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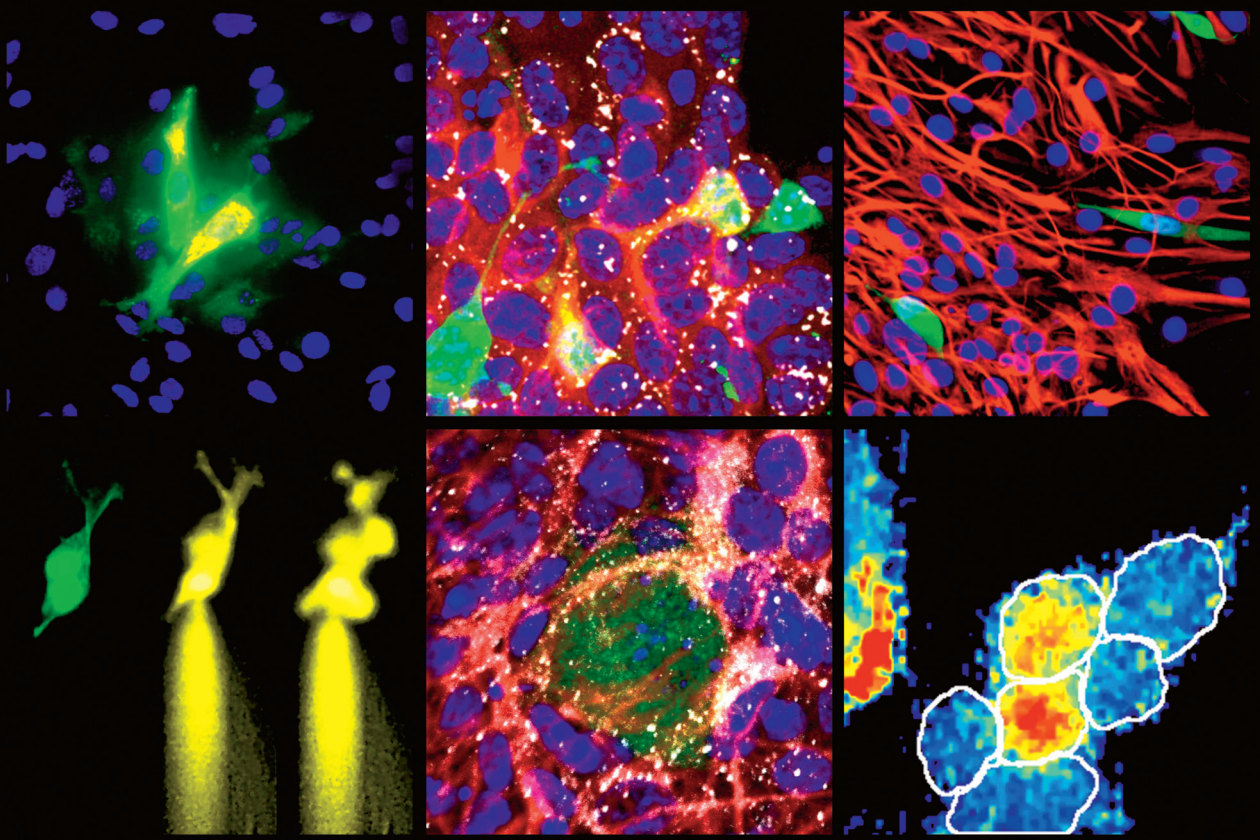
Neural stem cell engraftment

Functional interactions, brain repair and gap junctions



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Cover illustration

Top left panel | Direct intercellular communication between graft and host cells. Two functionally integrated engrafted murine neural stem cells (mNSCs) preloaded with the gap junction-permeable dye calcein (green) and labeled with the membrane dye DiI (red). Graft-host gap-junctional interaction is indicated by the spread of calcein to surrounding DiI negative host cells. Detailed in *Study II*, Fig. 2.

Top middle panel | Gap junctions containing connexin 26 are present between graft and host cells. Gap-junctional couplings consisting of connexin 26 (white) between grafted GFP (green) and Tuj1 (red) positive mNSCs and recipient host cells. DAPI nuclear stain is blue. Detailed in *Study II*, Fig. 1.

Top right panel | Gap-junctional couplings are necessary for the beneficial effects of neural stem cell engraftment. Suppression of connexin synthesis in mNSCs (green) by RNA interference blunted their ability to decrease OC gliosis estimated by GFAP immunohistochemistry (red) in a dose-dependent manner. DAPI is blue. Detailed in *Study II*, Fig. 3.

Bottom left panel | Grafted cells form functional couplings to host cells. Progressive spread of Lucifer Yellow (yellow) from patched and microinjected grafted mNSCs (green) to surrounding host cells evident of gap junction formation. Detailed in *Study II*, Supporting Information, Fig. S2.

Bottom middle panel | Gap junctions containing connexin 43 are present between graft and host cells. Gap-junctional couplings consisting of connexin 43 (white) between grafted GFP (green) and nestin (red) positive mNSCs and recipient host cells. DAPI is blue. Detailed in *Study III*, Fig. 3**.**

Bottom right panel | Grafted neural stem cells participate in functional host network activity. Graft and host cells loaded with the ratiometric calcium indicator Fura-2 to allow for characterization of calcium based communication between grafted mNSCs and endogenous host cells. Regions of interest are marked by white circles. Detailed in *Study II*, Supporting Information, Movie. S3.

ABSTRACT

Neural stem cell (NSC) engraftment therapies show increasing promise as potential cures for several of the diseases of the central nervous system. Traditionally, functional improvements after NSC engraftment have been attributed to replacement of lost neurons and astrocytes, remyelination and support of adjacent cells at risk. How grafted NSCs and their progeny integrate into recipient brain tissue and functionally interact with host cells is as yet not fully understood.

In this thesis we have investigated the early and beneficial interactions between grafted NSCs and neural host cells. Initially the use of organotypic striatal slice cultures as an *ex vivo* experimental model to study integration of NSCs grafted to striatal tissue was evaluated. Using this model we recognized that NSC engraftment had a positive impact on the host tissue estimated by reductions of host cell astrogliosis, apoptosis and necrosis. Growth factor overexpression and serum free culturing conditions affected both the differentiation of the exogenous NSCs as well as their interactions with the host.

We observed that one of the first and essential ways in which grafted murine as well as human NSCs integrate functionally into host neural circuitry and affect host cells, even before consummation of neuronal differentiation, is via gap-junctional coupling. In addition to providing a potential template for subsequent mature electrical coupling, the gap junctions permit exogenous NSCs to exchange ions and molecules with host cells and participate in host network activity, including synchronized calcium transients in fluctuating networks. In both murine and human NSCs grafted to slice cultures and rodent models of neurodegeneration, gap junction formation was associated with the rescue of host cells. Both *in vitro* and *in vivo* the beneficial actions of the NSCs were abrogated by suppressing gap junction formation and function via pharmacologic and/ or RNA-inhibition strategies.

After mechanical injury to the host tissue and successive NSC engraftment the expression of connexins, the substrate for gap junction formation, changed significantly in both graft and host cells. The temporal connexin expression pattern suggested that a window of opportunity for successful host cell rescue by added NSCs exists.

Finally we show that controlled hypoxic preconditioning of NSCs prior to engraftment is a reliable and clinically relevant method to increase NSC hemichannel expression and function and thereby improve the engrafted NSCs' ability to interact with and potentially rescue host cells at risk.

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*, 2010, In Press.

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, 107(11), 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*, 2010, In Press, doi: 10.1016/j.yexcr.2010.07.011.

IV. **Johan Jäderstad**, Hjalmar Brismar , Eric Herlenius. Hypoxic Preconditioning Increases Gap-Junctional Graft and Host Communication. *NeuroReport*, 2010, In Press, doi: 10.1097/WNR.0b013e328340a77b.

CONTENTS

LIST OF ABBREVIATIONS

18-α-GA	18-α-glycyrrhetinic acid
aCSF	Artificial cerebrospinal fluid
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BDNF	Brain-derived growth factor
BP	Basal progenitor
bFGF	Basic fibroblast growth factor
β-gal	β-galactosidase
BMP	Bone morphogenetic protein
CBX	Carbenoxolone
CC3	Cleaved caspase 3
CNS	Central nervous system
Cx	Connexin
Da	Dalton
DiI	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor–2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acicid protein
GFP	Green fluorescent protein
GJIC	Gap-junctional intercellular communication
GL	Granular layer
HBSS	Hanks balanced salt solution
hESC	Human embryonic stem cell
hNSC	Human neural stem cell

IGF	Insulin-like growth factor
iPSC	Induced pluripotent stem cell
LY	Lucifer yellow
Map2ab	Microtubule associated protein 2ab
mESC	Murine embryonic stem cell
ML	Molecular layer
MMC	Mitomycin-C
mNSC	Murine neural stem cell
NB/B27	Neurobasal™ medium supplemented with B27™
NeuN	Neuronal nuclei
nr mouse	Nervous mouse
NT-3	Neurotrophin-3
OC	Organotypic culture
PBS	Phosphate buffered saline
PL	Purkinje layer
PFA	Paraformaldehyde
PI	Propidium iodide
PN	Purkinje neurons
PNS	Peripheral nervous system
RD	Rhodamine dextran
R_m	Membrane resistance
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT-PCR	Reverse transcriptase polymerase chain reaction
SCA-1	Spinocerebellar ataxia type 1
SVZ	Subventricular zone
tPA	Tissue plasminogen activator
TrkC	Tyrosine kinase receptor C
Tuj1	Neuron-specific class III β-tubulin
VEGF	Vascular endothelial growth factor
V_m	Resting membrane potential
WT	Wild-type
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1 INTRODUCTION

Stem cells have, at least during the last two decades, generated more public and professional interest than almost any other topic in biology. One reason neural stem cells have gained such broad attention likely is the future potential to treat severe neurological disorders which today are incurable. Neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease and Huntington's disease, stroke and traumatic lesions such as spinal cord injury together each year affect several thousands of patients in Sweden and millions of patients worldwide. Although the symptoms and age of the affected patients vary considerably the disorders are commonly associated with great emotional and physical discomfort, functional impairment and a shorter life span as well as a high cost both for the individuals, their families and the society as a whole. Finding effective cures is therefore one of the primary goals of medical research today.

The introduction is divided into three major sections; I) Functional neural transplantation; briefly describing the field of CNS transplantation and discussing the major challenges that exist before these techniques can be broadly applied in clinical practice, II) Neural stem cells; summarizing some of the most important facts about neural stem cells – the protagonists of this thesis and III) Gap junctions; presenting an overview of the mechanism of communication we suggest is essential for the early interplay between graft and host cells.

1.1 FUNCTIONAL NEURAL TRANSPLANTATION

1.1.1 Historical view

Transplanting neural cells and tissue from one individual to another is a prototypical strategy to replace lost components of the central nervous system and thereby potentially restore function. This idea is much older than one would anticipate. The first known recorded reference of neural transplantation is found in the autopsy reports of the French surgeon Ambroise Paré published in 1564 (Finger 1990; Dunnett 2009). In his records Paré wrote:

> *"A gentlemen otherwise well, had the idea his brain was rotten. He went to the King, begging him to command M. le Grand, Physician, M. Pigray King's Surgeon-in-Ordinary and myself, to open his head, remove his diseased brain*

and replace it with another. We did many things to him but it was impossible to restore his brain."

More than three hundred years later, in 1890, Thompson conducted the first scientific CNS tissue grafting experiments (Thompson 1890). As the optimistic title of his seminal report "*Successful brain grafting*" suggests this time the outcome was more favorable. In his experiments, Thompson removed 8 mm pieces of neocortex of one cat or dog and then placed them in cavities prepared in another recipient cat's or dog's neocortex. He concluded that the transplanted tissue remained viable for up to 7 weeks and resembled "brain substance". Although the report focused on the survival of the grafted tissue and he reported that a significant degree of necrosis was present, interestingly he also investigated the graft-host couplings and noted that at no point he could trace communicating nerve fibers between grafted tissue cylinders and the host.

Almost 20 years later Tello showed that pieces of peripheral nervous tissue (already at this time known to have an intrinsic regenerative capacity) could be grafted to the CNS and regenerate after 2-3 weeks *in situ*. The regenerative capacity was attributed to neurotropic properties of the PNS tissue (Tello 1911).

In 1917, Dunn found that grafting immature tissue blocks from neonatal rats increased the survival and growth of the engrafted tissue (Dunn 1917).

All these early transplantation experiments used the neocortex as the site of engraftment. The reason for this is probably related to the accessibility of the neocortical surface. Engraftment deep in the brain would have needed extensive dissections and still resulted in less inaccurate engraftment sites.

The prevailing view throughout the first half of the 20th century was, as argued particularly by Ramon y Cajal (1928), that regeneration and growth do not take place in the adult mammalian brain. Therefore, reports of successful neural transplantation were received with skepticism.

After the work of Thompson, Tello and Dunn the research field lost speed with only a few important discoveries during twenty to thirty years. Le Gros Clark showed 1942 that a portion of the facial nerve could facilitate regeneration in the CNS if it was pushed into the brain parenchyma with forceps thereby confirming the data shown by Tello.

Then again neural transplantation was almost forgotten for approximately thirty years until 1971 when Björklund (Björklund et al. 1971) and Das (Das et al. 1971) independently began to elucidate the potential of neural grafting in a more systematic

manner focusing on increased graft viability, precision of graft placement in the brain and the functionality of the implanted cells. During 1960s the view regarding the absence of regeneration of central nervous tissues had also begun to be challenged (as described more in detail in section 1.2.2). In 1970s the field then rapidly progressed and neural transplantation outcome was greatly improved by the introduction of glass capillaries for graft delivery, cell suspension engraftment (compared to the previous tissue block grafting) and the development of improved stereotaxic injection techniques (Dunnett 2009).

Today the most common way to introduce exogenous cells *in situ* utilizes micropipettes and cell suspensions. This progressive development has during the last decades made it possible to implant and study neural and somatic cells and tissues in almost any part of the brain and importantly characterize graft-host interactions in disease states that engage specific defined host structures and neural populations such as Parkinson's and Huntington's disease (Martino et al. 2006; Lindvall et al. 2010).

One of the major goals of neural transplantation studies in recent years has been to better understand the functional interplay between grafted cells and host cells. Several techniques have been employed to demonstrate functional graft-host coupling. Some of the early techniques included anatomic tracing of the graft-host neural circuits by injection of wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP) (Ralston 1990) or FluoroGold (Grabowski et al. 1992). Recently, transplantation of NSCs that have been genetically modified to endogenously express truncated WGA has also been utilized to directly reveal establishment of neural circuits between transplanted NSCs and host cells (Yoshihara et al. 1999; Ourednik et al. 2002). Additionally, expression of synaptophysin has been suggested as an indirect proof of graft-host integration (Zeng et al. 1999). Other indirect methods are the induction of immediate early genes such as *cfos* in transplanted cells or contacted host cells (Sheng et al. 1990; Schulz et al. 1994) and increased metabolic activity in grafted regions measured e.g. by 2-deoxyglucose uptake or PET-imaging.

The possibility to transfect fluorescent labels such as green fluorescent protein (GFP) has recently facilitated use of electrophysiological recordings from living donor cells. Electrophysiological recording using single or double micropipette patching has been used to evaluate the integration of grafted NSCs in several studies e.g. (Auerbach et al. 2000; Englund et al. 2002; Kim et al. 2002).

The first neural transplantations to humans (excluding the 16th century work by Ambroise Paré) were conducted in two patients with Parkinson's disease in 1982. In these experiments by Backlund *et al*, cells from the adrenal medulla were grafted into the striatum by stereotaxic injection (Backlund et al. 1985). Although the symptomatic improvements following these initial transplantations were very limited the experiments were repeated by another group in 1987 with dramatically better outcome (Madrazo et al. 1987). This encouraged several additional clinical trials and the first use of human embryonic tissue as graft material (Lindvall et al. 1989).

NSC engraftment is also often associated with behavioral functional improvements e.g. (Isacson et al. 1986; Lindvall et al. 1990; Pluchino et al. 2003). These functional changes can be estimated with an array of different methods that test for instance motor function, cognitive changes and quality of life. A description of all these is beyond the scope of the current thesis.

1.1.2 Challenges before transition to clinical practice

Transplantation of stem cells as well as predifferentiated neurons and glia show increasing promise as potential therapies for neurodegenerative disorders, traumatic injuries and ischemic damage to the central nervous system. Recently stem cell engraftment has also been suggested as an alternative strategy for gene therapy (Muller et al. 2006b). Stem cell transplantation has been shown to reduce symptoms in several animal models of neuropathology including for instance Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, stroke, multiple sclerosis and spinal cord injury (for reviews, see for instance (Lindvall et al. 2004; Goldman 2005; Martino et al. 2006; Lindvall et al. 2010)). Although considerably more limited, data from human stem cell engraftment experiments also suggest significant improvements (Lindvall et al. 2010).

As discussed by Lindvall and Kokaia (Lindvall et al. 2010) several obstacles remain before stem cell transplantation can be broadly applied in clinical practice. First it should be emphasized that even though stem cell therapies appear to be broadly applicable, the conditions that are being treated have markedly different characteristics and unique functional requirements for the grafts which have to be met. At the same time the techniques for precise delivery of cells to the affected tissues also present very diverse challenges. For instance Parkinson's disease affects an isolated population of dopaminergic neurons predominantly in the substantia nigra whereas Alzheimer's

disease causes widespread plaque formation and neuronal loss in many parts of the brain. In stroke the entire tissue including blood vessels and intricate neural circuits is lost which makes total restoration very complex. In spinal cord injury long projections are broken which require extensive and intricate axonal path finding by the graft.

