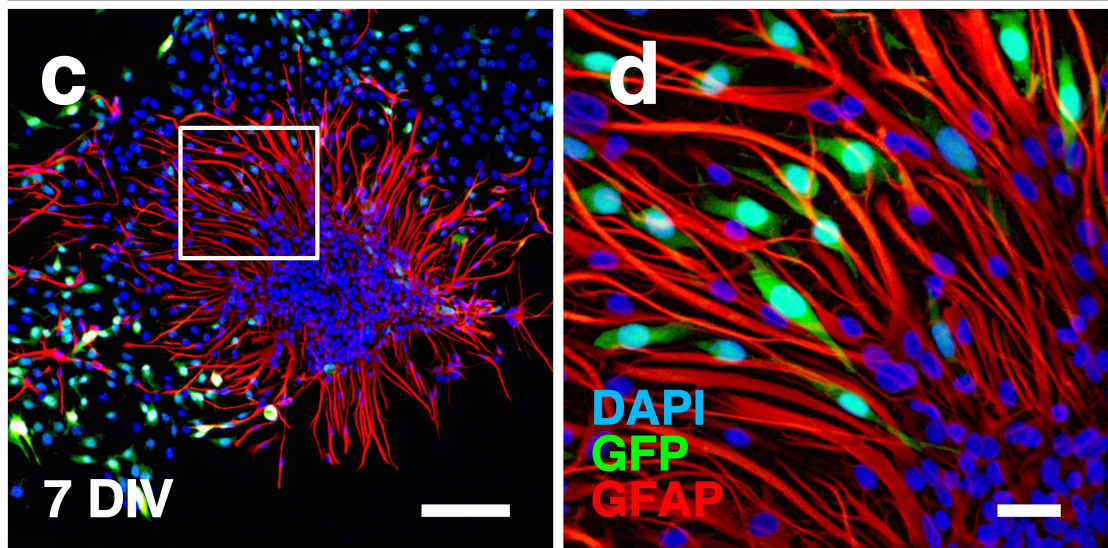
c 7 DIV

d DAPI GFP GFAP

Figure 1. **Grafted neural stem cells survived and integrated throughout the organotypic culture.** The striatal tissue slices used to establish the organotypic culture gradually thinned-down and spread out during the time in culture. At the first day in culture, the striatal tissue slice had an opaque appearance and well-defined boarder **[a]**. Subsequently, during progressive time in culture, it transformed to a thin layer of cells at 28 DIV **[b]**. Stem cells grafted within 1 mm from the organotypic culture migrated towards the slice and integrated into the host tissue **[c]**. The highlighted area in **[c]** is displayed at increased magnification in **[d]**. The grafted mNSCs were recognized by GFP fluorescence (green). DAPI nuclear stain is blue and GFAP is red. Scale bars: 200 μm in **[a]**, 100 μm in **[b, c]** and 20 μm in **[d]**.

for engraftment more prone to differentiate towards a neuronal linage and had started to express the early neuronal marker Tuj1 (9 ± 2%). NSC-NT-3 also expressed small amounts of GFAP. Besides being an astrocytic marker GFAP has also proven to be expressed by many postnatal and adult-derived NSCs (Alvarez-Buylla et al. 2001).

All examined cells retained their β-galactosidase expression revealed by the X-gal stainings. NSCs also had a strong GFP expression at the time for engraftment, which remained during the entire studied culturing period.

The MMC treatment did not affect the morphology of the NSCs and effectively halted the NSCs in the cell cycle. In a subset of experiments, we grafted non-MMC treated mNSCs to the OCs. This was associated with overgrowth and subsequently caused detachment of the striatal tissue and NSCs from the cover slip.

4.2.2 Neural stem cells after engraftment

Grafted mNSC gradually migrated towards and integrated into the striatal tissue **[Fig. 1c, d]**. After 28 DIV the mNSC were fully integrated into the striatal tissue and morphologically formed a part of the host parenchyma. The ratio of surviving mNSCs compared to the original NSC number grafted was 0.8 ± 0.02 after 28 DIV, which corresponded to a mNSC:OC cell ratio of 1:10.