The targeted CNS pathologies also have distinctly different timescales. In stroke and traumatic injury neural cells are lost within minutes to hours whereas in neurodegenerative disorders progressive host cell demise occurs over a period of years.

The potential use of stem cell grafts to treat neurological disorder should also be weighed against the risks that are involved, for instance tumor formation, immunosuppression and surgical complications. For example in Parkinson's disease several effective drug alternatives exist during the initial phases of the disease and symptomatic treatment is broadly available in advanced stages. The life expectancy is also virtually normal for patients with Parkinson's disease (Lindvall et al. 2010). In comparison patients with amyotrophic lateral sclerosis have no effective treatments and show a rapid disease progression with 50% of patients dying within three years of onset (Mitchell et al. 2007). In such cases stem cell approaches (including the associated risks) may offer substantial advantages.

Finally to optimize and securely use stem cell transplantation in a clinical setting there is still a need for much more extensive information on how the grafted cells give rise to the improvements seen in animal models and human patients and how to improve long time graft survival. Implanted stem cells may directly or indirectly affect the damaged tissue and both replace and rescue host cells at risk (Ourednik et al. 2002; Ourednik et al. 2005). The evaluation of the graft and host cellular interplay is therefore one of most important goals of stem cells research today.

1.2 NEURAL STEM CELLS

1.2.1 Definitions

The definition of a stem cell is “a cell that has the capacity to self-renew as well as the ability to generate differentiated cells” (Smith 2001; Weissman et al. 2001). Thereby a stem cell can give rise both to identical daughter cells and cells with more restricted potential to differentiate. The mode of cell division can be symmetrical which produces two identical cells or asymmetrical which leads to the formation of one new stem cell and one more differentiated cell.

The characteristics of stem cells are often described in terms of self renewal, clonality and potency (Melton et al. 2009). In regard to self-renewal, stem cells are often referred to as “immortal” or to have “unlimited” self renewal capacity. As suggested by Melton (Melton et al. 2009) this choice of words is a bit unfortunate since experiments to verify stem cell immortality per definition would newer end and would outlast the researchers trying to test this hypothesis. Somatic cells have been demonstrated to survive up to 80 cell divisions (Hayflick 1974; Shay et al. 2000). Alluding to this fact, cells capable of more than twice the number of cell divisions (>160) would correspond to cells with “extensive” proliferation (Melton et al. 2009). This kind of extensive proliferation has been reported in embryonic and neural stem cells (Morrison et al. 1997; Smith 2001). For adult stem cells, extensive proliferative capacity might also be defined as a potential to proliferate throughout the lifetime of the animal.

The term, clonality, refers to the ability to give rise to identical daughter cells. Finally, potency, describes the placement in the lineage hierarchy. The fertilized egg constitutes a totipotent cell capable of forming all types of cells including extraembryonic tissues. As the early development of the embryo progresses the blastocyst is formed with an inner cell mass surrounded by trophoblasts. The inner cell mass, which contain the embryonic stem cells, can give rise to all cell types in the body but not extraembryonic tissues and therefore is defined as pluripotent. Multipotent stem cells are formed even later and correspond to more lineage restricted tissue stem cells such as neural or hematopoietic stem cells.

NSCs can self-renew and differentiate into all types of cells in the nervous system including neurons, astrocytes and oligodendrocytes (Gage 2000). The term neural progenitor is sometimes used synonymously to neural stem cell but more correctly refers to NSC progeny with further restricted potential for differentiation (Seaberg et al.

2003; Kriegstein et al. 2009). Four main types of neural stem cells are present in the brain (Conti et al. 2010): I) neuroepithelial progenitors (NEPs) which are found in the early embryo and are responsible for neurogenesis in the neural tube. II) radial glia (RG) which originate from NEPs and serve both as neural progenitors and as support for migrating newborn neurons. RG is the dominating cell type in the developing brain. III) Basal progenitors (BPs) which are neurogenic precursors predominantly located to the subventricular zone (SVZ) in the developing telencephalon. BPs are generated from NEPs in the early stages of development and thereafter from RGs and IV) Adult progenitors which are multipotent neural cells mainly present in the subventricular zone (SVZ) of the lateral ventricle wall and the subgranular zone (SGZ) of the hippocampus of the adult mammalian brain as explained below.

1.2.2 Historical view

The term stem cell first appeared in the scientific literature in 1868 when the German biologist Ernst Haeckel presented the idea of a stem cell or "stammzelle" as the evolutionary ancestor of all multicellular organisms (Haeckel 1868; Ramalho-Santos et al. 2007). In 1874 Haeckel then expanded the term to also include the fertilized egg as a stem cell thereby transferring the concept from evolution (phylogeny) to embryology (ontogeny) (Haeckel 1874).

For several years considerable efforts were then made to characterize the formation of the blood cells. Although Alexander Maximow is often accredited for coining the term "hematopoietic stem cell" in 1909 (Maximow 1909), Artur Pappenheim, already in 1896, defined a potential stem cell that was giving rise to both red and white blood cells (Pappenheim 1896). The existence of hematopoietic stem cell was then experimentally proven 1961 by James Till and Ernest McCulloch (Till et al. 1961).

The description of a potential adult neural stem cell was initially met with doubt since the widespread view in the beginning of the 20th century was that the structures of central system were fixed after birth and that no new neurons could be formed. In a seminal report published in 1928 (y Cajal et al. 1928) Santiago Ramón y Cajal, one of the most influential neuroscientist of that time, wrote:

> *Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated.*

It was not until the 1960s that this view started to be seriously challenged. Using a marker for dividing cells, ^{3}H-thymidine, Joseph Altman and Gopal Das demonstrated the existence of dividing cells in the hippocampus in rats (Altman et al. 1965). After initial skepticism the field then expanded rapidly. Utilizing the thymidine analog 5-bromo-2-deoxyuridine (BrdU) adult neurogenesis has since been shown in mice (Kempermann et al. 1997), birds (Goldman et al. 1983), monkeys (Gould et al. 1998) and humans (Eriksson et al. 1998) as well as in several other mammals (Rakic 2002; Taupin et al. 2002). The first successful isolation and subsequent culturing of NSCs from the adult rat brain was done by Reynolds et al 1992 (Reynolds et al. 1992).

1.2.3 Neural stem cells during CNS development

The formation of the nervous system begins with the induction of the neuroectoderm which gives rise to the neural plate. This occurs at embryonic day (E) 7.5 in mice. At E8.5 in mice the neural plate folds to form neural tube. Both of these structures are composed from a single layer of neuroepithelial progenitors (NEPs). The central pore of the neural tube later forms the ventricular system and spinal canal.

Briefly, the patterning of neural tube along the dorsoventral axis is governed by gradients of morphogens, most importantly BMPs, Wnt and sonic hedgehog (Shh) while the rostrocaudal axis is foremost patterned by transcription of *Hox* genes (Lagercrantz et al. 2010).

The NEPs are elongated cells that stretch from the ventricular to the pial surface of the neural tube. The ventricular zone is the main location for NEP cell division. As the development progresses NEP cells divide symmetrically to form a pool of multipotent cells in the ventricular zone. Altogether this causes the neural tube to thicken (Merkle et al. 2006). By the onset of neurogenesis at E9-10 in mice the neuroepithelial cells transform into radial glial cells which like the NEP cells stretch from the ventricular to the pial surface of the neural tube (Kriegstein et al. 2009). The neural tube then contains several cell layers (Gotz et al. 2005). The RG cells express astroglial markers (for a review, see (Kriegstein et al. 2009)). The nuclei of both NEPs and RGs move up and down from the basal to the apical side during the cell cycle, so called interkinetic nuclear migration (Miyata 2008). Little is known about the functional significance of interkinetic nuclear migration although it has been suggested to control of neurogenesis via apical-basal Notch gradients (Del Bene et al. 2008). RG cells appear to serve both as neural precursors throughout the CNS and a scaffold for migrating neurons in the

developing cortex (Rakic 1988; Anthony et al. 2004; Kriegstein et al. 2009). The RG cells are more restricted than the NEP cells and each RG typically can form only one neural cell type; glia, oligodendrocytes or neurons (Gotz et al. 2005).

The progressive development of the nervous system creates a laminar structure in which newborn cells migrate from the ventricular zone to apical layers. This occurs in an inside-out pattern in which the first born neurons migrate to the inner layer and subsequently born neurons are placed in outer layers of the developing CNS (Rakic 1974). The migration has been suggested to be dependent on adhesive properties of gap junctions (Elias et al. 2007; Elias et al. 2008; Elias et al. 2010).

Except for RG cells basal progenitors (BPs) occur in the SVZ at the onset of neurogenesis (Noctor et al. 2004). During later stages of neurogenesis, BP cells form the subventricular zone (Haubensak et al. 2004). BPs might increase the number of generated neurons by allowing cell division to occur distant from the apical surface (Gotz et al. 2005).

At the end of neurogenesis the progenitor cells switch to form oligodendrocytes and astrocytes. Most RG cells finally migrate toward the cortical plate and transform into astrocytes (Misson et al. 1991).

A complete description of development of the nervous system is outside the scope of this thesis. For a more detailed presentation of these processes, see for instance (Lagercrantz et al. 2010).

1.2.4 Neural stem cells in the adult brain

In the adult brain NSCs and neurogenesis have been demonstrated in the subventricular zone (SVZ) of the lateral ventricle wall and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Gage 2000; Kriegstein et al. 2009). The SVZ contains the largest pool of dividing neural progenitors in the adult brain (Bordey 2006). Neurogenesis outside these two regions is controversial and appears to be extremely limited in the intact adult mammalian CNS but has been demonstrated primarily during pathological conditions in e.g. the neocortex, striatum, amygdala and substantia nigra (Ming et al. 2005; Gould 2007; Burns et al. 2009).

NSCs from the adult brain can be grown *in vitro* in the presence of the mitogens fibroblast growth factor–2 (FGF-2) or epidermal growth factor (EGF) (Gage 2000). Withdrawing the mitogens induces the cells to differentiate towards different neural

cell types (Gage 2000). The NSCs can be maintained either as neurospheres or as flat adherent cultures.

In vivo, ependymal cells in the SVZ have been suggested to function as adult NSCs (Johansson et al. 1999) although several studies have questioned this idea since ependymal cells are quiescent and do not exhibit properties of NSCs when grown *in vitro* (Doetsch et al. 1999; Capela et al. 2002). Instead cells within the SVZ itself appear to be neural stem cells. The SVZ contains at least four different cell types; type A - migrating neuroblasts, which migrate in chains to the olfactory bulb along the rostral migratory stream, type B – GFAP positive progenitors which ensheathe the migrating neuroblasts, type C - transit amplifying cells which are spherical and highly proliferative progenitors and type E - ependymal cells which line the ventricles (Doetsch et al. 1999; Bordey 2006).

The fact that adult NSCs exhibit several characteristics of mature astrocytes has gained a lot of attention and raised questions about the cell lineages involved in adult neurogenesis. NSCs in the adult brain have for instance been shown to express molecular glial markers and have ultrastructural resemblance to astrocytes (Barres 1999; Doetsch et al. 1999; Alvarez-Buylla et al. 2001). Similar to adult astrocytes the neuronal precursors may also support neurons and synaptic activity (Kriegstein et al. 2009). The electrophysiological properties of the precursors in the SGZ and SVZ also resemble that of astrocytes in the brain (Carleton et al. 2003; Fukuda et al. 2003). We still do not know the fundamental characteristics that distinguish neurogenic astrocytes from the more common population of non-neurogenic astrocytes in the brain (Merkle et al. 2006).

Neurons born in the adult SVZ migrate long distances through the rostral migratory stream and differentiate into interneurons in the olfactory bulb (Carleton et al. 2003). Neurons born in the adult SGZ migrate into the granule cell layer of the dentate gyrus and become dentate granule cells (van Praag et al. 2002). The newborn neurons integrate into the existing circuitry and receive functional input (Zhao et al. 2008).

Although synapses are not present in the SVZ (Liu et al. 2005) the progenitors progressively develop mature neuronal membrane properties and repetitive action potentials after migration has ended in the olfactory bulb (Carleton et al. 2003). Similarly progenitors in the SGZ go through several developmental stages before they become fully mature neurons (Esposito et al. 2005).

The regulation of the adult NSCs in the SVZ and the SGZ is not fully understood. The differentiation and fate specification of adult NSCs, in part, seem to be regulated by neighboring cells. The local tissue microenvironments that maintain and regulate stem cells are often referred to as “niches” (Doetsch 2003; Morrison et al. 2008). For instance, hippocampal progenitors in SGZ are located close to astrocytes which have been shown to induce neurogenesis (Song et al. 2002) in the SGZ by the Wnt signaling pathway (Lie et al. 2005). In the SVZ, adjacent ependymal cells express the protein Noggin that inhibits the neurogenic actions of bone morphogenetic protein (BMP) (Lim et al. 2000). Similarly dopaminergic signaling in the proximity of the SVZ precursor cells promotes proliferation *in vivo* (Hoglinger et al. 2004) and adjacent NMDA receptor activity modifies both hippocampal neurogenesis and SGZ proliferation (Nacher et al. 2006). Growth factors like brain derived growth factor (BDNF), neurotrophin-3 (NT-3), basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF) all elicit neurogenic effects (Lanza 2009). Even local endothelial cells might affect progenitor proliferation via the secretion of vascular endothelial growth factor (VEGF) which promotes proliferation both in the SVZ and SGZ (Cao et al. 2004). In the SVZ there is also evidence that direct cell-cell interactions control neurogenesis (Bordey 2006). For instance local non-synaptic GABAergic signaling between neuroblasts in the SVZ has been coupled to both migration and proliferation (Bordey 2006). In the olfactory bulb olfactory ensheating cells have been shown to directly affect the maturation of olfactory sensory neurons via gap-junctional contacts (Rela et al. 2010).

Adult neurogenesis is affected by a large number of external factors (for reviews, see e.g. (Ming et al. 2005; Zhao et al. 2008)). For example exercise enhances (van Praag et al. 1999) and stress and clinical depression suppresses hippocampal neurogenesis (McEwen 1999; Jacobs 2002). Elevation in plasma corticosterone is linked to decreased neurogenesis (Cameron et al. 1994). Neurogenesis also declines with age in both the SVZ and SGZ (Morrison et al. 2008).

1.2.5 Induced pluripotent stem cells

Some of the major remaining obstacles before stem cells can be used for neural transplantation therapies in clinical practice are the shortage of fetal graft material and the risk of graft rejection. Therefore the recently developed methods to induce stem cell properties in somatic cells, so called induced pluripotent stem cells (iPSC) has received

considerable attention. By transfecting mouse fibroblasts with four transcription factors (Oct3/4, Sox2, c-myc and Klf4) Takahashi and Yamanaka were able to produce cells similar to embryonic stem cells in terms of morphology, gene expression, proliferation and potency (Takahashi et al. 2006). Since then iPSCs has been generated also without c-myc using several other somatic cell types (Rashid et al. 2010). Human iPSCs were established in 2007 (Takahashi et al. 2007; Yu et al. 2007) making it possible to produce patient-specific iPSCs without the risk for immune rejection when applied in cell grafting therapies.

Another application for iPSC research is the potential to study disease development, progression and treatment in iPSCs generated from patients with for instance ALS, Parkinson's disease or type 1 diabetes (Dimos et al. 2008; Park et al. 2008).

Recently the potential of iPSCs has been questioned though since it has been demonstrated that induced pluripotent stem cells retain some residual DNA methylation signatures characteristic of their somatic tissue of origin (Kim et al. 2010). This "epigenetic memory" might facilitate differentiation along lineages related to the donor cell and restrict other alternative cell fates (Kim et al. 2010; Zwaka 2010).

1.3 GAP JUNCTIONS

1.3.1 Historical view

82 years ago, the observation of dye transfer between stomach epithelial cells was probably the first indication of gap-junctional intercellular communication (GJIC) although it was not interpreted as such at that time (Schmidtmann et al. 1928; Shibata et al. 2001). Cell-to-cell communication and electrical coupling in invertebrate neurons was then first clearly suggested 1959 (Furshpan et al. 1959). In 1964, Loewenstein and Kanno showed that the hydrophilic molecule fluorescein could pass between cells (Loewenstein et al. 1964). Another important discovery was that glia form a functional syncytium of interconnected cells by cell-cell couplings (Kuffler et al. 1966; Mugnaini 1986). The term “gap junction” was first established in 1967 from histological studies of rat heart and liver tissue using electron microscopy (Revel et al. 1967). Finally, electrophysiological techniques combined with emerging molecular biological techniques made the field expand rapidly during the 1980s with identification and characterization of several gap junction complexes. Connexins, the substrate for gap junction formation were first cloned in hepatocytes in 1986 (Paul 1986).

1.3.2 Structure and function

As shown in **[Fig. 1]** gap junctions are hydrophilic channels made up of two hemichannels also termed connexons which are localized in the membrane of neighboring cells. Each connexon consists of six tetraspan membrane proteins termed connexins (Goodenough et al. 2009). Connexons can be assembled from either a single type of connexins (homomeric) or multiple types of connexins (heteromeric). In turn, the gap junction can either, consist of two identical connexons (homotypic) or two differently composed connexons (heterotypic).

The common nomenclature for connexins is according to molecular mass (e.g. connexin 43 weighs approximately 43 kDa and is denoted Cx43) (Sohl et al. 2003). Based on amino acid similarities, connexins have also been classified into two main subgroups α and β (Goodenough et al. 1996; Willecke et al. 2002). 21 different connexin genes have been identified in rodents and humans (Goodenough et al. 2009). All known connexins represent structurally conserved non-glycosylated transmembrane proteins and are highly related (50-80% structural identity), differing mainly in the length of their cytoplasmic C-terminal domain (Goodenough et al. 1996; Segretain et al. 2004). With a few exceptions (for instance Cx50) connexins are furthermore highly

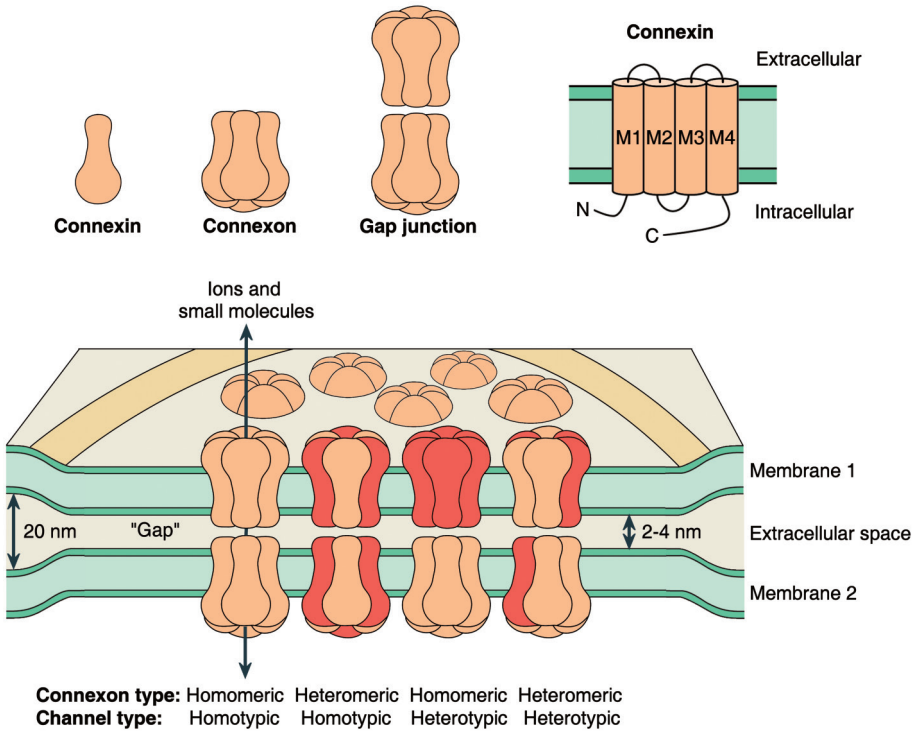

Figure 1 | **Structure and function of gap junctions.** Gap junctions are formed by the apposition of two hemichannels also known as connexons. Each connexon is composed of six connexin (Cx) subunits that assemble in the cytosol and then is trafficked along microtubules to the cell membrane (Segretain et al. 2004). Connexins are tetrameric membrane spanning proteins with cytosolic C- and N-terminals and four membrane located domains (M1-M4). The connexin nomenclature is related to the molecular mass for instance connexin 43 weighs 43 kDa and connexin 26, 26 kDa (Sohl et al. 2003). Connexons can contain only one type of connexin subunits (homomeric connexons) or a mixture of different connexins (heteromeric connexons). Gap-junctional channels can consist of two of the same connexons (homotypic channels) or of connexons with different subunit compositions (heterotypic channels). The gap-junctions are commonly located in clusters or plaques on the cell surface each of which can contain ~10 to more than 10,000 intercellular channels (Musil et al. 2000). Together the two connexons form a pore that bridge the cytoplasm of two neighboring cells over a gap of 2-4 nm and allows direct intercellular passage of ions and small molecules. The permeability of the gap-junctional channel is selectively regulated as explained in the text. Bottom part of the figure is modified with permission from (Bloomfield et al. 2009).

conserved among species, which makes interspecies comparisons reliable. Importantly this is also true for the here investigated Cx26 (Cruciani et al. 2006) and Cx43 (Goodenough et al. 1996).

Invertebrates express topologically and functionally similar channel forming proteins, designated as innexins, which interestingly show no sequence homology to connexins (Phelan et al. 2001). A third group of channel proteins is the pannexin family which is found in humans but is homologues to innexins. The exact function of pannexins is as yet not fully understood (Panchin 2005). Pannexins are able to form channel structures and can be blocked by the gap junction blocker carbenoxolone (Bruzzone et al. 2005) but appear not to form functional intercellular channels in the brain (Huang et al. 2007).

In vertebrates the expression of the different connexins varies between cell types and organs. Virtually all cells in solid tissues are joined by gap junctions. Only a few differentiated cell types in vertebrates do not communicate through gap junctions. These include erythrocytes, platelets, sperm cells and fully differentiated skeletal muscle (Evans et al. 2002).

Connexins have been identified at protein or mRNA levels in the developing and mature nervous system; Cx26, Cx29, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45 and Cx47 (Rouach et al. 2002; Cina et al. 2007). Of these, Cx43 is the most abundantly expressed and is predominantly found in astrocytes, activated microglia, immature neurons, and in the smooth muscle and endothelial cells of cerebral blood vessels (Nagy et al. 2000; Naus et al. 2001; Contreras et al. 2002).

In the mature nervous system astrocytes typically express Cx43 but have been reported to also express Cx26 and Cx30 (Nagy et al. 2004). In oligodendrocytes Cx29, Cx47, and Cx32 have been shown (Nagy et al. 2004). Neurons express several connexins, but mainly Cx26, Cx36, and Cx45 and Cx57 (Sohl et al. 2005; Elias et al. 2008).

Several molecules are gap junction permeable including small ions (Na^+, K^+, Ca^{2+}, H^+, Cl^-), second messengers (cAMP and $InsP_3$), amino acids (glycine, glutamate), metabolites (glucose, glutathione, adenosine, AMP, ADP, ATP), short interfering RNA and small peptides (Alexander et al. 2003; Neijssen et al. 2005; Valiunas et al. 2005). The transfer of ions and small molecules through gap junctions is mediated by passive diffusion (Elias et al. 2008; Goodenough et al. 2009). The maximum size limit for gap junction permeable molecules has previously been defined as 1 kDa (Kumar et al.

1996) although recent evidence suggest that larger molecules can pass provided that they have a more elongated three dimensional structure. Both bystander MHC class I peptides (Mw ~1800 Da) which enable crossprentation of antigens in neighboring cells (Neijssen et al. 2005) and calmodulin (Mw ~17 kDa), a calcium dependent mediator of several intracellular reactions (Curran et al. 2007; Cieniewicz et al. 2010) have been demonstrated to pass gap junctions.

Hemichannels can also exist in an unopposed form on the cell membrane and then mediate exchange of molecules (e.g. ATP) with the extracellular space (Goodenough et al. 2003).

Intercellular communication occurring through gap junctions has been linked to several vital cellular processes, including intercellular buffering of ions, electrical synchronization, cell migration, cell proliferation, cell differentiation, metabolism, apoptosis and carcinogenesis (De Maio et al. 2002; Mesnil et al. 2005; Parekkadan et al. 2008; Todorova et al. 2008).

Intercellular calcium waves have been shown to propagate between cells by the spread of $InsP_3$ through gap junctions. Supporting this, pharmacological gap junction blocking inhibits astrocyte calcium waves (Finkbeiner 1992; Venance et al. 1997; Goodenough et al. 2003). ATP released from hemichannels and then binding to P2 purinergic receptors and eliciting $InsP_3$ production and calcium release in neighboring cells has been suggested as an alternative mechanism for wave propagation (Goodenough et al. 2003; Elias et al. 2008) (see also **[Fig. 5]**).

In excitable cells GJIC increase the speed in synaptic transmission and synchronize groups of cells for coordinated electrical activity (Goodenough et al. 2009). The synchronization of excitable cells by gap-junctional communication has several consequences. These have been summarized into five general classes: speed, synchrony, switching, symbiosis and stimulus/suppression (Simon et al. 1998).

Gap junctions play central roles in several disease states and mutations in the different Cx genes cause significant clinical impairments affecting both nervous and somatic tissues (Rouach et al. 2002; Alldredge 2008). In humans these include for instance X-linked Charcot-Marie-Tooth disease, skin disorders, congenital cataracts and more than half of cases of hereditary deafness (Goodenough et al. 2003).

1.3.3 Regulation

Gap-junctional intercellular communication is regulated at multiple levels and at several different time scales. Compared to other membrane channels the regulation is surprisingly complex. The most rapid regulation occurs by changing the conductance of single channels or altering their probability of opening. Many connexons are voltage gated and can display multiple voltage-dependent conductance states (Neyton et al. 1985; Chen et al. 1992). The different conductance states are simultaneously regulated by connexin phosphorylation (Lampe et al. 2000). Phosphorylation of Cx43 has been shown to decrease channel conductance (Lampe et al. 2000) whereas dephosphorylation cause a channel conductance increase (Kim et al. 1999).

Intracellular acidification is an additional factor that decreases gap-junctional conductance. This occurs by direct binding of protons to amino acid residues in the cytoplasmic loops of the connexin proteins. All known connexins have been shown to be pH sensitive and the binding of protons result in a total closure of the channel (Spray et al. 2002).

A third mechanism related to fast changes in the channel conductance is the intracellular calcium concentration. Calcium was the first intracellular factor suggested to control GJIC (Loewenstein 1981; Kumar et al. 1996). Calcium thereby serves a dual role since it is both transferred via gap junctions and has the potential to regulate them. Intracellular $[Ca^{2+}]_i$ levels below 100 µM is associated with little or no direct action on gap junction channels whereas markedly increased intracellular calcium levels to non-physiological levels as seen under pathological conditions reduce GJIC (Kumar et al. 1996; Spray et al. 2006).

The conformational changes related to closing and opening of connexon hemichannels morphologically resemble a camera shutter (Willecke et al. 2002) **[Fig. 2]**.

Slower regulation is achieved by altering the number of channels present in the membrane by changing rates of synthesis and assembly, posttranslational modification and/or protein degradation (Segretain et al. 2004). The turnover for connexins is much faster than most other cell surface proteins. For instance Cx43 has been shown to have a turnover of only 1-5 hrs (Musil et al. 2000; Segretain et al. 2004).

Phosphorylation is an important process also in this longer time scale. For instance gap junction assembly contains several phosphorylation steps (Musil et al. 1991; Segretain et al. 2004) and the trafficking of connexins via the Golgi to the membrane is

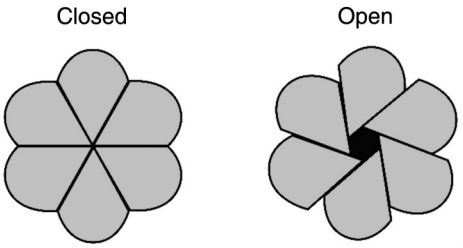

Figure 2 | **Control of hemichannel conductance.** The connexon hemichannels, here shown from above, open and close by conformational changes to the connexin structures with cause an opening of the central pore. The process is regulated at multiple levels as described in the text. The change between the opened and closed states morphologically resembles a camera shutter (Willecke et al. 2002).

dependent on phosphorylation (Solan et al. 2007). Phosphorylation is also used by different connexins to both block and enhance degradation (Laird 2005). Connexin degradation occurs through lysosomal as well as proteosomal pathways (Segretain et al. 2004). Pharmacological blocking of proteosomal breakdown is associated with increased dye coupling between neighboring cells (Musil et al. 2000).

Several techniques have been suggested to actively enhance the expression of connexins and subsequently enhance gap junction formation and intercellular coupling (Heng et al. 2004). For instance, in cortical progenitors cultured *in vitro* treatment with bFGF enhances the expression of Cx43 (but not Cx26) and increases dye coupling between cells (Elias et al. 2008). In fetal neurons foskolin (an agent which stimulate the adenylyl cyclase - cAMP pathway) increases Cx43 expression and cellular coupling (Dowling-Warriner et al. 2000). Similarly retinoic acid enhances connexin expression and GJIC in neuroblastoma cells (Carystinos et al. 2001).

Several common drugs have also been shown to affect gap junction permeability by different pathways some of which include the mechanisms describe above (for reviews, see (Rouach et al. 2002; Spray et al. 2002; Spray et al. 2006)) .

1.3.4 Connexins during development

Already the pre-implantation embryo expresses several connexin genes, including Cx30, Cx30.3, Cx31, Cx31.1, Cx36, Cx40, Cx43, Cx45 and Cx57 (Nishi et al. 1991; Davies et al. 1996; Houghton et al. 2002). mRNA encoding Cx31, Cx40, and Cx43 is

found as early as at the two- to four-cell stage of the pre-implantation embryo in rodents (Davies et al. 1996; Reuss et al. 1997). Functional gap junctions with widespread intercellular coupling are prominent from the eight-cell stage (Lo et al. 1979). At embryonic day 6.5-7.5 the GJIC between the inner cell mass and the trophectoderm is progressively lost which gives rise to two different functional compartments (Kalimi et al. 1988; Kalimi et al. 1989). Coupling also diminishes between germ layers, although cells within each germ layer remain highly coupled to each other (Kalimi et al. 1988). Additional compartmentalization then occurs progressively in both the embryo and extra-embryonic tissues (Kalimi et al. 1988; Kalimi et al. 1989). This mediates specific intercellular signaling between similar cell types (Wong et al. 2008). After implantation gap junctions then continue to serve as key regulators of embryonic patterning and morphogenesis. This is potentially related to the creation of morphogenic gradients and synchronization of electrical and/or metabolic activities in coupled populations of cells (for a comprehensive review of these mechanisms, see (Levin 2007)).

Like the inner cell mass, mouse and human embryonic stem cells (mESC and hESC) demonstrate extensive connexin expression and form functional GJIC (Dale et al. 1991; Hardy et al. 1996; Worsdorfer et al. 2008). Similarly, neural stem cells isolated from the developing rat brain have been shown to express at least Cx26, Cx43 and Cx45 (Dermietzel et al. 1989; Bittman et al. 1999; Cai et al. 2004). The gap-junctional communication in both ESCs and NSCs is required to maintain a proliferative state (Cheng et al. 2004; Todorova et al. 2008).

Connexins serve prominent roles in the development of the nervous system (for a review, see (Bruzzone et al. 2006)). In the rodent embryonic cerebral cortex Cx26, Cx36, Cx37, Cx43 and Cx45 have been found (Nadarajah et al. 1997; Cina et al. 2007; Elias et al. 2008). Gap-junctional coupling in the developing cerebral cortex might play an important role in controlling the pattern of neurogenesis both by forming clusters of coordinated active cells (Lo Turco et al. 1991) as well as affecting migration of newborn neurons along radial glia by a recently suggested adhesive property of connexins unrelated to their channel activity (Elias et al. 2008; Elias et al. 2010). Hemichannel-mediated spread of Ca^{2+} waves also helps to fine tune and shape neural networks during later phases of neurogenesis (Spitzer et al. 2004; Elias et al. 2008). Cx43 appears to play a more important role than Cx26 in the mediation of Ca^{2+} waves (Elias et al. 2008).

A prototypical system for activity dependent development is the spontaneous retinal calcium waves during the development of sight which are institutional for the formation of ocular dominance columns in the visual cortex. These waves are also dependent on gap junctions and hemichannels (Katz et al. 1996; Cook et al. 2009).

1.3.5 Gap-junctional communication during cellular stress

Except for being key players during development gap junctions are essential elements in the cellular reactions to harmful stimuli (Chew et al. 2010). Evolutionary this potentially reflects an endogenous protective strategy in which alteration of the number of metabolically connected and spatially buffered cells will provide means to reduce the spread of the injury and at the same time support neighboring cells at risk. This mechanism is present in several tissues although only the reactions in the CNS will be discussed here. Changes in both spatial and temporal Cx43 protein expression are seen following various types of CNS pathologies for instance ischemia, traumatic injury and neurodegenerative disorders (Rouach et al. 2002).

In rodent models of brain ischemia, increased Cx43 protein levels have been demonstrated in for instance the hippocampus (Rami et al. 2001), striatum (Hossain et al. 1994) and cortex (Haupt et al. 2007). Similarly more dense Cx43 immunoreactivity is found in the penumbra in human brain tissue following focal brain ischemia (Nakase et al. 2006). Both astrocytic (Cotrina et al. 1998) and neuronal (Thompson et al. 2006) gap junctions and hemichannels are open during ischemia enabling transfer of ions and metabolites between damaged and healthy cells (Chew et al. 2010). The increased probability of Cx43 hemichannel opening likely result from the reduced extracellular and increased intracellular Ca^{2+} concentrations associated with global ischemia (Retamal et al. 2007; Chew et al. 2010) although direct effects of reactive oxygen species have been suggested to open hemichannels as well (Contreras et al. 2003; Retamal et al. 2006).

A similar response is seen after CNS trauma. Compression injury to the spinal cord is associated with elevated Cx43 expression for up to seven days (Theriault et al. 1997). Increased Cx43 expression has also been reported in microglia in a brain stab wound injury model (Eugenin et al. 2001) and in the cortex and hippocampus following lateral fluid percussion injury (Ohsumi et al. 2006).

GJIC also increase in several neurodegenerative disorders (Rouach et al. 2002). Augmented connexin levels have been reported in the striatum of both the Huntington-

diseased (Vis et al. 1998) and the Parkinson-diseased brain (Rufer et al. 1996) as well as in Alzheimer's disease (Nagy et al. 1996).