The NSC-GFP clone reduced its nestin and GFAP expression, while the Tuj1 and Map2ab remained constant during time *in vitro*. The neural maturation could in part be induced by culturing in NB/B27 medium, which favored the Map2ab expression, but had little effect on the Tuj1 expression. After 28 DIV in OC medium the Map2ab expression was 6 ± 1% and the Tuj1 expression 44 ± 3%. When culturing in NB/B27 medium the NSC-GFP cells expressed 67 ± 2% Map2ab and 41 ± 3% Tuj1. Why the Map2ab expression but not the Tuj1 expression was enhanced by the serum-free medium may be explained by altered transcription of mNSC genes induced by the mix of external cues.

The NSC-NT-3 clone reduced its nestin and GFAP expression during the time *in vitro*. After 28 DIV 0.2 ± 0.1% and 0.6 ± 0.4% of the NSC-NT-3 cells were reactive for nestin and GFAP while 70 ± 2% and 19 ± 4% expressed Tuj1 and Map2ab. The Map2ab expression could further be induced by culturing in the NB/B27 medium, which after 28 DIV resulted in a Map2ab expression of 68 ± 3%.

Neither culturing in serum-free NB/B27 medium nor endogenous NT-3 over-expression could however promote a complete differentiation of the grafted cells

towards fully mature neurons. During the studied 28 day time period none of the grafted cells displayed positive synaptotagmin stainings.

We also concluded that the strong neuronal differentiation of the NSC-NT-3 clone, reflected in Tuj1 and Map2ab expression, made it especially suitable for studies of interactions between grafted young neurons and host cells.

4.3   ENGRAFTMENT PROVED BENEFICIAL FOR THE HOST

In *Study I and II* we evaluated the influence of the grafted NSCs on the host cells after implanting mNSCs to striatal organotypic cultures. For this we employed nestin and GFAP immunostainings to estimate cellular stress, PI and CC-3 stainings to quantify the amount of necrotic and apoptotic cells and the overall survival of the OC as a general measurement of the outcome. Host cell stress, depicted by the nestin and GFAP expression, was reduced by 25-30% during the first and second week in culture when the OCs were grafted with the NSC-GFP clone. A similar impact on the host cells was observed when OCs were grafted with the NSC-NT-3 clone, with a 28% reduction in nestin reactivity and a 43% decline in GFAP expression after 14 DIV. We also noticed that host cell necrosis, estimated by PI staining at 7 DIV, was reduced by 86% when OCs were grafted with the NSC-GFP clone and by 71% when grafted with the NSC-NT-3 cells. In addition, the CC-3 expression was reduced by 78% and 64%, respectively. The same parameters were investigated in *Study IV* at 21 DIV after hESC engraftment. A similar beneficial impact was witnessed using hESCs. In this case, GFAP was reduced by one fourth while CC-3 expression was decreased by two thirds. The OC cell necrosis was however unaffected.

The overall survival increased by 43% after engraftment with the NSC-GFP clone. The timing of the NSCs implantation turned out to be of importance. Grafting mNSCs to the OCs 0-24 hours after establishment resulted in an increased overall survival. However, grafting mNSCs 48-72 hours after establishment was not associated with an increased overall survival and grafting cells 96-144 hours after establishment, unexpectedly, had a negative effect on OC survival. This indicate that grafted mNSC, which entered to the acutely damaged tissue were able to aid cell survival but when grafted to a fully matured injury they were unable to assisting continued existence of host cells. Engraftment with the more neuronally predestined clone, NSC-NT-3, was not associated with any improvement in overall survival. Since engraftment of both the NSC-GFP and NSC-NT-3 clone was associated with comparable reductions in host cell gliosis, necrosis and apoptosis we concluded that the beneficial effects observed in this

model were not dependent of growth factor overexpression. NT-3 is known to be a neuroprotective agent (Connor et al. 1998) and exerts its effect by TrkC receptor activation. These receptors are present in the striatum (Escandon et al. 1994; Lamballe et al. 1994; Jung et al. 1996) so the lack of a more pronounced beneficial effect on striatal cells must be explained by another mechanism than altered NT-3/TrkC activation. A possible explanation might be coupled to naturally secreted growth factors like BDNF and GDNF, which are produced by the native mNSCs. Likely due to the increased NSC differentiation induced by NT-3 overexpression, GDNF and BDNF secretion is decreased in NSC-NT-3 cells (Lu et al. 2003). Since both GDNF and BDNF are potent neuroprotective factors (Deierborg et al. 2008) the limited effects exhibited by the NSC-NT-3 clone might instead be explained by the decreased GDNF/BDNF secretion.

4.4 GRAFT AND HOST COMMUNICATION

In *Study II* the electrophysiological properties of NSC derived cells and host cells were determined. Non-grafted mNSCs displayed limited sodium/ potassium (Na^+/K^+) channel activity and had a less pronounced resting membrane potential (V_m) and high membrane resistance (R_m). After engraftment to OCs the mNSCs showed significant developmental differences in both V_m and R_m. After 28 DIV 66% of the mNSC displayed active membrane properties and ability to fire single action potentials, indicative of differentiation towards a neuronal phenotype. The number of Na^+/K^+ channel was however insufficient to generate repetitive action potentials characteristic of fully mature neurons. This indicated that the four weeks of culturing in the organotypic culture system, was not enough to allow complete maturation of the grafted mNSCs. Previous studies have shown that neuronal maturation is a time consuming process, where synaptic connectivity takes up to 60 days to form (Toni et al. 2007). Despite the absence of neurons capable of repetitive firing, extensive functional communication between graft and host cells was present in the form of synchronous calcium (Ca^{2+}) fluctuations.

Spontaneous fluctuations were commonly observed in both grafted and non-grafted OCs. Both ATP and glutamate could provoke such fluctuations. In half of all the examined grafted OCs, synchronous Ca^{2+} fluctuations between grafted mNSCs and striatal host cells were noticed. The temporal overlapping Ca^{2+} fluctuations indicated that mNSC were able to participate in Ca^{2+} mediated host networks and become a functional part in host Ca^{2+} mediated circuitry. Because calcium transients is known to

be spread through the P2 purinergic receptors (P2R) upon ATP activation and since application of ATP evoked transient and sustained Ca^{2+} elevations in the majority of both mNSCs and OC cells, the P2R antagonist suramin was applied in order to determine the mechanism underlying the synchronous graft host Ca^{2+} activity. Suramin did however not affect the overlapping Ca^{2+} events, suggesting that the interplay between mNSCs and OC cells was not dependent on ATP-P2R interactions. It is known that during normal CNS development oscillatory patterns of electrical activity, rather than mature chemical synapses, interpret the biological blueprint and orchestras the formation of the cortex (Weissman et al. 2004). The presence of calcium wave signaling between grafted mNSCs and OC cells seemed to recapitulate events from embryonic development. We knew that gap junctions are widely expressed during embryonic development and provide a route for cell-to-cell contact and communication that can mediate the spread of Ca^{2+} waves across cell populations (Elias et al. 2007; Elias et al. 2008). Therefore, we hypothesized that formation of gap junctions underlies the coupled Ca^{2+} fluctuations and the early functional integration of grafted mNSCs.

4.5 GAP JUNCTION FORMATION BETWEEN GRAFT AND HOST CELLS

Gap junctions are formed by the consolidation of two connexons on adjacent cells. Each connexon consists of six connexin subunits. These intracellular bridges connect the cytoplasm of neighboring cells and allow the exchange of small molecules less than 1000 Da through passive diffusion (Goodenough et al. 2003). However, larger molecules can evidently pass through if they have the correct three-dimensional structure (Neijssen et al. 2005; Cieniewicz et al. 2010).

Since Cx26 and Cx43 are important during neural development (Cina et al. 2007; Elias et al. 2008; Goodenough et al. 2009) and increased Cx43 expression is a hallmark of injured neural tissue (Rouach et al. 2002) we choose to focus on these and investigated their expression in both host and graft cells. Both connexin subtypes were commonly seen in OC cells. Cx43 often co-localized with nestin and GFAP, while Cx26 co-localized with Tuj1 expressing host cells. Before engraftment, 49 ± 14% of the mNSCs expressed Cx43 and 9 ± 2% expressed Cx26. After grafting, the mNSCs started to form functional gap junctions with host cells already two hours post engraftment. After 3 DIV there was a marked increased in connexin-expression, 92 ± 1% of the mNSC expressed Cx43 and 24 ± 1% expressed Cx26.

During the 28 DIV we observed that the Cx43 expression declined whereas the Cx26 expression in mNSCs increased. Since the mNSCs initially had a nestin/GFAP

positive phenotype which successively changed into a Tuj1 positive phenotype during the 28 DIV, it seemed that the changes in connexin expression paralleled the maturation of the mNSCs. We concluded that Cx43 expression was associated with immature mNSCs and non-neuronal cells, while the Cx 26 expression was associated with neuronally differentiated mNSCs.