The reasons why changes in connexin expression accompany many CNS pathologies is not fully understood. Under certain conditions the intercellular diffusion of necrotic or apoptotic signals has been suggested to propagate cell death to surrounding cells (Lin et al. 1998; Thompson et al. 2006). However, this view is disputed by evidence suggesting that gap-junctional channels help to buffer harmful levels of substances by allowing diffusion of ions and molecules to less injured cells, which in turn may counteract cytotoxicity and contribute to cell survival in the initially affected cells (Siushansian et al. 2001; Nakase et al. 2004; Leis et al. 2005; Nakase et al. 2006). Increased connexin 43 expression has furthermore been suggested as a mediator of the neuroprotective activity of corticotropin-releasing hormone (Hanstein et al. 2009). The positive outcomes related to changes in GJIC are also likely explained by spread of health-promoting molecules such as metabolites (Pitts 1998; Magistretti 2006; Elias et al. 2008), small peptides (Neijssen et al. 2005) or genetic material (Valiunas et al. 2005), in the opposite direction, from less to more impaired cells, leading to rescue of injured and distressed neighbors.

2 AIMS

The overall aim of this thesis was:

- To identify mechanisms that underlie early functional and beneficial interactions between grafted neural stem cells and the host.

The specific objectives were:

- To characterize the progressive development of functional neural properties in engrafted neural stem cells.

- To establish an *ex vivo* experimental model to study functional integration of neural stem cells grafted to striatal tissue.

- To investigate the role of growth factors and culturing conditions in the differentiation of exogenous neural stem cells and their interactions with striatal tissue.

- To characterize potential mechanisms by which gap junctions formed between the grafted neural stem cells and the host mediate early functional and beneficial interactions.

- To elucidate how the formation of gap-junctional couplings between graft and host cells varies at different time points after NSC engraftment.

- To evaluate the use of preconditioning of NSCs before engraftment as a method to improve the NSCs' functional interaction with the host.

3 METODOLOGY

This section contains a discussion and brief descriptions of the methods and materials used. Details can be found in each of the four studies that are included in this thesis.

3.1 NEURAL STEM CELLS

To model the behavior of NSCs in these studies, five different preparations of NSCs were employed, that varied by species and/or method of isolation and propagation in order to test the broad applicability of the mechanisms being studied:

3.1.1 Murine neural stem cells (mNSCs)

3.1.1.1 The C17.2 clone

Three clones of a previously generated and well-characterized murine neural stem cell line, C17.2 (Snyder et al. 1992), were used in *Study I-IV*. No additional transfections or alterations of the original clones were made by us.

Briefly, the C17.2 NSC clone was originally derived from the external germinal layer of P4 mouse cerebellum and retrovirally transfected with the stemness gene myc to preserve multipotency, self-renewal, and an undifferentiated state *in vitro* (Snyder et al. 1992). The *myc* gene is spontaneously down-regulated upon contact, engraftment and/or differentiation (Snyder et al. 1992; Flax et al. 1998). Cells were further engineered via retroviral-mediated transduction to express the β-galactosidase (β-gal) encoding *lacZ* gene (Snyder et al. 1992). The original C17.2 NSCs were used in *Study IV* and as control cells in *Study III*. The clones used in *Study I-III* were also previously transfected with Green Fluorescent Protein (GFP) as a reporter gene to enable recognition of grafted cells (Ourednik et al. 2002).

To study the effect of grafting cultures with NSCs at various stages of differentiation as well as the effects of excessive amounts of secreted NT-3 (*Study I-III*), we also utilized an NSC subclone that routinely yields a high proportion of clonally related young neurons (Lu et al. 2003). This clone had been genetically modified to produce Neurotropin-3 (NT-3) using a 950-bp cDNA encoding human NT-3 (Lu et al. 2003). The NT-3 operates upon the NSC (and host) TrkC receptors in an autocrine/ paracrine fashion (Lu et al. 2003).

Throughout the thesis the NSCs clone only expressing GFP is termed "**NSC-GFP**" and the NSC clone also over-expressing neurotrophin-3 is termed "**NSC-NT-3**".

The C17.2 NSC clone was chosen for several specific reasons; (I) these cells are confirmed to be clonal which is difficult to achieve with primary NSC lines, (II) demonstrate stem cell gene expression profiles (Parker et al. 2005), (III) fulfill functional definitions of a somatic stem cell (Snyder et al. 1995a; Snyder et al. 1995b; Yandava et al. 1999; Park et al. 2006a), (IV) have a long history of successful transplantation, integration, and therapeutic benefit in many animal models *in vivo* (Snyder et al. 1992; Rosario et al. 1997; Snyder et al. 1997; Ourednik et al. 2002; Teng et al. 2002; Lu et al. 2003; Snyder et al. 2004; Yan et al. 2004; Li et al. 2006a; Park et al. 2006b; Lee et al. 2007) and most importantly, (V) have such extensive self-renewal capacity that these NSCs could be grown in sufficiently large, homogenous and healthy quantities to enable reproducible patterns of engraftment, making comparisons between large numbers of cultures possible and eliminating confounders such as variability in engraftment, migration, differentiation, cell number and survival.

Prior to engraftment the NSCs were cultured as monolayers in stem cell medium (SC medium) (Lu et al. 2003) containing 83% Dulbecco's Modified Eagle Medium (DMEM) and 0.11g/L Pyruvate with pyridoxine, 10% Fetal Bovine Serum (FBS), 5% horse serum, 1% L-glutamine (200mM), and 1% Penicillin/ Streptomycin/ Fucidin. Before grafting, when the NSCs reached ~50% confluence, the alkylating agent mitomycin C (1 µM) was added for 48 hrs to promote pharmacological cell cycle arrest and to induce differentiation. Passage number never exceeded seven.

3.1.1.2 Primary murine NSCs

To evaluate interactions after NSC engraftment to the spinocerebellar ataxia type 1 (SCA1) mouse model in *Study II* primary NSCs were isolated from the subventricular zone (SVZ) of 4–8 week-old FVB/N mice, expanded as neurospheres and engineered to express GFP as previously described (Gritti et al. 1999). *These experiments were performed by Satyan Chintawar and Massimo Pandolfo.*

3.1.2 Human neural stem cells (hNSCs)

Human NSCs (used in *Study I-II*) were obtained from three different sources. First from a stable population (“HFB2050”) initially isolated from the telencephalic ventricular germinal zone of a 13-week-old human fetal cadaver (Flax et al. 1998; Imitola et al. 2004b) and expanded and maintained as detailed elsewhere (Imitola et al. 2004b; Lee et al. 2007). Briefly these hNSCs were initially grown as a polyclonal monolayer

population in serum-supplemented media. Thereafter cells that respond to both EGF and FGF and re-enter the cell cycle were selected using a serial growth factor selection procedure (Flax et al. 1998). The small population of hNSCs selected from the larger heterogeneous population of cells was then expanded and maintained in medium consisting of Neurobasal/B27 supplemented with N2 and recombinant human basic fibroblast growth factor (bFGF; 20 ng/ml), heparin (8 µg/ml), leukemia inhibitory factor (LIF; 10 ng/ml), L-glutamine and 1% Penicillin/ Streptomycin/ Fucidin.

These cells have not been genetically manipulated, carry no transgenes, and are propagated with mitogens alone as in monolayer. Their ability to engraft and integrate has been previously documented (Flax et al. 1998; Ourednik et al. 2001; Imitola et al. 2004a; Bjugstad et al. 2005).

In addition, two additional hNSC lines ("HFB11ws6" and "HCX11ws6") were previously created from human cerebral cortex and subcortical regions from 11 week-old human fetal cadavers (Carpenter 1999; Castelo-Branco et al. 2003; Piao et al. 2006). These cells were collected in a manner largely similar to that described above, maintained in neurospheres and characterized in *Study II*.

3.2 ORGANOTYPIC CULTURES

Organotypic striatal cultures utilized to study graft and host interactions in *Study I-III* were prepared using the roller drum technique as previously described in detail (Gahwiler 1981b; Gahwiler 1988). Roller drum cultures provide unmatched optical features compared to semi-permeable membrane explant cultures due to the thinning of the slice from an initially opaque slice to an almost transparent and homogenous layer of cells which makes the model highly suitable for functional studies. In comparison, interface conditions using semi-permeable membrane produces an intact host tissue suitable for studies in which preservation of the original cytoarchitectural relationships are prioritized. For a more detailed description of the protocols as well as a discussion regarding advantages and disadvantages of organotypic culture system, see (Herlenius et al. 2010).

Briefly, newborn Sprague Dawley rats were killed by decapitation at postnatal day (P) 2-6 and the striatum was isolated. The striatal tissue was cut into 250 µm thick coronal slices and immersed in Gey's Balanced Salt Solution to allow for tissue recovery. After 40 minutes the cut slices were placed on 12 x 24 mm glass coverslips, embedded in reconstituted chicken plasma and coagulated by bovine thrombin for 30

minutes. Thereafter coverslips containing the attached OCs were transferred to 15 ml Falcon tubes filled with 1.1 ml culture medium in which they were maintained throughout the studied time period.

Grafted and non-grafted cultures were maintained in organotypic culture medium containing 55% DMEM with 25 mM HEPES, Sodium Pyruvate and 1000 mg/L glucose, 32.5% Hanks Balanced Salt Solution, 10% Fetal Bovine Serum (FBS), 1.5% D-(+)Glucose 20% solution, 1% HEPES, and 1% Penicillin/ Streptomycin/ Fucidin (Gahwiler 1981b). Throughout the text this is termed "organotypic culture medium", abbreviated as "**OC medium**".

To test whether defined serum-free conditions could be applied to direct differentiation and to promote a neuronal fate in the grafted cells, a subset of 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 (Brewer et al. 1993) 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 text this culture medium is abbreviated as "**NB/B27 medium**". In both culturing conditions the medium was changed every third day throughout the studied time period.

3.3 HYPOXIC PRECONDITIONING

Preconditioning with hypoxia was utilized in *Study IV* as a method to increase connexin 43 expression. To create a standardized preconditioning procedure NSC cultures were transferred to a custom build airtight chamber through which 0.5% of oxygen, 5% of carbondioxide and 94.5% of nitrogen, preheated to 37°C, was passed. The chamber was kept inside the cell culturing incubator. Ctrl cells were maintained for the same time outside the chamber.

3.4 NSC ENGRAFTMENT AND RECOGNITION OF GRAFTED CELLS

NSCs were grafted to organotypic cultures as a cell suspension. In summary NSCs were trypsinized and resuspended twice in SC medium to yield a final concentration of 300-500 cells/µL. 10 µL of a cell suspension (3000-5000 cells) were dispersed within 1

mm of the striatal slice. To enable the NSCs to attach, the grafted cultures were left in a horizontal position at 37°C in 5% CO_2 for 30 minutes, and then were returned to Falcon tubes and placed in the roller-tube device.

Several techniques exist to label NSCs and enable identification of graft derived cells following transplantation. In this thesis we utilized transfection with the reporter gene GFP (green fluorescence) (Tsien 1998; Ourednik et al. 2002) and pre-labeling with the fluorescent lipophilic carbocyanine membrane dye DiI (red fluorescence). Other examples include the reporter gene lacZ (Snyder et al. 1992; Sanchez-Ramos et al. 2000) and pre-treatment with nucleoside analogues BrdU (Gratzner 1982; Yu et al. 1992) or [3H]-thymidine (Taylor et al. 1957; Yu et al. 1992). The family of carbocyanine membrane dyes also include DiI (orange fluorescence), DiO (green fluorescence), DiD (red fluorescence) and DiR (far red fluorescent) (Honig et al. 1986). Briefly, the pitfalls of transfection based techniques are downregulation of reporter gene expression over time while pre-labeling is limited by the dilution of the dye during each cell division.

3.5 SURVIVAL, PROLIFERATION AND DIFFERENTIATION

3.5.1 Necrosis and apoptosis staining

The effects of NSCs engraftment on the host cell well being was initially characterized by estimation of NSC necrosis and apoptosis (*Study I-II*). Similarly the cell death inducing effects of hypoxia on expanded NSCs was delineated in *Study IV*.

Propidium Iodide (PI) staining was used to detect dying cells (both necrotic and apoptotic cells). PI is a fluorogenic compound that binds to double-stranded DNA but only cross the plasma membrane of non-viable cells (Riccardi et al. 2006; Krysko et al. 2008). Cells that have lost their membrane integrity will consequently show red fluorescent PI staining throughout the nuclei. Briefly cell and tissue cultures were rinsed in PBS and then incubated in PI (20 µg/mL) for 10 minutes at room temperature. Finally the specimens were fixated in 4% PFA and counterstained with DAPI nuclear stain.

For specific estimation of apoptosis in *Study I* immunohistochemical staining against cleaved caspase-3 (CC3) (Srinivasan et al. 1998; Thornberry et al. 1998) was performed using primary CC3 antibodies (1:500 concentration) and fluorescent secondary labels.

3.6 IMMUNOHISTOCHEMISTRY FOR NEURAL MARKERS

Standard immunohistochemical stainings were performed to characterize NSC differentiation, host cell responses and connexin expression. In short, cultures were fixed in 4% PFA in 0.1 M PBS. After rinsing twice with PBS sections were permeabilized in 0.1% Triton X-100 in PBS for 1 hr and then washed again in PBS. Non-specific binding was blocked with 5% non-fat dry-milk in PBS for 1 hr. Cultures were incubated overnight at 4°C in a humid chamber with primary antibodies. Nestin (1:500), GFAP (1:200), βIII-tubulin (Tuj1; 1:500), Map2ab (1:60) and synaptotagmin I (1:100) were used to characterize the presence and differentiation of different neural cell types. Hsp27 (1:500) enabled tracing of grafted human NSCs. Antibodies against Cx26 and 43 were employed for quantification of connexin expression. Following incubation the cultures were washed three times in 0.1% Triton X-100 in PBS. The stainings were visualized with fluorescent secondary antibodies labeled with Alexa 546 or 647 and counterstained with DAPI nuclear stain. All antibodies were diluted in PBS.

For temporal studies, the expression of immunomarkers in grafted organotypic cultures was evaluated after 7, 14, 21, and 28 DIV (*Study I-III*).

Purkinje neuron (PN) density *in vivo* was determined by calbindin staining of slice preparations (see below).

3.7 IMAGING TECHNIQUES

The expression of immunomarkers in striatal and NSC-derived cells was assessed at 40X magnification using a Nikon Eclipse E 800 epifluorescence microscope in a blinded fashion. Subcellular localization of the investigated antigens and three-dimensional integration of grafted NSCs were estimated using confocal laser scanning microscopes - Leica DM IRBE and Carl Zeiss 510 Meta. Confocal stacks were collected using a 40X, oil-immersion objective and saved as 512x512 pixel, 8-bit images. For stacks a z-step of 0.1-0.2 µm was used. All image processing and analysis was carried out with ImageJ software (rsb.info.nih.gov/ij/) except for 3D rendering which was performed using Huygens Essential (www.svi.nl). Time-lapse microscopy techniques are described in section 3.10.1.

3.8 FLOW CYTOMETRY

To record temporal changes in connexin expression in very high numbers of graft and host cells (thousands of cells per second) flow cytometry recordings were performed (Jaroszeski et al. 1999).

Cell fluorescence signals were determined using a FACSort flow cytometer (Becton Dickinson). A primary gate based on physical parameters (forward and side light scatter, FSC and SSC, respectively) was set to separate NSCs from debris and dead cells. Cx-negative and positive NSC-derived cells and OC cells within the primary gate were sorted based on their GFP (*FL-1*; 530 nm) and Alexa 546 fluorescence (*FL-2*; 585 nm).

Negative and positive controls were used to set the detection range. For the green spectrum NSCs without GFP expression (C17.2) and OC cells were used as negative controls and NSCs with a well-known and microscopically verified strong GFP expression in all cells (C17.2-NT-3) were used as positive control (Lu et al. 2003). For the red spectrum the background levels in both NSCs and OC cells were estimated by treating the cells with the secondary Alexa 546 antibody but 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 decade were considered as positive for the investigated parameters.

Analyses of expression levels were performed using FlowJo software (Tree Star, Inc. Ashland, OR, www.flowjo.com) and custom-written analysis scripts.

3.9 WESTERN BLOTTING

Immunoblotting (Towbin et al. 1979) was performed to estimate the level of connexin protein in RNAi treated NSCs in *Study II*. Briefly, a sample of the NSCs prepared for transplantation was denatured by heating in 5% sodium dodecyl sulfate (SDS) sample buffer. Total proteins were separated by 12% SDS–polyacrylamide gel electrophoresis (PAGE), transferred onto nitrocellulose filters by electro blotting, and blocked overnight in PBS with 0.1% Tween-20 and 5% non-fat dry milk. The filters were then immunostained using primary antibodies against connexins (1:250). For signal detection, anti-rabbit antibodies conjugated with horseradish peroxidase were added and binding was identified using enhanced chemiluminescence (ECL, 1:7500) and then detected on photographic film.

To allow for semiquantitative estimates of the amounts of Cx43 in RNAi-suppressed cells compared to Ctrl cells (see below) the total amount of protein in each lane was measured before separation on the SDS-gel using a standard Bradford method (Bradford 1976). The samples were also normalized using the intensity of a non-immunostained background band detected with Coomassie Brilliant Blue (Bio-Rad) staining. Employing this correction procedure semiquantitative intensity measurements from each band were carried out using ImageJ software. Relative Cx 43 protein expression was calculated in percent compared to transfected control cells (Ctrl-RNAi).

3.10 EVALUATION OF FUNCTIONAL GRAFT AND HOST INTERACTIONS

3.10.1 Calcium Imaging

Calcium imaging was performed in *Study II* to investigate how grafted NSCs participate in and affect host calcium based network activity. A large number of fluorescent Ca^{2+} indicators are available for the study of intracellular calcium concentration $[Ca^{2+}]_i$ in cells (Thomas et al. 2000). Fluorescent Ca^{2+} indicators change their light absorption and/or emission characteristics upon Ca^{2+} binding. Most calcium indicators are based on the Ca^{2+} chelators EGTA or BAPTA that have been modified to incorporate fluorescent reporter groups. (Neher 1995; Thomas et al. 2000).

We used the calcium indicator Fluo-4 (Gee et al. 2000) to evaluate cell-cell communication in non-grafted cultures and overall activity. Ratiometric imaging utilizing the Fura-2 indicator (Grynkiewicz et al. 1985) was employed to study the interaction of grafted cells recognized by their GFP expression with host cell astrocytic and neuronal networks in the striatal culture.

Briefly, grafted and non-grafted organotypic cultures were incubated with 1 µM Fluo-4 AM or 2 µM Fura-2 AM and 0.2 ‰ Pluronic F-127 for 45 minutes in artificial cerebrospinal fluid (aCSF). The aCSF contained 124 mM NaCl, 5.0 mM KCl, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$ and 30 mM glucose and was equilibrated with 95% O_2 and 5% CO_2 at 26-27 °C to give a pH of 7.4. After incubation cultures were moved to fresh aCSF for 30 min to allow dye de-esterification. The coverslips were then mounted in a 2.5 ml chamber and placed on the microscope. Measurements were made at 37°C with constant aCSF perfusion (1ml/min) via gravity and a peristaltic pump. During Fluo-4 experiments, a Leica DM IRBE confocal laser scanning microscope equipped with a 40X/1.25 epifluorescence oil-immersion objective was used. Fluo-4 was excited at wavelength 488 nm. Images were collected

every 1.7 seconds, corresponding to a Nyquist frequency of 294 mHz. Fluo-4 responses were normalized.

Ratiometric Fura-2 imaging was carried out with a Zeiss Axiovert 135 microscope using a 40X/1.4 epifluorescence oil-immersion objective. Fura-2 AM was excited at wavelength 340 nm and 380 nm. NSC-derived cells were recognized using 470 nm excitation (GFP). Data were recorded with a GenIISys image intensifier system connected to a CCD camera at recording cycles of 3 seconds. Responses were measured after application of 30 µM ATP and 50 µM glutamate. To capture spontaneous $[Ca^{2+}]_i$ oscillations no chemo-transmitters were added during the first 400 seconds and the same time interval was left between each new application. To avoid the effects of phototoxicity, each experiment had a maximum total duration of one hr. Coupled fluctuations were defined as $[Ca^{2+}]_i$ fluctuations occurring in two or more neighboring cells with chronologically overlapping fluorescence peaks. A peak was defined as an arbitrary fluorescence ratio increase at least 3 times higher than the noise level for the same cell. Wave propagation speeds were measured as the time for $[Ca^{2+}]_i$ elevations in one cell to reach another distal cell in the direction of the wave. To quantify activity in different areas of the visual field, activity plots were created off line by calculating the standard deviation of pixel brightness over time. Changes in $[Ca^{2+}]_i$ were expressed in arbitrary units.

3.10.2 Dye transfer techniques

Gap-junctional communication in cultured NSCs as well as between grafted NSC derived cells and host cells can be studied with an array of different dye transfer techniques most of which utilize the gap-junction's permselectivety for molecules smaller than ~1 kDa (larger molecules have been shown to pass gap junctions although this is limited to molecules with very narrow and elongated three dimensional structures, see section 1.3.2). Examples of such techniques are scrape loading (El-Fouly et al. 1987), dye transfer from pre-loaded NSCs (Goldberg et al. 1995), dye transfer following microinjection (Ek-Vitorin et al. 2005), electrocoupling (Loewenstein 1979), transfer of radioactive metabolites (Hooper 1982) and FRAP analysis (fluorescence recovery after photobleaching) (Wade et al. 1986). We have successfully employed the first three in *Study II-IV*.

3.10.2.1 Scrape loading

Scrape loading was used in *Study II-III* to investigate the presence of functional gap-junctions in expanded NSCs prior to engraftment. The basic concept of scrape loading is the introduction of a low molecular weight (Mw) fluorescent dye, such as Lucifer yellow (Mw 457.2 Da) (Stewart 1981) by creating a transient tear in the cell culture (El-Fouly et al. 1987). In cells connected via gap junctions dye transmission then occurs within minutes after loading. To rule out involvement of cytoplasmic bridges cells are simultaneously loaded with a high Mw marker dye conjugate, such as rhodamine dextran (Mw 10.000 Da). Once introduced intracellularly the rhodamine dextran is unable to cross the relatively narrow gap-junctional pore (see also Fig. 2a in *Study III*).

Briefly NSCs grown on 35 mm coverslips were rinsed twice with 0.1 M PBS and then immersed in a dye solution containing 0.5 mg/ml Lucifer Yellow (LY) and 0.5 mg/ml Alexa 546 Dextran dissolved in PBS with Ca^{2+} and Mg^{2+}. A transient tear was made with a surgical blade, and after one minute the dye solution was discarded and the culture was returned to cell culture medium (DMEM) after initial washing with 0.1 M PBS. The cells were then left for 30 or 60 minutes to allow dye spread and thereafter fixed in 4% PFA for 5 min and subsequently mounted with DAPI Vectashield. In the presented studies the amount of dye spreading (reflecting the abundance of gap-junctional communication) was expressed as the ratio of Lucifer Yellow- and Alexa 546 Dextran-filled cells. Extensive spreading of Lucifer Yellow consequently resulted in a higher ratio, and limited spreading resulted in lower ratio.

3.10.2.2 Dye transfer from pre-loaded NSCs

To study gap-junctional coupling between grafted NSC derived cells and host cells in a non-invasive fashion, dye transfer from pre-loaded NSCs was characterized in *Study II and IV*. Based on a previously published protocol (Goldberg et al. 1995) NSCs were washed twice in Hanks Balance Salt Solution (HBSS) and then loaded with 50 µM calcein AM (Mw 995 Da) in for 15 minutes in HBSS. To distinguish grafted cells from host cells NSCs were simultaneously labeled with the lipophilic carbocyanine membrane dye DiI (5 µM). NSCs were then trypsinized and grafted onto cell cultures. When gap junctions are established, the cytosolic gap junction permeable calcein tracer is transferred from the DiI positive cell to neighboring DiI negative cells. Similar to

above, the amount of dye spreading was expressed as the ratio of calcein- and DiI-filled cells.

3.10.2.3 Dye transfer following microinjection

To further confirm the results from the functional tests above 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 using the same whole patch clamp procedure as detailed below. 0.1% LY and RD were added to the intracellular patch pipette solution. After patching the grafted cells, dye-spread was photographed continuously over a 30 minute period. Dye-spread was also evaluated in fixed slices using a Leica DM IRBE confocal laser scanning microscope and digital three-dimensional reconstruction. A limitation of this technique is the application of mechanical force when creating the membrane seal which may cause the patching itself to disrupt the gap-junctional hemi-channels. This possibly leads to an underestimation of intercellular contacts.

3.10.2.4 Calcein dye efflux

The hemichannel function was estimated in *Study IV* with calcein dye efflux experiments as previously described (Thompson et al. 2006). In short, NSCs were washed twice with HBSS and then loaded with 150 μM calcein AM dissolved in HBSS for 15 minutes at room temperature. Calcein fluorescence was then visualized by excitation at 488 nm using a Leica DM IRBE confocal laser scanning microscope. Images were collected every 10 s. During live imaging experiments cells were kept in cell culture medium at 37 ºC. For temporal analysis of the dye efflux speed calcein fluorescence signals were normalized and expressed as F/F_0.

3.10.3 Whole cell patch clamp

To determine neuronal membrane properties, basic whole-cell patch-clamp recordings (Sakmann et al. 1984) were performed in expanded NSCs and NSC-derived cells and cells from the organotypic culture after 7, 14, 21 and 28 DIV (*Study II*). NSC derived cells were identified using combined differential interference contrast and epifluorescence microscopy with 488 nm filter sets at 40X primary magnification with a water-immersion objective. Experiments were performed at 37°C in perfused (1 ml/min) oxygenated aCSF (see calcium imaging section above). A few initial

measurements were done in cell culture medium. The pipette solution contained 125 mM K-gluconate, 20 mM KCl, 4 mM ATP-Mg, 10 mM Na-phosphocreatine, 0.3 mM GTP-Na, and 10 mM HEPES, pH 7.3 (310 mOsm/ l). Biocytin (0.5 mg/ml) was added to the pipette solution to allow subsequent identification of recorded cells. Microelectrodes were pulled from borosilicate glass and had a resistance of 3.5-5.0 MΩ. Electrical signals were recorded with a PC-ONE 200B amplifier, digitized at 20 kHz by an analog-to-digital converter and controlled by Igor patch clamp software.

GigaOhm seal and rupture of the membrane was performed during voltage clamp conditions. Subsequent measurements were all done in current-clamp mode. After establishing whole-cell patch clamp configuration and measuring the resting membrane potential (V_m) the membrane potential was adjusted to -75 mV by holding currents. Input resistance plots were constructed after 20, 100 and 500 ms stimulation with step-wise increasing somatic current injections (10–350 pA). The ability to generate action potentials was determined after brief and prolonged stimulation. Each experiment had a maximum total duration of one hr per specimen. Cultures were subsequently fixed in 4% PFA and kept in PBS until immunostaining was carried out.

Data were analyzed off-line using Igor Pro with custom written procedures. The spike threshold was defined as the membrane potential at which the slope of the voltage trace increased abruptly when charging the membrane with positive current pulses. The spike amplitude was measured as the voltage difference between the peak of the action potential and the action potential threshold.

3.11 GAP JUNCTION INHIBITION

Gap junction formation and function was blocked in order to verify the hypothesized functional and protective mechanisms using both pharmacological blockers and RNAi inhibition strategies.

3.11.1 Pharmacological blockers

Several pharmacological gap junction blockers exist for example 18-α-glycyrrhetinic acid (18-α-GA) and its derivative carbenoxolone (CBX), fenamates, the lipophils heptanol, octanol, arachidonic acid and quinine and its derivatives (Spray et al. 2002). A limitation is the absence of completely selective pharmacological gap junction inhibitors. For instance inhibitory off-target effects have been described on P2X7-induced dye uptake (Spray et al. 2006) and Ca^{2+} channels (Vessey et al. 2004) at

similar concentrations used for connexon hemichannel inhibition which might lead to misinterpretation of the results. This underlines the need for employing several different inhibition techniques (Spray et al. 2002; Spray et al. 2006). In the current studies we utilized 18-α-GA (50 µM; *Study II*) and CBX (100 µM; *Study II-IV*) for pharmacological blocking and RNA interference techniques for specific reduction of connexin synthesis (*Study II*).

3.11.2 RNA interference

To guard against these non-specific off-target effects, use of the two different pharmacological drugs was in *Study II* complemented by the more specific RNA interference methods targeted to reduce connexin 26 and 43 expression (Fire et al. 1998; Dykxhoorn et al. 2003; Becker et al. 2007).

Briefly, NSCs were transfected as cell suspensions with the siPORT-Amine transfection agent from the SilencerR siRNA transfection kit II (Ambion). A scrambled cRNA without significant homology to known murine genes, and GAPDH were used as controls. Transfection efficiency was determined by using fluorescent labeled Cy3 siRNAs and confirmed by real-time PCR. A detailed description of this method is found in *Study II* supplemental information. *The RNAi transfections were carried out by Carmen Salto and Ernest Arenas.*

3.12 IN VIVO MODELS

To determine whether gap junctions may mediate translationally relevant neuroprotection *in vivo*, three representative rodent models of neurodegeneration and trauma where host cells are rescued by direct contact with NSCs (despite the fact that the insult in each model results from different pathophysiological triggers) were examined in *Study II*.

3.12.1 Spinocerebellar ataxia type 1 (SCA1) mouse model

Spinocerebellar ataxia type 1 (SCA1) is caused by the expansion of a polyglutamine repeat within the disease protein, ataxin-1 (Atxn1). It is characterized by ataxia due most prominently to the loss of cerebellar Purkinje neurons (PNs). A transgenic mouse, the B05/+ SCA1 mouse (Clark et al. 1997; Chintawar et al. 2009) models the human disease. PN dysfunction and ataxia are noted at 5 weeks postnatally, abnormalities in PN dendritic trees are seen at 12 weeks and significant PN loss follows at 24 weeks,

progressing to severe reduction of molecular layer (ML) thickness and the ectopic location of the remaining PN bodies.

Murine neurospheres were transplanted into the cerebellar cortex of 24-week old SCA1 or wild-type mice under stereotaxic guidance under deep anesthesia. Injections were done using 10 µl a Hamilton syringe and a microstereotaxic injection system. Mice were given three 2 µl deposits of suspended mNSCs (50,000 cells/µl) into the cerebellar white matter. The needle was placed in situ for 2 min pre and post-injection before being slowly removed.

Accelerating rotarod test (see below) was performed four weeks post-transplantation in sex- and weight-matched grafted and ctrl SCA1 mice. Mice were sacrificed and the cerebella analyzed after 2 months. *These experiments were carried out by Satyan Chintawar and Massimo Pandolfo.*

3.12.2 Nr mouse model

The nervous (nr) mutant mouse is characterized by degeneration of most of its PNs by 22-35 days of age as a consequence of a series of dysregulated intracellular signaling pathways downstream of tissue plasminogen activator (tPA) (Li et al. 2006b). The nr homozygotes (*nr/nr*) are symptomatic by P21 (hyperactivity and ataxia). They also have a retinal phenotype.

Balb/c mice homozygote for the *nr* mutation (Li et al. 2006b) were bred and maintained as previously detailed (Li et al. 2006a; Li et al. 2006b). For transplantation into the neonatal mouse cerebellum of cryoanesthetized newborn mouse pup (P0) three 2 µl injections of a well-triturated suspension of mNSCs (containing 104 cells/µl dissolved in HBSS with 0.05% trypan blue) were delivered via a glass micropipette through skin and skull into the external germinal layers of the left cerebellar hemisphere, right cerebellar hemisphere, and vermis, respectively. The cerebellum was identified by transillumination. HBSS alone similarly injected served as a “sham” control. Pups were returned to maternal care until weaning (Snyder et al. 1992; Parker et al. 2005; Li et al. 2006a). *These experiments were carried out by Jianxue Li and Richard L. Sidman.*

3.12.3 Model of contused cervical spinal cord in adult rats

As previously detailed (Choi et al. 2005), cervical spinal cord hemicontusion injury was created by a 3.0 mm left-sided hemilaminectomy encompassing the caudal end of

vertebral level C4 and rostral end of vertebral level C5 in female Sprague-Dawley rats after anesthesia with intraperitoneal ketamine (75 mg/kg) and xylazine (10 mg/kg; (Choi et al. 2005). hNSCs (prepared as a 2 µl cell suspension containing, 105 cells/µl) were slowly injected into the injury epicenter 3 days after SCI under anesthesia (Teng et al. 2002; Choi et al. 2005). *These experiments were carried out by Yang D. Teng.*

3.12.4 Assessment of motor behavior

Cerebellar-mediated motor skills of mice were assessed on a rotating rod ("rotarod") which underwent linear acceleration from 4 to 40 rotations per minute (Hamm et al. 1994; Chintawar et al. 2009). Animals were scored, by a blinded observer, for their latency to fall or until they made two consecutive revolutions while holding onto the rod. Each trial lasted for a maximum of five minutes. Mice underwent four trials per day for four consecutive days and the mean was considered for statistical analysis.

3.13 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.

3.14 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 DISCUSSION

This section summarizes the findings described in the four studies included in the thesis. It is also a description of the road we travelled, beginning at the investigation of potential model systems for the study of functional NSC differentiation and engraftment and ending at a novel optimization technique to enhance graft and host interplay.

PART 0: THE EARLY STAGES

Initially we characterized the functional properties of murine NSCs (C17.2 cell line) grown *in vitro*. Using whole cell patch clamp and calcium time-lapse recordings we recognized that the cultured NSCs had functional characteristics of undifferentiated neural cells although they did not develop more mature neuronal membrane properties when cultured *in vitro* (as detailed in section 4.3.1) **[Fig. 3]**. We therefore next studied the electrophysiological maturation of NSCs after transplantation to the lateral cerebral ventricles of newborn (P1) mice (strain C57BL). The mice were sacrificed at 3-12 weeks after transplantation and the engrafted NSC were investigated in acute brain slice preparations. The engrafted NSCs survived, integrated and formed cells with neuronal morphology **[Fig. 3]**. However, these experiments were associated with considerable technical difficulties. At the same time there were limited possibilities to control and evaluate the mechanisms involved in the NSC differentiation and the cell level graft and host interactions. For these reasons we decided to focus on *ex vivo* organotypic cultures for the investigation of the cellular processes involved in the early graft and host interplay thereby taking advantage of the accessibility of the slice culture system but still retaining many of the developmental cues present *in vivo* (Jäderstad et al. 2003b; Jäderstad et al. 2003a).