To investigate whether the presence of connexins and functional gap junctions in mNSCs, was a unique finding or if it could have a wider applicability, we studied the same variables in hNSC grafts. Similar results were obtained. The hNSCs also expressed Cx43 and formed gap junctions upon initial contact with OC cells. As for mNSCs the hNSCs were also able to functionally integrate into host Ca^{2+} mediated networks.

To evaluate the functionality of the gap-junctional couplings, standard dye loading experiments were performed. Scrape loading, dye transfer from pre-loaded NSCs and dye transfer following microinjection (as described in section 3.7.2) showed extensive spreading of the gap-junctional permeable dyes LY and calcein, indicative of functional gap-junctional couplings between both grafted human and murine NSCs and host striatal cells.

4.6 BLOCKING GAP-JUNCTIONAL COMMUNICATION

The role of gap-junctional couplings in the graft-host interplay was verified using a combination of inhibition strategies. Transfer of LY and calcein from grafted NSCs to neighboring OC cells as well as coupled graft-host Ca^{2+} transients was effectively suppressed by the pharmacological gap junction channel blockers CBX and 18-α-GA. In addition, pharmacological inhibition of gap junction function abrogated the beneficial impact grafted mNSCs and hNSCs exerted on host cells. Following CBX treatment of grafted cultures, the gliotic response in OC cells was comparable with that of the non-grafted cultures. To more specifically investigate the impact of gap-junctional communication on host cell well-being and to avoid off target effects caused by the pharmacological inhibitors, we complemented the gap junction blocking studies by suppressing Cx26 and Cx43 in the mNSCs using RNAi techniques. Blocking the Cx43 formation by 30-70% abrogated the beneficial effects of NSC grafting in a dose dependent manner, i.e. the antigliotic actions were more prominent when engrafting mNSCs with 70% of the Cx43 expression retained compared to when grafting mNSCs with only 30% of the original Cx43 expression. Blocking Cx26 alone had little effect

on host cell gliosis, suggesting that the therapeutic action exhibited by the mNSC was mainly mediated by Cx43.

4.7 HOST CELL RESCUE *IN VIVO*

In order to investigate whether the intracellular cross-talk held translational relevance (i.e. was relevant in other model systems) we examined the interactions between grafted mNSCs and host cells *in vivo*. We employed two mouse models where transplanted cells had proven to rescue imperiled host neurons.

In the SCA1 mouse model, the mutated protein ataxin-1 causes selective death of PNs. The mutant mice exhibit at 24 weeks of age a significant loss of PNs which results in severe ataxia and behavioral changes. When transplanting NSCs, originated from the subventricular zone of adult SCA1 mice a promoted survival of PNs, behavioral amelioration, improved cerebellar morphology and a restored PN excitability was observed, even though no grafted cells adopted the morphological and immunohistochemical characteristics of PNs. Rather, this near-normal histological, anatomical and behavioral findings was due to preserved host PNs (Chintawar et al. 2009). The mNSCs mediated host cell rescue was witnessed only when there was a direct contact between transplanted cells and host PNs and immunohistological analysis revealed an extensive presence of Cx43 at the interface between rescued PNs and transplanted mNSCs. This local connexin reactivity was not detected in sham-transplanted or wild-type animals.

In the *nr* mouse model the same PNs degenerate through another mechanism, but are likewise saved by transplanted NSCs. When NSCs are transplanted to the premorbid *nr* mouse the mNSCs can rescue a majority of the PNs from an impending death if there is a direct cell-to-cell contact between the transplanted cells and the host cells (Li et al. 2006a). Immunohistochemistry showed abundant Cx43 but no, or very little, Cx26 expression between rescued PNs and exogenous mNSCs. Wild type and non-transplanted *nr* mice did not express the same abundant immunoreactivity for Cx43. To determine if suppression of gap junction formation would abrogate the mNSCs mediated rescue, we inhibited Cx43 expression in donor mNSCs with RNAi. This strategy yielded surviving PN numbers as poor as in non-transplanted *nr* mouse. Again, the rescuing effect was dependent on the level of RNAi suppression. When transplanting mNSC transfected with non-functional RNAi the mNSCs continued to promote PN survival.

In the contused cervical spinal cord rat model grafted hNSC promote recovery of motor and respiratory function. Immunohistological analysis revealed Cx43 between graft and host cells. Cx43 expression was not observed in the absence of engraftment.

In summary, we observed that grafted mNSC reduced OC gliosis, necrosis and apoptosis and increased overall survival, integrated functionally in Ca^{2+} mediated host networks and were extensively dye coupled. These features could be hindered either by employing pharmacological gap junction blockers or by suppressing connexins by RNAi, especially Cx43. Observations from three different *in vivo* models indicated that the findings had a translational relevance.

We concluded that gap junction formation appeared to be a crucial early step and set the stage for the functional interplay between grafted cells and host cells that ultimately lead to a beneficial outcome for the host.

4.8   CONNEXIN EXPRESSION CHANGED AFTER ENGRAFTMENT

In the second study we noticed that there were clear temporal aspects affecting the outcome of mNSC grafting. The time point for the NSC implantation greatly influenced the overall survival of the OCs and an early engraftment proved beneficial for the host while late engraftment even was associated with a decreased overall survival. Data from *Study II* indicated that gap junctions underlie functional and beneficial interactions between the grafted neural stem cells and the host. In S*tudy III* we therefore choose to investigate the dynamics in connexin expression and gap junction formation in mNSCs and OC cells at different time points after engraftment in order to determine if there existed an optimal time window for engraftment.

Estimated by flow cytometry recordings the NSC-GFP and the NSC-NT-3 clone expressed high levels of Cx43 (92% and 94%, respectively) and lower levels of Cx26 (21% and 24%, respectively) before engraftment. After engraftment, the connexin expression in mNSCs displayed significant changes. At 7 DIV the immunoreactivity for Cx43 in NSC-GFP and NSC-NT-3 was $95 \pm 1\%$ and $85 \pm 1\%$, respectively After 28 DIV the expression had decreased to $47 \pm 4\%$ and $28 \pm 3\%$, respectively. As previously described, the Cx43 immunoreactivity was typically co-localized with nestin and GFAP and decreased during the progressive neuronal differentiation and progression towards a Tuj1 positive phenotype. The lower Cx43 expression in the NSC-NT-3 clone could hence be explained by its intrinsic predestination towards a neuronal linage (Lu et al. 2003). The pattern of Cx43 expression could also be altered by exogenous growth

factors. Culturing mNSCs in NB/B27 medium reduced the Cx43 expression through its neuronal inducing properties (Brewer 1995).

In the acutely isolated striatal tissue, approximately one fourth of the examined cells expressed Cx43. After 7 DIV nearly all of the examined cells expressed Cx43. At later stages (14-28 DIV) the OC cell connexin expression returned to lower levels. The Cx26 expression in grafted mNSCs and host cells did not display consistent temporal and growth factor mediated changes comparable to those evident for Cx43. This might reflect a different function of Cx26 consistent with our findings in *Study II*, where we conclude that Cx43 played a more prominent role as a health promoting substrate. When aligning the Cx43 expression profiles in mNSCs and OC cells, we realized that there were a synchronous peak in graft and host Cx43 expression at 7 DIV. The overlapping elevations of Cx43 could set the stage for beneficial graft-host interactions and thereby potentially create a "window of opportunity" for optimal stem cell grafting. Within this time window, there are optimal conditions for host cell rescue by direct health promoting interaction with grafted cells is at and subsequently mNSC mediated rescue via gap junction channels.

4.9   ENHANCING THE GRAFT AND HOST COMMUNICATION

In *Study IV*, we investigated if pharmacological pretreatment of the hESCs could be used to increase connexin expression and thereby enhance the beneficial graft and host interplay. The Rho-associated kinase inhibitor (ROCKi) Y-27632, Fausidil®, is clinically used as a migraine medicine and has been showed to augment gap-junctional couplings in retinal epithelial cells (Anderson et al. 2002). It has been applied in clinical practice for nearly 20 years (Zhao et al. 2006). We employed ROCKi as a strategy to increase connexin expression both pre- and post engraftment. An eight hours exposure to ROCKi increased the Cx43 expression in expanded hESCs by 61% (from $40 \pm 3\%$ to $66 \pm 3\%$) and was accompanied by a 250% more extensive dye spread compared to non-treated hESCs. The dye spread could be blocked by the pharmacological gap junction blocker CBX. When grafting ROCKi treated hESCs, host cell gliosis was reduced to $16 \pm 1\%$ compared to $33 \pm 3\%$ when grafting native hESCs. Surprisingly, the host cell necrosis in OCs grafted with ROCKi treated hESCs was higher compared to cultures grafted with non-ROCKi treated hESCs. This may be due to off-target effects since even relatively selective ROCK inhibitors such as Y-27632 have been shown to inhibit other kinases than ROCK (Anderson et al. 2002). Nevertheless, Rho-associated kinase inhibition presents a promising strategy to increase graft and host

interactions. This might be important for the optimization of therapeutic stem cell applications.