PART I: BEING A GOOD HOST AND A GUEST WITH POTENTIAL

4.1 AN EX VIVO MODEL TO STUDY GRAFT AND HOST INTERACTIONS

After the initial experiments, we investigated the use of organotypic striatal slice cultures as a model to study graft and host cell interactions after engraftment of murine (C17.2 cell line) as well as human NSCs. The organotypic culture system allowed us to control and evaluate environmental factors such as addition of growth factors in the

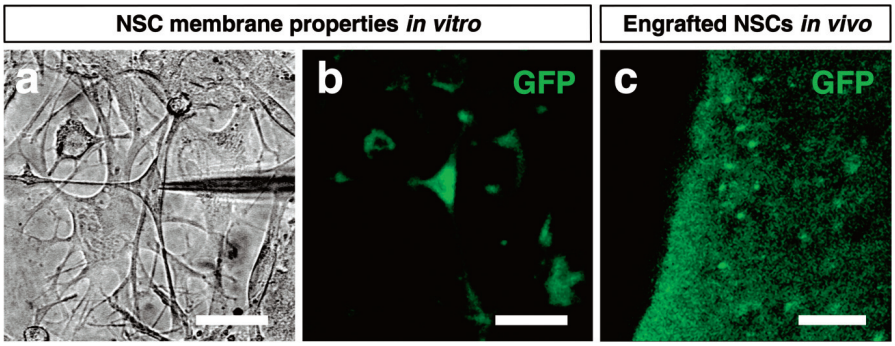

Figure 3 | **Initial investigation of membrane properties and NSC engraftment *in vivo.*** In the beginning we investigated electrophysiological characteristics of murine NSCs grown *in vitro* and after transplantation to the lateral cerebral ventricles of newborn mice. Cultured GFP positive NSCs demonstrated membrane properties of immature neural cells [a, b]. The NSC shown in [a-b] had a resting membrane potential of -52 mV, a membrane resistance of 306 MΩ but no spontaneous pre- or postsynaptic potentials or sodium-potassium channel activity. Mice were sacrificed at 3-12 weeks after transplantation and the engrafted NSCs (recognized by their GFP expression) were investigated in acute slice preparations [c]. The transplanted NSCs survived, integrated and formed cells with neuronal morphology *in vivo* [c]. Scale bars: 20 µm in [a-b] and 100 µm in [c].

extracellular milieu in a manner unachievable *in vivo*. This made it possible to study the behavior of grafted cells at different stages of differentiation and their interactions with the host.

In *Study I* we recognized that during the investigated 28 day culturing period the organotypic culture slice gradually thinned from initially 250 µm to about 50 µm, likely due to cell death and migration as previously reported (Gahwiler et al. 1997). Due to this flattening the organotypic culture changed from an opaque slice to an almost transparent and homogenous layer of cells. This provides unmatched optical features compared to semi-permeable membrane explant cultures and has given the model recognition for use in functional studies. (Gahwiler 1981b; Gahwiler 1981a; Gahwiler et al. 1997; Mohajerani et al. 2005). Because of a tissue organization that closely resembles that observed *in vivo* the organotypic culture model is often referred to as being an *ex vivo* system.

NSCs grafted to slice cultures maintained in interface conditions using semi-permeable membranes has previously been suggested as a suitable *ex vivo* platform for studying graft and host interactions in hippocampus (Benninger et al. 2003; Scheffler et al. 2003) and in corticocospinal explants (Kamei et al. 2007). Generally the interface

condition produces an intact host tissue suitable for studies in which preservation of the original cytoarchitectural relationships are prioritized (Gahwiler et al. 1997; Thonabulsombat et al. 2007).

Depending on the application the slice culture systems have some limitations. Except for being an *ex vivo* model lacking for instance the influence of the immune system, blood circulation and potentially several not as yet described factors, the roller drum technique is associated with difficulties in studying migration of NSCs as the continuous rotation and influence of gravitational forces could possibly bias the evaluation of cell movement. Nonetheless, the advantages of the method have enabled studies of human embryonic and fetal neural stem cells ability to respond to local environmental cues as well as functional characterization of several fetal, and adult rodent and human neural stem cell lines and primary cells, see (Joannides et al. 2007) and *Studies I-III*.

4.2 INTEGRATION AND DIFFERENTIATION OF GRAFTED NSCS

NSCs expansion as well as survival, integration and differentiation of grafted NSCs were investigated in *Study I-III*.

Thawing and expansion of NSCs in stem cell medium generated large quantities of undifferentiated mainly nestin positive cells. The treatment with mitomycin C did not change the morphology of the NSCs compared to nontreated cells. Estimated by Tuj1 stainings, the NSC-NT-3 clone was already prior to engraftment significantly more neuronally differentiated than was the NSC-GFP clone.

After engraftment within 1000 µm of the striatal slice NSCs migrated towards the organotypic culture and gradually integrated in the host. At 28 DIV grafted neural stem cells were found throughout the thickness of the striatal tissue and formed a natural part of the host structure. The ratio of surviving NSCs during the 28 DIV compared to the number of originally engrafted NSCs was between 0.6 and 0.9. GFP expression was initially estimated by flow cytometry and thereafter observed in 92-93% of NSCs throughout the studied time period. The stable GFP expression enabled reliable graft identification.

When organotypic cultures were grown in OC medium on average half of the grafted NSC-GFP cells and one third of NSC-NT-3 cells expressed nestin at 7 DIV. During the time in culture the stem cell progeny differentiated towards a mix of neural cell types and the number of nestin positive NSC-GFP cells decreased to one out of five

at 28 DIV and in NSC-NT-3 cells to almost none. GFAP expression that, except for being a marker for mature astrocytes, has been suggested to be a characteristic of immature cells (Alvarez-Buylla et al. 2001; Kriegstein et al. 2009), exhibited the same pattern. At 7 DIV half of NSC-GFP cells and one fifth of NSC-NT-3 cells expressed GFAP. However, at 28 DIV this expression had decreased to one fourth in NSC-GFP and to 0.6% in NSC-NT-3 cells. Several of the GFAP positive grafted NSCs simultaneously expressed nestin at 7 DIV. The coexpression which could explain the high total number of nestin and GFAP positive cells likely results from the mechanical injury during OC establishment as explained below (Clarke et al. 1994; Pekny et al. 1999; Pekny et al. 2005).

Similar to what has previously been reported (Lu et al. 2003) NSC-NT-3 cells were more prone to form neuronal progenitors and neurons detectable at 21 DIV and at 28 DIV. These NSCs expressed almost twice as much Tuj1 as NSC-GFP cells when grown in OC medium. Quantification of Map2ab expression revealed similar differences, especially after 21 and 28 DIV. At 28 DIV NSC-NT-3 expressed twice as high levels of Map2ab as did NSC-GFP cells.

To investigate if the grafted NSC-NT-3-derived cells could be advanced to even later stages of neuronal differentiation, serum free culturing in NB/B27 was explored. In grafted organotypic cultures maintained in NB/B27 significant but inconclusive differences in nestin and GFAP expression were seen but Tuj1 expression in NSC-NT-3-derived cells somewhat surprisingly remained unchanged compared to cultures grown in OC-medium. However, when neuronal differentiation was estimated using Map2ab, NB/B27 had significant effects on differentiation. Throughout the 28 DIV culture period the NB/B27 medium generated higher expression of Map2ab in NSC-NT-3-derived cells compared to when the grafted cultures were grown in OC medium.

None of the investigated grafted cells expressed synaptotagmin, indicating that the studied 28 day time period was not sufficient to consummate the later stages of neuronal differentiation, not even when propagated in NB/B27 medium. The absence of synapse formation within four weeks is supported by previous *in vivo* findings using the current clone (Lu et al. 2003) as well as predifferentiated dopaminergic neurons (Clarke et al. 1988). The immature neuronal phenotype might suggest that the NSC-NT-3 clone is a suitable model for investigations of the interaction between grafted young neurons and host cells.

Taken together both NSC NT-3 over-expression and serum-free Neurobasal / B27 culturing conditions turned out to be successful means to direct the NSC differentiation towards a neuronal lineage, in accordance with previous reports (Brewer et al. 1993; Brewer 1995; Lu et al. 2003).

Grafted hNSCs, mainly studied in the context of gap-junctional graft-host communication at 7 DIV (see below), survived and integrated morphologically in the host structure similar to what was evident using rodent NSCs.

PART II: TALKING TO THE HOST

4.3 GRAFTED NSCS PARTICIPATE IN FUNCTIONAL HOST ACTIVITY

A hallmark of normal neural development is the formation of electrically-active functional networks characterized by communication between neurons (Spitzer 1994; Katz et al. 1996), between glial cells (Cornell-Bell et al. 1990; Theis et al. 2005) and between glia and neurons (Nedergaard 1994; Pasti et al. 1997; Haydon 2001; Hertz et al. 2004).

The initial aim of *Study II* was to characterize the development of classic sodium-potassium channel activity and chemical synapse formation between graft and host cells. During the initial experiments however, it became clear that the added NSCs instead mainly communicated via mechanisms preceding the more mature forms of action potential driven neuronal interplay but still progressively developed characteristics of functional neural cells that participated in the host neuronal and glial network activity. The description of these findings is found below and continued in section 4.4.

4.3.1 Development of neuronal membrane properties

Grafted mNSCs displayed significant developmental differences in both resting membrane potential (V_m) and membrane access resistance (R_m).

Between 7 and 14 DIV, the mean resting potential in grafted cells decreased from -23 ± 4 mV to -35 ± 3 mV (P=0.02). The membrane access resistance decreased from 566 ± 218 MΩ to 180 ± 64 MΩ between 7 and 21 DIV (P=0.03). The progressive decrease in resting membrane potential and access resistance of the NSCs after engraftment is consistent with the increase in sodium/potassium channels as previously reported (Cai et al. 2004). The change to a more differentiated and process-rich neuronal morphology is also reflected by the decrease in R_m.

Host cells within the organotypic slices showed no change in resting membrane potential or membrane access resistance during the culturing period

66% of the grafted cells displayed active membrane properties due to sodium/potassium channel activity, but none of the immature NSC did so before grafting. Single overshooting action potentials could be generated by current injection. Such active membrane properties seen in recordings from grafted cells indicated that the progeny of the donor mNSCs started to differentiate toward a neuronal phenotype after implantation (Carleton et al. 2003). Notably, even after 28 days in culture, the number of formed sodium/potassium channels in the neuronal progeny of the mNSCs was still insufficient to cause repetitive action potentials as those seen in completely mature striatal neurons, again suggesting that consummation of the neuronal maturation process of engrafted mNSC-derived neurons requires greater than one month. Despite these findings, mNSCs were able to functionally integrate into and influence calcium-mediated host networks, as detailed below.

4.3.2 Grafted cells integrate into functional neural networks

In *Study II* $[Ca^{2+}]_i$ homeostasis and fluctuations were examined in 91 grafted and 36 non-grafted OCs after 7, 14, 21 and 28 DIV. Spontaneous fluctuations were commonly seen in young transplanted and non-transplanted OCs and application of ATP or glutamate elicited responses from both grafted and non-grafted OCs. Responses to ATP were typically mono- or bi-phasic while glutamate application typically resulted in bi-phasic responses or repetitive fluctuations. There was no significant difference in response patterns over time or between groups. Calcium waves were found to propagate between cells at speeds of 24±4 μm/second.

Ratiometric Fura-2 imaging demonstrated that grafted mNSCs, recognized by GFP expression, integrated in fluctuating functional networks **[Fig. 4]**. Fluorescence peak analysis revealed temporal overlaps of grafted cells and neighboring organotypic culture cells in 50% of the examined specimens. Overlapping peaks were seen in both slow (33%; arbitrarily defined as fluctuations of 151-to-500 second intervals between peaks) and fast (67%; arbitrarily defined as fluctuations of 5-to-150 second intervals between peaks of calcium transients) fluctuating networks. No significant difference in

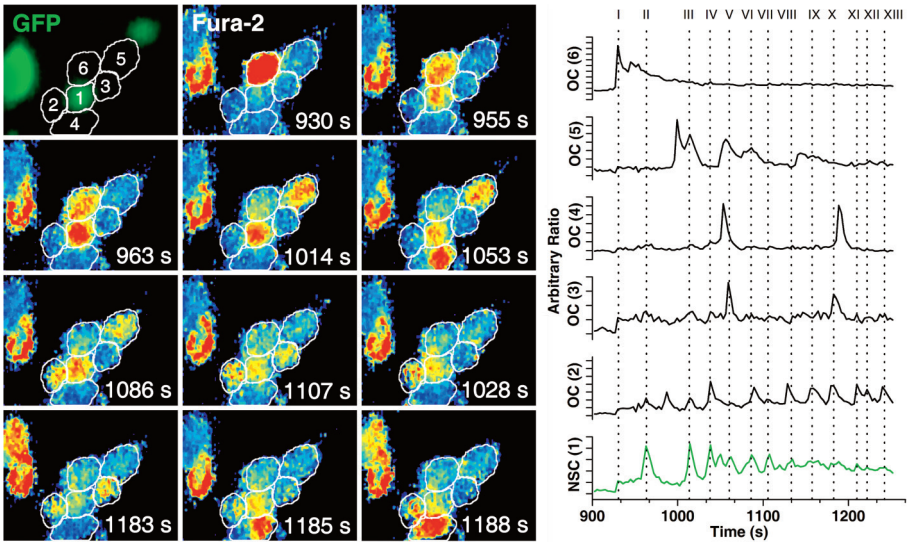

Figure 4 | **Communication between grafted cells and endogenous organotypic culture cells revealed by ratiometric calcium imaging.** Overlapping calcium fluctuations in NSC progeny recognized by their GFP fluorescence (green) and endogenous striatal host cells was investigated using the ratiometric calcium indicator Fura-2.

The depicted experiment shows a typical response after 14 DIV in cultures grafted with NSC-NT-3 cells. Higher levels of $[Ca^{2+}]_i$ are displayed as warmer colors. No $[Ca^{2+}]_i$ events were detected during control conditions or during application of 30 µM ATP (not shown). Application of 50 µM glutamate caused an instantaneous rise in $[Ca^{2+}]_i$ and induced temporary coupled oscillations in both grafted (cell #1) and endogenous cells (cells #2-6). The line chart show the overlapping $[Ca^{2+}]_i$ fluorescence peaks in the graft and host cells after application of glutamate. Traces are numbered corresponding to the cell numbers and placed above each other to facilitate comparison. The grafted cell trace is green. Overlapping events are marked with dotted lines I-XIII. The period between peaks was 81±41 seconds. (Note that the engrafted cell to the left also frequently communicated with its neighboring host cell).

the spontaneous or inducible $[Ca^{2+}]_i$ activity was seen in OCs during the different DIVs. Application of ATP could evoke transient or sustained responses in several, but not all, of the examined OC and NSC cells, suggesting a role of P2-purinergic receptor activation in calcium waves. However the P2-purinergic receptor antagonist suramin (100 µM) did not affect the synchronized calcium waves that were detected between graft and host cells, indicating that, in this system, these short range waves are not dependent on ATP-purinergic receptors.

hNSCs exhibited functional intercellular communication, and demonstrated a similar pattern of spontaneous and inducible Ca^{2+} flux with Ca^{2+} waves that propagated between cells at 30±6 µm/sec, comparable to that observed in OC cells.

4.4 GAP JUNCTIONS MEDIATE EARLY FUNCTIONAL INTEGRATION

While the participation and influence of NSCs on calcium waves within the host network might emanate from fully mature integrated donor-derived neurons, we knew from the data above that the NSC progeny had not yet (within the first 4 weeks) achieved a level of maturity that permitted repetitive action potentials or formed chemical synapses to the host. Therefore, there appeared to be an earlier, preceding mechanism that allowed a more rapid institution of intercellular communication. We hypothesized that this earlier mechanism for integration involved the formation of gap junctions (Jäderstad et al. 2006; Highlight 2010), a process that seems to be vital for endogenous progenitor cell survival and neurogenesis *in vivo* (Rozental et al. 1998; Cai et al. 2004; Cheng et al. 2004; Montoro et al. 2004; Elias et al. 2007; Elias et al. 2008; Wong et al. 2008). A potential mechanism for the calcium based intercellular signaling between grafted neural stem cells and the host is described in **[Fig. 5]**.

4.5 GAP JUNCTIONS ARE PRESENT AT THE GRAFT-HOST INTERFACE

To test the hypothesis that gap-junctional coupling underlies the early graft and host communication we next examined the expression of connexins in the NSCs and striatal host cells by immunohistochemistry and their function by dye transfer studies (*Study II*). As explained in the introduction gap junctions are intercellular channels formed by the apposition of two hemichannels which allow passage of small molecules between neighboring cells. Each hemichannel is formed by six connexin (Cx) subunits. Gap junctions serve vital roles both in the adult and developing nervous system (Cai et al. 2004; Montoro et al. 2004; Elias et al. 2008; Goodenough et al. 2009).

Cx43 was expressed in about half of NSC-NT-3 cells prior to implantation while less than 10% expressed Cx26. As early as 2-18 hrs after grafting, mNSCs started to form functional gap junctions with host cells via Cx43. By 7 days post-implantation into the OCs, connexin 43 was expressed in 35% of NSC-NT-3 derived cells **[Fig. 6]**. By 28 DIV, however, during which time differentiation of the donor NSC-NT-3 cells towards a predominantly neuronal phenotype had occurred, very few (n=2 of 44) of the grafted cells but all of the surrounding astrocytes (n=62) expressed Cx43. hNSCs also

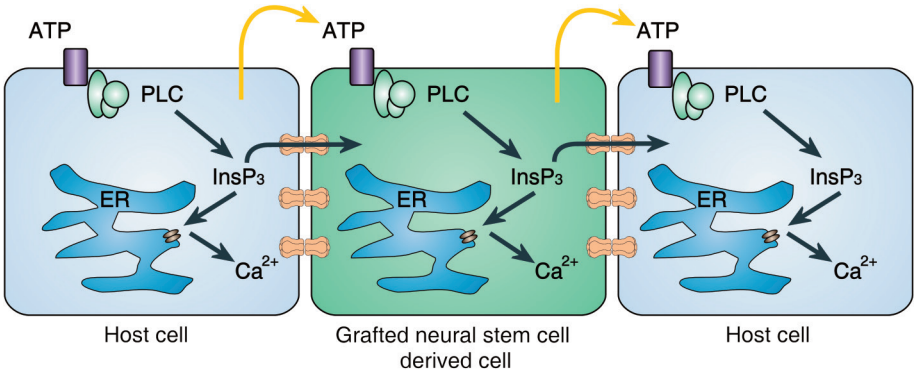

Figure 5 | **Gap-junctional couplings permit grafted NSCs to participate in host network activity.** After engraftment NSC progeny may both participate in and initiate coupled host calcium signaling. The calcium signaling patterns in the host, some of which potentially are orchestrated by the grafted cells, might control such diverse processes as cell-cycle regulation, gene expression, differentiation, migration, neurite and dendritic outgrowth, synaptic fine tuning and apotosis (Berridge et al. 2000; Bootman et al. 2001; Uhlén et al. 2010).