4.10 GENERAL DISCUSSION

The main finding in this thesis is that the formation of gap junctions is a pivotal first step where graft and host cells establishes functional communication which ultimately leads to increased host well-being. We observed that the formation of gap junctions goes through a series of dynamic changes after the NSCs engraftment which can be altered by the use of pharmacological agents.

Gap junctions are widely expressed during embryonic development and provide a mean of cell-to-cell contact and communication and can also act as adhesive molecule which aids neuronal migration in the cortex (Elias et al. 2008). The wide distribution and conservation of connexins in different neural cells underline their fundamental role for cell function and health (Kumar et al. 1996). The importance of gap junctions in brain pathology is reflected by observations that aberrations in connexins and gap junctions are associated with disorders like stroke (Rouach et al. 2002), AD (Nagy et al. 1996), PD (Rufer et al. 1996) and HD (Vis et al. 1998). Whether alteration in gap junction expression is a cause or a consequence in different brain pathologies is a matter of scientific dispute. The relationship between brain pathologies and modulations in gap-junctional couplings is highly complex and current data present a contradictory picture (Rouach et al. 2002). Alteration in gap junction function can ultimately lead either to "bystander death" by the propagation of the injury through gap junctions to neighboring cells or alternatively to a "good Samaritan effect" where healthy cells rescue dying cells by buffering ions and harmful metabolites or by directly supplying beneficial metabolites (Farahani et al. 2005; Leis et al. 2005; Nakase et al. 2006). The establishment of functional gap junctions between grafted stem cell progeny and host cells at risk permits direct delivery of factors that promote survival like ATP, ions (De Maio et al. 2002), small interfering ribonucleic acid (siRNA) (Valiunas et al. 2005), glutathione (Alexander et al. 2003), second messengers (Niessen et al. 2000; Bedner et al. 2005) and polypeptides (Neijssen et al. 2005) as well as neutralize pathological molecules or processes like ROS and excitotoxicity. Gap junctional coupling between graft and host cells might also permit implanted stem cells to directly participate and influence host cell calcium basted activity. Calcium fluctuation serve as a key regulated of many cellular vital processes **[Fig. 2]**. We believe that these mechanisms in part explain the beneficial effects seen after stem cell grafting to the injured CNS.
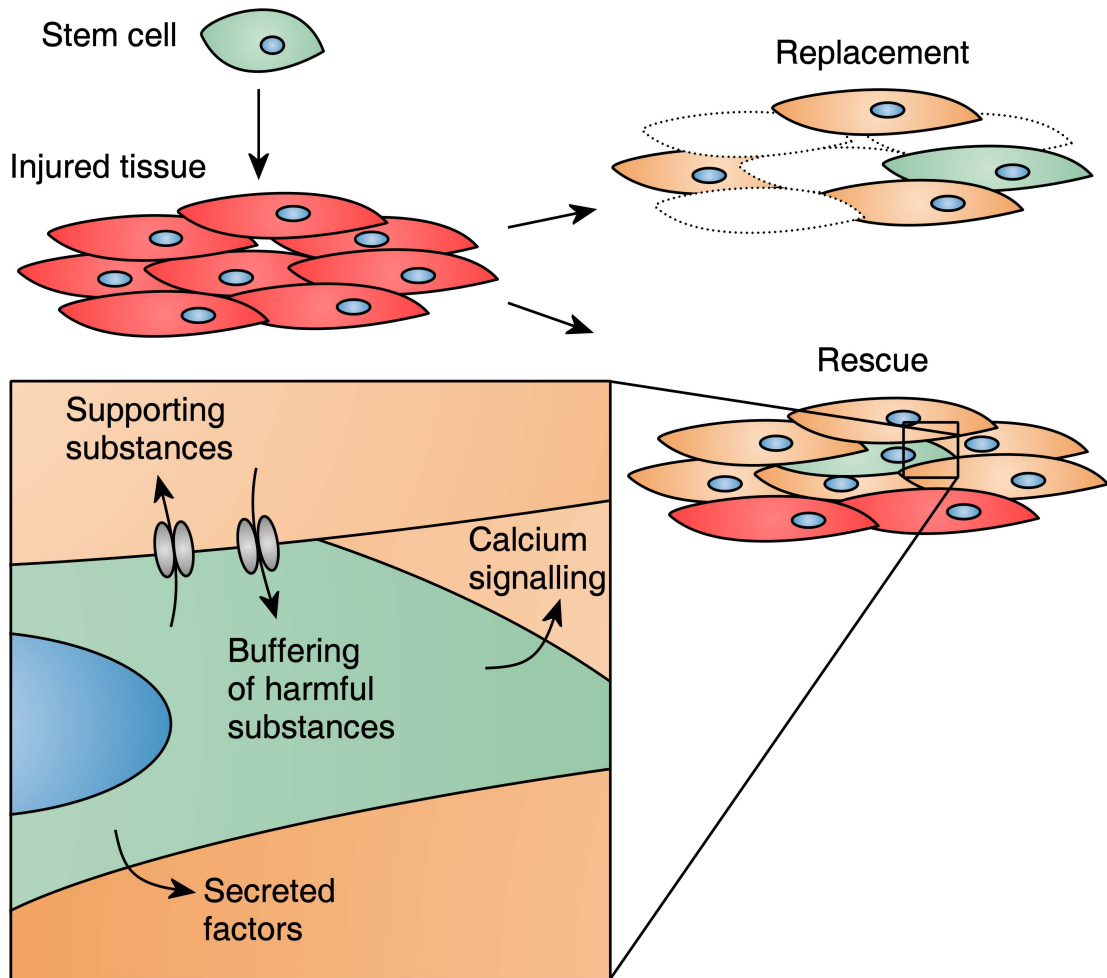
Figure 2. Stem cell interactions with the injured brain. Grafted stem cells interact with the host tissue through a multitude of mechanisms. Injured central nervous system (CNS) tissue **(top left, red cells)** is typically characterized by ion dyshomeostasis, energy depletion, excitotoxicity, altered gene transcription and accumulation of reactive oxygen species (ROS) and other harmful metabolites. Stem cells **(green cells)** which are grafted into this environment, can either replace lost cells **(top right, lost cells marked by dotted contours)** or rescue host cells at risk **(bottom right, orange cells)**. This might occur by a combination of support by secreted factors and direct beneficial interactions via gap junctions. Through established graft-host gap junctions, added stem cells can supply host cells with supporting molecules such as adenosine, ADP, ATP, glutathione, genetic material and second messengers and simultaneously "buffer" harmful events such as ion dyshomeostasis and production of ROS and other harmful substances. The direct gap junctional link also enables a direct route for calcium-based communication, which potentially could change the host cells internal calcium fluctuations toward a more beneficial pattern. Calcium signaling patterns govern diverse cellular processes such a cell differentiation, proliferation, migration, gene expression, exocytosis and apotosis.