Our data suggest that gap-junctional coupling is an important substrate for calcium based reciprocal graft-host communication. In the depicted model calcium ions (Ca^{2+}) are released from internal stores (most importantly the endoplasmic reticulum; ER) in response to elevated internal inositol-1,4,5-trisphosphate ($InsP_3$) in the NSC derived cell (green). $InsP_3$ then diffuse to a neighboring host cell (blue) through gap junctions and cause a new release of Ca^{2+} in the host cell, thereby creating a functional short-range signaling with potential functional consequences in the recipient cell. In parallel release of ATP from graft and host cells enable longer-range signaling via binding to phospholipase C (PLC) coupled purinergic P2Y receptors which cause a new production of $InsP_3$ (Stout et al. 2004). ATP release may involve several different pathways including release from hemichannels (Kang et al. 2008) as well as a range of other membrane channels and vesicles (Fields et al. 2002; Fields et al. 2006). Although, the described coupled short-range activity between graft and host cells did not seem to be dependent on purinergic signaling (*Study II*) we cannot rule out the involvement of ATP in longer-range graft and host interplay. Modified with permission from (Haydon 2001).

expressed Cx43 upon initial contact with OC cells. The murine and human NSC as well as host cell expression of Cx43 is consistent with reports suggesting that connexin 43 is important both in early CNS development and neural progenitor survival (Rozental et al. 1998; Cai et al. 2004; Cheng et al. 2004; Weissman et al. 2004) as well as in adult astrocytes (Goodenough et al. 1996; Rouach et al. 2002). However, the story unraveled regarding Cx26 was somewhat different. Immunohistochemical analysis in *Study II* showed a higher proportion of grafted NSC-NT-3 cells that expressed Cx26 at 7 days (n=21 of 81) than prior to grafting, with a further significant increase by 28 days post-

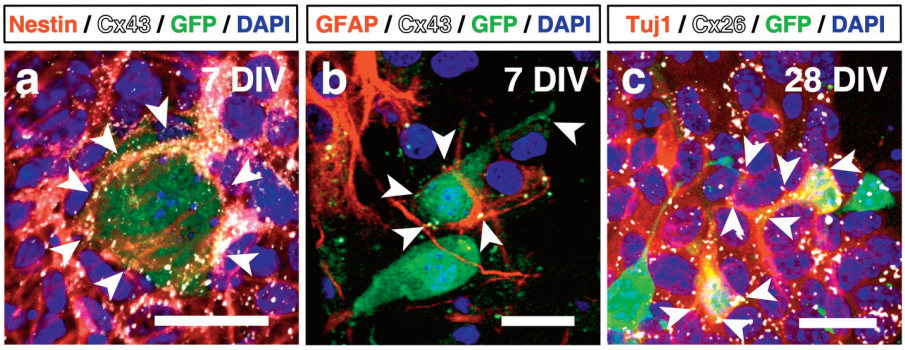

Figure 6 | **Gap-junctional couplings are present at the interface between graft and host cells.** After engraftment of murine NSCs (NSC-NT-3) to organotypic cultures intercellular gap-junctional contacts containing Cx43 and Cx26 (white punctuate immunoreactivity) were seen between GFP positive mNSC progeny (green) and OC cells, between host OC cells themselves and between grafted NSCs themselves. 7 days after engraftments exogenous nestin and GFAP positive NSCs commonly expressed Cx43 [a-b]. The Cx43 expression typically decreased with time in culture as the NSCs differentiated. Cx26 was localized primarily to graft and host cells expressing the neuronal marker Tuj1 [c]. Scale bars 20 µm.

implantation (n=58 of 58), by which time the majority of the NSC-NT-3 cells had begun to pursue a neuronal phenotype. Connexin 26 is an important component in precursor cell gap junctions (Montoro et al. 2004) and highly expressed in the rodent embryonic cerebral cortex (Elias et al. 2008). Within the slices, nearly all of the NSC-derived Tuj1 positive cells (98±2%) and the host-derived Tuj1 positive cells (96±4%) co-expressed Cx26 **[Fig. 6]**. A relationship seemed to exist between expression levels of Cx26 and a neuronal fate. Gap junctions containing Cx26 were seen between grafted cells and host striatal cells (n=48), as well as between grafted cells themselves (n=51) and between host striatal cells themselves (n=189).

To further evaluate if the gap-junctional couplings between murine and human NSC-derived cells and host cells were functional, several different dye transfer experiments were carried out. Dye transfer from grafted cells to host cells indicative of functional gap junctions was seen both after injecting Lucifer Yellow via micropipettes into the grafted murine cells as well as from grafting calcein preloaded mNSCs and hNSCs to striatal cultures. The transfer could be suppressed by pharmacologic gap junction blocking agents (carbenoxolone or 18-α-glycyrrhetinic acid). Pharmacological inhibition of gap junctions not only blocked dye-transfer from murine and human NSCs to host cells *in vitro* but also inhibited coupled Ca^{2+} transients, again suggesting that

gap-junctional coupling was integral to early intercellular cross-talk and coordination.

In conclusion the formation of gap junctions appears to be widespread phenomenon and one of the first steps by which grafted murine and human NSCs, their progeny, and host cells establish communication even before electrophysiological maturation has been consummated.

4.6 NSC ENGRAFTMENT WAS BENEFICIAL TO THE HOST

In *Study I* we employed overall survival, host cell necrosis and apoptosis and changes in nestin, GFAP and Tuj1 expression in striatal cells as quantifiable metrics to study the impact of NSC engraftment on striatal host tissue.

We first recognized that host cell necrosis, estimated by PI staining at 7 DIV, was significantly decreased if cultures were grafted with stem cells. Compared to non-grafted cultures the number of necrotic cells were reduced by 86% in cultures grafted with NSC-GFP cells and 71% in cultures grafted with NSC-NT-3 cells.

Similarly, host cell apoptosis, estimated by CC3 immunohistochemistry at 7 DIV, was decreased in grafted cultures. Compared to non-grafted cultures, the number of apoptotic host cells were reduced by 78% in cultures grafted with NSC-GFP cells and 64% in cultures grafted with NSC-NT-3 cells.

Early engraftment (0-24 hrs) with the less differentiated NSCs improved the overall survival of the organotypic cultures. If NSC-GFP cells were grafted early following OC establishment the overall survival increased by 43%. Late NSC-GFP engraftment, four or more days (96 and 144 hrs) after OC establishment, did not improve survival. In contrast, NSC engraftment during this time frame seemed to have a negative influence on OC survival. Grafting of both NSC clones at these time points decreased the overall survival by roughly one-third compared to in non-grafted cultures (P=0.002-0.008).

The mechanical stress inflicted upon the tissue during establishment of the model system (by the automated and reproducible tissue slicing and to a lesser degree by the subsequent manual handling of the slices) caused increased nestin and GFAP expression which in part was ameliorated by NSC engraftment. Following engraftment with NSC-GFP cells the nestin expression was reduced by 30% at 7 DIV (P<0.001), by 26% at 14 DIV (P=0.04) and by 35% at 21 DIV (P=0.006) compared to in non-grafted cultures. The impact of NSC engraftment on host cells was similar when NSC-NT-3 cells were used. Compared to striatal cells in non-grafted cultures, host cells in cultures

grafted with NSC-NT-3 cells expressed 27% less nestin at 7 DIV (P=0.012) and 28% less nestin at 14 DIV (P=0.01).

Similar reductions in the gliotic reaction caused by the above-described mechanical stress during OC establishment were evident using both clones. Compared to non-grafted cultures, cultures grafted with NSC-GFP cells revealed 30% less GFAP expression at 7 DIV (P<0.001). When NSC-NT-3 cells were grafted to the OCs, gliosis decreased by 44% at 7 DIV (P<0.001) and by 43% at 14 DIV (P<0.001).

A small but non-significant (P=0.08) loss of host neurons, detected by Tuj1 staining, was recorded in non-grafted OCs with time in culture. The host neurons appeared to be saved by grafted NSCs. The differentiation state of the grafted NSCs was important for this effect - cultures grafted with less differentiated NSCs (the NSC-GFP clone) displayed a 28% reduction in neuronal loss compared to non-grafted cultures after 28 days in culture (P=0.02).

4.6.1 Gap junction formation and host cell rescue

We hypothesized that the previously shown functional integration via gap junction formation between graft and host cells was also associated with the observed beneficial effects. This hypothesis was tested by suppressing gap junction formation and function and observing the associated effects on host cell rescue (*Study II*) .

Application of CBX in the culturing medium abrogated the beneficial impact of hNSCs on striatal cell gliosis. The proportion of GFAP-expressing host cells was 30±6 % following engraftment of hNSCs but rose to 51±4% if CBX (50 µM) was applied during the first 3 days post-engraftment, a percentage similar to that seen in non-grafted OCs. However, continuous exposure to CBX at doses that block or reduce the function of gap-junctional channels (Orellana et al. 2006), also compromised the well-being and proliferation of the murine and human NSCs. At the same time pharmacological gap junction blockers are also notorious for being unspecific drugs (Spray et al. 2006). We, therefore, confirmed the pharmacological experiments by suppressing gap junction formation by silencing connexins 26 and 43 in mNSCs through the use of RNA interference (RNAi), a technique with specificity superior to available pharmacological agents (Spray et al. 2006). We first established an optimal level of RNA interference such that mNSC viability was not compromised yet Cx expression at both the message and protein levels (as assessed by RT-PCR and Western analysis, respectively) was adequately suppressed for at least 7 DIV. We next affirmed that transducing RNAi

constructs into the mNSCs under these conditions did not alter their cell numbers or differentiation profile compared to mock or control-RNAi transfected NSCs.

When Cx43 interfering primers was transfected, with a total Cx43 suppression of 30%, the NSCs' beneficial effect in blocking gliosis was abrogated compared to nontransfected NSCs or cells transfected with a scrambled cRNA by 15±3%, P=0.02. When RNAi was further increased, such that a 50% suppression of Cx43 was achieved, NSC blockade of OC gliosis was even further inhibited compared to controls (by 37±9%, P<0.001). Interestingly, Cx26 suppression alone had little impact on blunting this mNSC action, suggesting that NSCs' therapeutic actions are principally mediated by Cx43.

4.6.1.1 Gap junction-dependent rescue of host cells neurons in vivo

To determine whether there is an *in vivo* correlate to this particular type of NSC-host interaction i.e. whether gap junctions may mediate clinically-relevant neuroprotection, we, together with collaborators, investigated the nature of the interaction between engrafted NSCs and host neurons in the brains or spinal cords of three well-characterized models of neuropathology in which stem cell-mediated rescue of compromised host cells had been observed (Teng et al. 2002; Li et al. 2006a; Chintawar et al. 2009) (*Study II*).

4.6.1.1.1 Spinocerebellar ataxia type 1 (SCA1)

When dissociated neurospheres (obtained from adult murine subventricular zones) were transplanted into the cerebella of SCA1 mice at the onset of PN loss (at 24 weeks of age) and the mice examined 2 months later, the PNs and the Purkinje cell layer (PCL) in adult B05/+ SCA1 mice more closely resembled those of wild-type adult mice and were significantly improved compared to those of untreated age-matched SCA1 mutant mice (Chintawar et al. 2009).

In no case was this improvement accompanied by the differentiation of donor neurosphere-derived cells into PNs. Rather, the Pukinje cell layer in SCA1 grafted appeared to be populated entirely by rescued host Purkinje neurons (PNs). Neurosphere-mediated PN rescue was also seen only under conditions of direct cell contact between transplanted cells and host PNs. Because this situation appeared so reminiscent of the *in vitro* data described above the presence of gap junction formation was investigated. Indeed, rescued SCA1 PNs demonstrated apparent Cx43 expression

at the interface to transplanted murine neurosphere-derived cells. This morphological connection was also reflected in functional cerebellar improvement estimated by longer latency to fall in rotarod tests.

4.6.1.1.2 Nervous (nr) mutant mouse

To determine whether the gap junction presence and function might have even broader applicability, we next examined another mouse model of neurodegeneration in which the same cell type (PNs) degenerated but by an entirely different pathophysiological etiology and at an earlier age and more rapid tempo. In addition, we employed murine NSCs prepared using a different method compared to SCA1 experiments (clone C17.2).

As recently reported that mNSCs transplanted at birth into the pre-morbid nr mouse cerebellum can rescue most PNs from death in adulthood (also here reflected by preserved rotarod function) if direct cell-cell contact is made between the NSCs and the PNs (Li et al. 2006a). Due to the graft-host contact pathologically high levels of tPA within the PN are returned to normal following transplantation. The normal tPA level, in turn, restored the downstream intracellular pathways regulating neurotrophic factor processing and mitochondrial function (Li et al. 2006a; Li et al. 2006b).

We again characterized the gap junction formation in the interaction between engrafted NSCs and host PNs. In the adult cerebellar cortex of transplanted nr mice, in the regions where exogenous mNSCs had integrated and were interfacing with rescued host PN somata, Cx43 immunoreactivity was abundant. Although some integrated mNSCs expressed Cx26 in their cell bodies, no Cx26 protein was detected in the mNSC processes or on the surface of rescued PNs, consistent with our finding *in vitro* that Cx43 appeared to play a more pivotal role in host well-being than did Cx26.

Employing RNA interference similar to above, a similar Cx43 dose-dependent impact on rescue of host PNs was observed in nr cerebellar organotypic slice cultures.

4.6.1.1.3 Cervical spinal cord contusion

A similar gap junction-associated Cx43 expression appeared in the interactions of human NSCs (prepared as neurospheres) grafted to the cervical spinal cord and saving host cervical spinal motor neuronal fibers of adult rats subjected to cervical spinal cord contusion (Teng et al. 2002). In this model respiratory and motor function improved following hNSC transplantation.

In conclusion the NSC-associated gap junction-mediated improvements were present in two different *in vitro* preparations and three different rodent models of neurodegeneration, each with a different pathophysiological trigger, hence potentially suggesting a broad applicability of our observations.

Possible cellular effector molecules active in this interplay and responsible for the positive effects (*in vitro* and *in vivo*) are discussed below.

4.7 CONNEXIN EXPRESSION CHANGE AFTER ENGRAFTMENT

The Cx immunostainings in *Study II* indicated temporal changes in the expression of connexins in graft and host cells. To further investigate these processes and possibly get clues to how grafted cells change their communication with the host and how to optimize the outcome of NSC engraftment procedures in this regard we next utilized flow cytometry to quantify the proportion of connexin expressing cells in a very large scale (millions of cells) in *Study III*.

Before engraftment, the two investigated murine NSC clones NSC-GFP and NSC-NT-3 expressed high levels of Cx43 (92 and 94 % respectively). Cx26 was detected in fewer cells in both clones (21 and 24 %).

After engraftment the NSC connexin expression displayed significant changes. As the grafted NSCs became more differentiated the expression of Cx43 decreased. At 7 DIV 74-95 % of grafted NSC-derived cells expressed Cx43. This early expression was typically co-localized with nestin or GFAP **[Fig. 6]**. At later time points the NSC expression of Cx43 decreased. These changes might be paralleled by a switch in the grafted NSCs action from a primarily rescue-based host cell interplay to a process in which grafted cells replace lost host cells and then gradually become less dependent on gap-junctional communication.

The NSC Cx43 expression could be modulated by changing the culturing conditions both via NT-3 overexpression in grafted NSCs and use of serum-free Neurobasal medium supplemented with B27. Four weeks after engraftment most of the grafted NSC-NT-3 cells had differentiated towards neurons. At the time, the expression of GFAP and Cx43 had decreased. More undifferentiated NSCs (NSC-GFP grafted to OCs maintained in OC medium) expressed higher levels of Cx43 at all time points compared to NSCs in growth factor rich and/or serum free conditions.

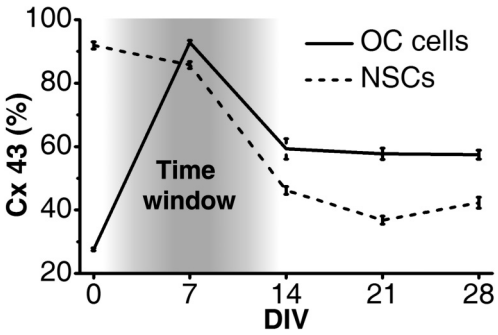

Figure 7 | **A time window for successful NSC engraftment.** The Cx43 expression in OC cells increased markedly during the first days following OC establishment (demonstrated here by the mean value of all OC cell flow cytometry recordings). At later stages when the tissue had recovered, Cx43 expression again returned to lower levels. This was paralleled by the initially high and then decreasing Cx levels in the undifferentiated grafted cells (demonstrated here by the mean value of all NSC flow cytometry recordings). The aligned Cx expression profiles potentially enable extensive and temporally matched formation of gap-junctional channels between graft and host cells and thereby set the stage for the beneficial interactions that lead to the rescue of imperiled host cells. The data might thereby suggest a time window (shaded grey area) for successful NSC engraftment when the reciprocally high Cx expression make host cells more susceptible for graft cell interactions and rescue via gap-junctional channels.

In striatal cells Cx43 expression was, as described previously, typically colocalized with nestin or GFAP. None of the Tuj1 positive host cells co-expressed Cx43. In acutely isolated striatal tissue approximately one quarter of the examined cells expressed Cx43. At 7 DIV following establishment of the organotypic culture the striatal cell Cx43 expression had increased almost four-fold (90-99%) in all examined conditions. This likely reflects a response to the mechanical and chemical injury associated with the establishment of the organotypic culture. At later stages (14-28 DIV) Cx43 again returned to lower levels.

Taken together with the initially high and then decreasing Cx levels in grafted cells due to their ongoing differentiation this might suggest a “window of opportunity” during which damaged host cells are more susceptible to graft cell interaction and rescue via gap-junctional channels **[Fig 7]**. As illustrated by *Study I-II* and (Ourednik et al. 2002; Madhavan et al. 2005), early transplantation is important for the grafted stem cells ability to rescue host cells this finding might in part explain these findings.

Cx26 was present in the graft-host interface during the entire studied time period but did not display consistent temporal and growth factor-mediated changes comparable to

those evident for Cx43. Cx26 expression was typically co-localized with Tuj1 in both graft and host cells **[Fig. 6]**. Between 30% and 60% of the striatal cells expressed Cx26 at 0-28 DIV. At the investigated time points differences were seen between OC cells or NSC derived cells in the investigated groups however no consistent trend was apparent. As described above, Cx43 seems to play a more prominent role in the supportive graft and host interplay than does Cx26 (*Study II*). Possibly Cx26 also does not respond to the same developmental and pathological cues and therefore does not follow the same temporal pattern as Cx43.

PART III: HYPOXIA MAKES STEM CELLS TALK

4.8 HYPOXIC PRECONDITIONING AUGMENTS NSC HEMICHANNELS

Finally, in the fourth and last study, we wanted to examine if the previously demonstrated functional gap-junctional graft-host coupling could be actively enhanced to promote more extensive and potentially beneficial interplay after NSC engraftment.

Several reports suggest that connexin levels in the brain increase following ischemic stroke (Rouach et al. 2002; Nakase et al. 2006; Talhouk et al. 2008) and that the function of hemichannels is of key importance (Cotrina et al. 1998; Thompson et al. 2006). Accordingly gap junction blockade minimizes brain damage (Talhouk et al. 2008). The mechanism causing this effect is not fully understood. The presence of GJIC in the ischemic tissue might both mediate spread of cytotoxic signals between cells (Lin et al. 1998) as well as allow intercellular passage of neuroprotective factors (Giaume et al. 2007).

We hypothesized that controlled short time hypoxic preconditioning of NSCs is a reliable method to actively and non-invasively increase NSC hemichannel expression and function and thereby improve the engrafted NSCs' ability to interact with and potentially rescue host cells at risk.

This hypothesis was tested in murine NSCs (C17.2) using immunohistochemical and necrosis quantifications as well as functional dye transfer experiments prior to and after NSC engraftment. Hypoxic exposure was achieved by culturing the NSCs in airtight chamber through which 0.5% O_2, 5% CO_2 and 94.5% N_2 gas, preheated to 37°C, was passed.

Initially the maximum time NSCs could be exposed to the hypoxic preconditioning stimulus without causing increased necrosis and apoptosis was evaluated. Up to three hr exposure was not associated with increased cell death. This is consistent with

previous findings showing that neural stem cells are resilient to hypoxia-ischemia (Romanko et al. 2004).

After 3 hr exposure to hypoxia the number of Cx43 aggregates in NSCs had increased 31% compared to Ctrl cells (P=0.02). Longer exposure (6 and 24 hrs) amplified the number of Cx43 aggregates even more but induced NSC cell death. Therefore 3 hr hypoxia was chosen as the standard treatment for functional investigations. The increased Cx43 levels following hypoxic exposure likely result from slowing of the rate of protein degradation (VanSlyke et al. 2005; Lin et al. 2008). Cx43 has a rapid turnover with a half-life of only 1–5 hrs (Segretain et al. 2004). Together with regulation of Cx43 function by changes of the phosphorylation state as well as numerous other gating mechanisms at a minute-to-minute basis (Goodenough et al. 2009) this makes the preconditioning a very dynamic process (see also section 1.3.3).

Evaluated by calcein efflux experiments hypoxic preconditioning increased the NSC hemichannel function. In NSCs exposed to 3 hr hypoxia the speed of dye efflux was higher compared to NSCs maintained under control conditions. The calcein efflux could be blocked by addition of the hemichannel blocker CBX.

Engraftment of NSCs preloaded with calcein indicated that NSCs exposed to 3 hr hypoxic preconditioning earlier established functional couplings to host cells. 4 hrs after engraftment transfer of dye to adjacent cells, indicative of GJIC, was more than six times as common in preconditioned NSCs as in Ctrl NSCs. 18 hrs after engraftment no difference was detected between groups. At the same time point calcein had however spread to a higher number of host cells in cultures grafted with preconditioned NSCs. The ability to interact with host cells more rapidly following preconditioning might in part be explained by the increased likelihood of gap junction formation due to the larger number of hemichannels. It might also be attributed to the adhesive functions of connexins (Elias et al. 2008). The earlier graft-host connection potentially enhances the “bystander cell rescue” phenomenon during the 4-6 hr time window within which neuroprotection seems to be most effective (Ginsberg 2008). The fact that preconditioning is accomplished by reversible alteration of physiologically occurring oxygen and not addition of non-physiological compounds makes the suggested preconditioning method an attractive candidate for future clinical NSC applications.

4.9 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The main findings reported in this thesis are that communication via gap junctions underlies early functional and beneficial interactions between grafted neural stem cells and the host, that this form of communication changes dynamically after NSC engraftment and that it can be enhanced by preconditioning of the NSCs before grafting.

It is becoming recognized that grafted NSCs may salvage or protect endogenous neural cells and their connections (Ourednik et al. 2002; Ourednik et al. 2005:Martino, 2006 #1866; Lindvall et al. 2010) e.g. dopaminergic neurons in Parkinsonian models (Ourednik et al. 2002; Redmond et al. 2007), cortical neurons following ischemic insult (Park et al. 2002), motor fibers following spinal cord injury (Lu et al. 2003) and Purkinje neurons in models of cerebellar neurodegeneration (Li et al. 2006a). However, the mechanisms underlying these effects remain poorly characterized. While, in some cases, this action can be attributed to the release of diffusible neurotrophic and/or plasticity-modulating substances from the NSCs (e.g. glial-derived neurotrophic factor, brain-derived neurotrophic factor and vascular endothelial growth factor) (Ourednik et al. 2002; Lu et al. 2003; Ourednik et al. 2005), we suggest that direct interaction of the NSCs with host cells and the host microenvironment via gap junctions might be responsible as well **[Fig. 8]**.

Gap-junctional intercellular contacts with host cells may permit NSCs to participate in endogenous ion homeostasis and influence intracellular calcium oscillations that enhance the well-being and function of host cells **[Figs. 4, 5 and 8]**. Gap junctions directly bridge the cytoplasm of cells allowing for the direct exchange of ions and small molecules such as metabolites and second messengers, as well as for electrical coupling between neighboring cells (Elias et al. 2008; Goodenough et al. 2009) (see also section 1.3.2).

Since GJIC seem to serve a prominent role especially during the early graft and host communication, treatment of acute disorders such as stroke, perinatal hypoxia and traumatic brain injury might be particularly valid targets for future investigations of this mechanism **[Fig. 8]**. Altered gap-junctional communication and/or ion dyshomeostasis are hallmarks of these and many other neuropathological processes (Rouach et al. 2002). Connexin levels in the brain increase following ischemic stroke (Rouach et al. 2002; Nakase et al. 2006; Talhouk et al. 2008) and the associated neuronal excitotoxicity and ion dyshomeostasis (Leis et al. 2005) during prolonged brain

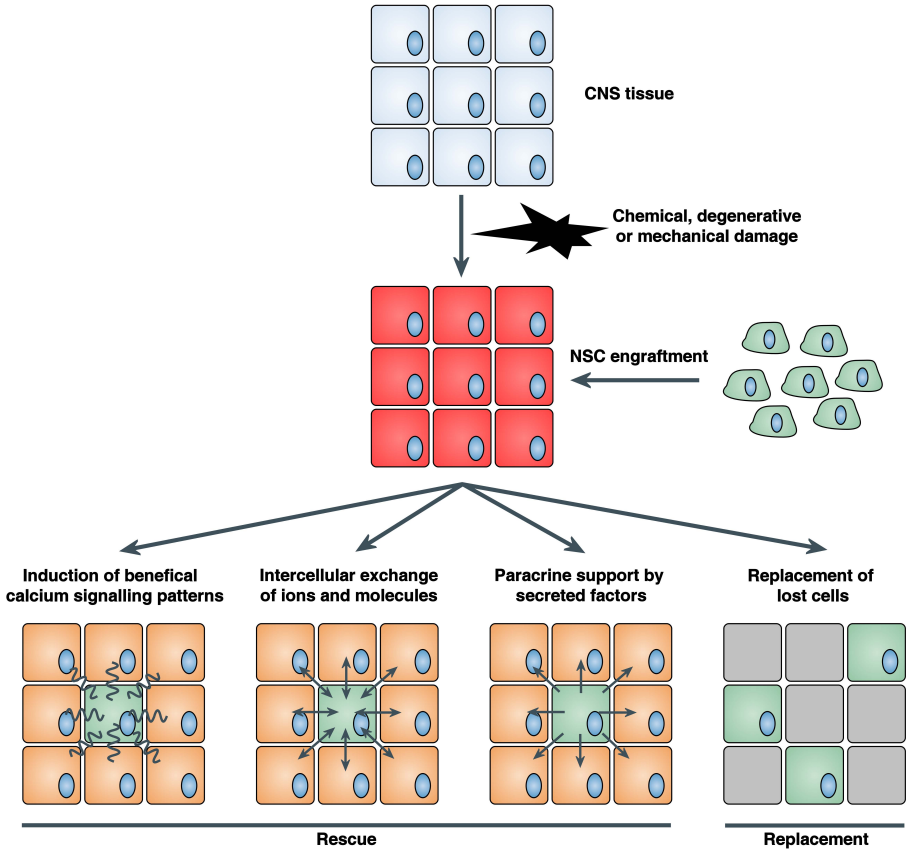

Figure 8 | **Grafted NSCs rescue endangered host cells via a multitude of mechanisms.** Following mechanical or chemical injury to the central nervous system such as external trauma or brain ischemia a multitude of pathological cellular events occur. These events include severe disturbances in cellular ion homeostasis, energy depletion, oxidative stress, alterations in transcriptional activity and initiation of cascading events leading to necrosis and/or apoptosis. Successively the pathological processes lead to cell impairment and demise with associated functional deficits. NSC transplantation has been recognized as a promising future treatment for these conditions. Traditionally recovery of function following NSC engraftment has been attributed to replacement of lost cells. Recently it has also been suggested that grafted NSCs rescue impaired cells in the host tissue. Rescue of host cells might occur by a combination of mechanisms. NSCs are known to produce and secrete growth factors. By paracrine action on host cells these help to preserve cellular health. In this thesis we have suggested that the formation gap-junctional couplings underlies a substantial portion of the early functional and beneficial interactions between grafted NSCs and host cells. Via this form of interplay imperiled host cells might be saved by induction of beneficial calcium signaling patterns, by buffering of harmful levels of ions and molecules and by transfer of metabolites and genetic material. The expression of connexins, the substrate for gap junction formation, changes dynamically in both graft and host cells after engraftment and can be actively enhanced by NSC preconditioning.

ischemia is accompanied by the opening of neuronal gap junction hemi-channels and calcium dysregulation (Thompson et al. 2006). As discussed above, the presence of GJIC in the ischemic tissue might both mediate spread of cytotoxic signals between cells (Lin et al. 1998) as well as allow spatial buffering and intercellular passage of neuroprotective factors (Giaume et al. 2007).

After traumatic injury to the spinal cord glial and neuronal connexin expression is enhanced (Lee et al. 2005). Mechanistically, the reactive gliosis associated with CNS injury is also accompanied by altered potassium buffering capacity in astrocytes and adjacent cells (Holthoff et al. 2000; Bordey et al. 2001; Pekny et al. 2005).

Furthermore, increased intracellular calcium levels have been postulated to be responsible for the toxicity seen in the SOD1 transgenic mouse model of amyotrophic lateral sclerosis (Roy et al. 1998). Similarly, in Huntington's disease Cx43 expression increases and has been suggested to be linked to enhanced endogenous spatial buffering capacity in turn helping to promote neuronal survival (Vis et al. 1998). Increased connexin levels have also been reported in the Parkinson-diseased brain (Rufer et al. 1996) as well as in Alzheimer's disease (Nagy et al. 1996).

In all such conditions, grafted NSCs, through the establishment of functional gap junctions with the host, could plausibly help restore homeostasis in endogenous cells by buffering potentially harmful levels of ions such as potassium and calcium.

The finding that grafted NSCs may both participate in and initiate host calcium signaling is potentially linked to a multitude of fascinating host effects **[Figs. 4, 5 and 8]**. Intracellular and intercellular calcium signaling patterns, might control such diverse processes as cell-cycle regulation, gene expression, differentiation, migration, neurotransmitter release, neurite and dendritic outgrowth, synaptic fine tuning and apotosis (Berridge et al. 2000; Parpura et al. 2000; Bootman et al. 2001; Uhlén et al. 2010).

The positive outcomes related to changes in GJIC may also be explained by the spread of other health-promoting molecules such as metabolites (Pitts 1998; Magistretti 2006; Elias et al. 2008; Kang et al. 2008), second messengers (cAMP and $InsP_3$) (Spray et al. 2006; Kanaporis et al. 2008) and small peptides (Neijssen et al. 2005), from exogenous NSCs to impaired host cells, thereby mediating the "bystander-rescue" phenomenon.

Yet another implication of the graft-host GJIC is coupled to the recent realization of short RNAs as key regulators of gene expression (Dykxhoorn et al. 2003; Gangaraju et

al. 2009). Thousands of target genes in vertebrates have been identified and can be downregulated by RNA silencing (Sontheimer et al. 2005). Interestingly, Cx43 (but not Cx26 or Cx32) channels has been shown to allow cell-to-cell transfer of short interfering RNA by gap junctions (Valiunas et al. 2005) thus potentially allowing a grafted cell to inhibit gene expression in an adjacent host cell directly. Gap-junctional coupling between endogenous cells and host cells thereby possibly creates a new approach for delivery of regulatory genetic material with potential for clinical gene therapy applications (Snyder et al. 1996; Sontheimer et al. 2005; Muller et al. 2006a).

Finally, the possibility to alter the calcium initiated processes described above, transfer active molecules as well as directly control gene transcription in neighboring cells taken together with the reported homing of grafted NSCs to malignant tumors (Benedetti et al. 2000; Brown et al. 2003) might make the graft-host gap junctions an attractive new approach to cancer gene- and chemotherapy for primary and metastatic intracranial and systemic tumors of both neural and non-neural origin.

Although all these implications are beyond the scope of the current thesis the ability for exogenous NSCs to directly affect vital host processes such as those described above may now be investigated in future studies.

5 CONCLUSIONS

This thesis describes early and beneficial interactions between grafted neural stem cells and the host. Most importantly it identifies a novel and clinically relevant mechanism by which exogenous murine as well as human NSCs integrate functionally into host neural circuitry and affect host cells via gap-junctional coupling.

Initially we concluded that engraftment of NSCs to organotypic striatal cultures is a suitable model system for the study of interactions between graft and host cells and that NSC differentiation can be directed by growth factors and serum free culturing conditions. NSC engraftment itself appeared to be beneficial to the host and a significant part of this interaction was attributed to the formation of gap junctions. In addition to providing a potential template for subsequent mature electrical coupling, the gap junctions permitted grafted NSCs to participate in host network activity, transfer molecules intercellularly and protect host cells from astrogliosis and cell death. Suppressing gap junction function and formation by pharmacological blockers and RNA interference supported their essential role in stem cell-host interactions both in slice cultures and rodent models of degeneration where host cells are rescued by NSCs.

NSC connexin 43 expression was controllable by growth factor overexpression and serum free culturing conditions and followed a clear temporal pattern after engraftment. In parallel, connexin expression in injured host cells peaked after traumatic stimuli. Taken together, this suggested a window of opportunity for successful host cell rescue by NSC engraftment.

Finally, data indicated that preconditioning by exposure to hypoxia presents a noninvasive method to increase connexin 43 expression and hemichannel function in NSCs before engraftment. This in turn facilitates earlier functional and potentially beneficial communication between grafted NSCs and the host.

The here reported early gap-junctional coupling might explain some of the recently described modulatory, homeostatic, and protective effects seen on host systems after NSC transplantation. Several molecules such as second messengers, metabolites, ions, amino acids and even polypeptides and regulatory genetic material are gap junction-permeant and may influence host homeostasis and possibly directly control host gene expression. Although beyond the scope of this thesis the stage may now be set for future studies to identify the trans-cellularly passed molecules responsible for the neuroprotective actions and the precise mechanisms involved in saving the brain.

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