5 CONCLUSIONS

This thesis illustrates that organotypic striatal cultures (OC) provide a suitable model system to study graft and host interactions. After engraftment to the OCs the NSCs survived, differentiated and gradually became morphologically and functionally integrated into the host parenchyma. The NSC differentiation could be directed towards a neuronal lineage by serum free culturing conditions and endogenous over expression of growth factors.

Gap-junctional couplings preceded the formation of chemical synapses, and this form of communication appears to be one of the first steps whereby grafted cells and host cells establish functional and beneficial interactions. The intracellular cross talk between coupled graft and host cells was associated with a beneficial impact on the damaged host cells, as estimated by metrics like host cell astrogliosis, necrosis, apoptosis and overall OC survival. The gap junction expression pattern was highly dynamic in both in graft and host cells. The co-incidence of gap junction expression peaks in graft and host cells early after engraftment indicated a window of opportunity for optimal NSC engraftment. Finally, a pharmacological Rho-associated kinase inhibitor could be used to actively increase the gap junctional communication in hESC. The effect was paralleled by a more pronounced beneficial impact upon the damaged host cells after grafting ROCKi treated stem cells.

The exact mechanisms underlying graft host gap junctional communication and the exact identity of the transferred molecules that mediate the beneficial effects however remain to be investigated. This in itself opens the field for a multitude of future studies.

6 ACKNOWLEDGEMENTS

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